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Oligofructose, 2'fucosyllactose and β-glucan in combination induce specific changes in microbial composition and short-chain fatty acid production compared to sole supplementation

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Abstract

Aims: In this study, we explored the effects that the prebiotic inulin-type fructans, and prebiotic candidates: 2'fucosyllactose and β-glucan from barley, singular and in combination had on microbial load, microbiome profile, and short-chain fatty acid production. This was carried out as a prescreening tool to determine combinations that could be taken forward for use in a human intervention trial.

Methods and results: Effects of inulin-type fructans, 2'fucosyllactose and β-glucan from barley in singular and combination on microbial load and profile and short-chain fatty acid production (SCFA) was conducted using *in vitro* batch culture fermentation over 48 h. Changes in microbial load and profile were assessed by fluorescence *in situ* hybridization flow cytometry (FISH-FLOW) and 16S rRNA sequencing, and changes in SCFA via gas chromatography. All substrates generated changes in microbial load and profile, achieving peak microbial load at 8 h fermentation with the largest changes in profile across all substrates in *Bifidobacterium* ($Q < 0.05$). This coincided with significant increases in acetate observed throughout fermentation ($Q < 0.05$). In comparison to sole supplementation combinations of oligofructose, β-glucan and 2'fuscosyllactose induced significant increases in both propionate and butyrate producing bacteria (*Roseburia* and *Faecalibacterium praunitzii*), and concentrations of propionate and butyrate, the latter being maintained until the end of fermentation (all $Q < 0.05$).

Conclusions: Combinations of oligofructose, with β-glucan and 2'fucosyllactose induced selective changes in microbial combination and SCFA namely *Roseburia*, *F. praunitzii*, propionate and butyrate compared to sole supplementation.

Significance and importance of the study

Our results indicate that compared to sole supplementation, combining oligofructose with 2'fuscoyllactose and β-glucan could provide a novel approach for targeted manipulation of the gut microbiota—namely, increasing numbers of butyrate and propionate bacteria (*Roseburia* and *F. praunitzii*) and should be studied *in vivo* to determine their real-world effects.

Keywords: Inulin-type fructans, β-glucan, 2'fucosyllactose, gut microbiota, short-chain fatty acids

Introduction

The term prebiotic was first defined by (Gibson and Roberfroid 1995) as a "nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon". The purpose of prebiotics is to target human and animal-associated microbiota and consequently improve health. Since then, the definition of a prebiotic has evolved where today prebiotics are categorized as a "substrate that is selectively utilized by the host microorganisms conferring health benefit to the host" (Gibson et al. 2017).

Whilst much of the traditional prebiotic literature has focussed on increasing *Bifidobacterium* spp. as an outcome, it is clear that different carbohydrates have different effects on the gut microbiome (Carlson et al. 2017, Collins et al. 2021), resulting in differences in microbial populations and short chain fatty acids produced from the complex cross-feeding that occurs in the gut microbiome (Flint et al. 2012, Louis and Flint 2017, Reichardt et al. 2014). We hypothesize that blending combinations of prebiotics and prebiotic candidates will result in the stimulation of a wider range of organisms, resulting in the increased and sustained generation of SCFA, particularly propionate and butyrate, compared to sole supplementation.

The most substantiated prebiotics are oligofructose (OF) and inulin, which belong to a class of nondigestible carbohydrates referred to as inulin-type fructans (ITF) (Scott et al. 2019). One of the key concepts in prebiotics is the targeted manipulation of the gut microbiota. The selectivity of ITF to stimulate changes in *Bifidobacterium* has been extensively investigated and documented in both human intervention studies and *in vitro* model systems (Wang and Gibson 1993, Kolida et al. 2007, Vandeputte et al. 2017a). However, while substantial increases in *Bifidobacterium* have been demonstrated

in vivo with both OF and long-chain inulin (Gibson et al. 1995), considerable differences are detected *in vitro* with OF appearing to be more readily utilized compared to long-chain inulin (Ghoddusi et al. 2007). Bifidobacteria express a range of enzymes and transport systems with specificity for low-molecular-weight oligosaccharides making them very competitive on OF, whereas the expression of inulinase among bifidobacterial strains is less consistent (Lee and O'Sullivan 2010).

Several other oligosaccharides have been investigated for their prebiotic potential, including HMOs, which is a classification given to a group of structurally diverse and complex unconjugated glycans present in human breast milk (Ninonuevo et al. 2006). Yet, to date, although there are around 200 known HMOs, very few are currently produced on a commercial scale; namely 3-sialyllactose (3'sl), 6-sialyllactose (6'sl), lacto-N-tetarose (LNT), 3-fuscosyllactose (3'fl), 2'fl, and lacto-N-neo-tetraose (LNnT). The most common of these currently used in a commercial setting are 2'fl and LNnT.

The efficacy of 2'fl to manipulate gut microbiota composition is somewhat unclear, with several *in vitro* studies reporting that 2'fl promotes the growth of several *Bifidobacterium* and *Bacteroides* species and strains, including *Bifidobacterium longum* subsp. *infantis*, *Bacteroides fragilis*, and *Bacteroides vulgatus* (Marcobal et al. 2010, Li et al. 2012, Gotoh et al. 2018). In contrast, several species and strains of microorganisms found within the gut microbiota, including *B. adolescentis* and *B. animalis* do not grow well in the presence of 2'fl (LoCascio et al. 2010, Marcobal et al. 2010, Xiao et al. 2010, Sela et al. 2012, Lawson et al. 2020). Furthermore, while there is increasing interest in the effects of 2'fl and other HMOs in shaping both the healthy and diseased microbiota, much remains unknown regarding the efficacy of 2'fl to manipulate changes in the adult microbiota due to the limited number of clinical studies undertaken to date (Suligoj et al. 2020, Ryan et al. 2021).

Other potential prebiotic candidates include β-glucan—a nonstarch polysaccharide composed of β-D-glucose monomer units linked by glycosidic linkages at the β (1,3),(1,4) position, either in a branched or in an unbranched manner (Rahar et al. 2011). One of the most prominent sources of β-glucan is barley, which is also a major source of arabinoxylans, the main polysaccharide present in the cell wall of whole grain cereals, including oats and barley and are composed of a backbone of β-(1,4)-linked xylose residues (Izydorczyk and Dexter 2008), substituted with arabinose residues on the C(O)-2 and/or C(O)-3 position (Knudsen and Laerke 2010). They are also considered prebiotic candidates (Sanders et al. 2019). Within the gut, several bacterial genera, including *Bacteroides* and *Prevotella* possess a plethora of loci with the potential to target the β-(1,3),(1,4) linkages of β-glucan (Dejean et al. 2020). Furthermore, increases in *Roseburia spp.* counts and propionate production have been associated with β-glucan supplementation *in vitro* (Fehlbaum et al. 2018). Whereas the ability of *Bifidobacterium* to utilize β-glucan as a carbon source appears to be somewhat mixed (Shoukat and Sorrentino 2021). This is likely due to not all bifidobacteria possessing the necessary loci able to utilize high molecular weight and complex carbohydrates.

During the fermentation of these complex carbohydrates, the SCFAs acetate and lactate are formed as products of fermentation by members of *Bifidobacterium*. However, *Bifidobacterium* does not produce either propionate or butyrate.

Several bacteria within the gut, including *Bacteroides*, *Roseburia* spp., *F. prausnitzii*, and *Clostridium* cluster IX can utilize either or both acetate and lactate via selective fermentation pathways to produce propionate and/or butyrate (Louis and Flint 2017). However, solely relying on cross-feeding to result in the generation of propionate and/or butyrate leads to unpredictability in the formation of these beneficial metabolites due to the complex and often competitive nature of microbial interactions. Thus, there is interest in combining prebiotics ITF with other potential prebiotics substrates including β-glucan and 2'fl may result in a more selective and controlled approach to regulating the microbial composition and SCFA production. Resulting in a greater generation of propionate and butyrate production bacteria and subsequent metabolites.

As a result, in this study, we investigated the fermentation properties (changes in microbial load, composition and SCFA) of OF and inulin, 2'fl and β-glucan from barley in singular and combination using pH-controlled *in vitro* batch culture fermentation over 48 h.

Methods

Materials

Prebiotics

The ITF used was oligofructose (OF) (Orafti® P95, DP 3–9, average DP 4; BENEO-Orafti, Tienen, Belgium) and oligofructose-enriched inulin (OFI) (50%±10% DP 3–9, 50%±10% DP ≥ 10; Orafti® Synergy 1, BENEO-Orafti, Tienen, Belgium) and an ITF-mix with shorter and longer chains (degree of polymerization (DP) ≥11 ~25%–30% (on g/100 g DM), average DP of 7–8, 15-30 wt.-% (dry matter, based on the total mass of carbohydrates) of Fm compounds with m = 2 to 9, BENEO-Orafti, Tienen, Belgium).

Prebiotic candidates

2'fucosyllactose (2'fl) is a human milk oligosaccharide (HMO) produced commercially via genetically modified yeasts and bacteria. 2'fucosyllatose (96%–98% pure) is a fucosylated HMO composed of L-fucose, D-galactose, and D-glucose. 2'fl was supplied by BENEO-Orafti, (Tienen, Belgium).

BENEO β-glucan rich barley flour was supplied by BENEO-Orafti, Tienen, Belgium). Per 100 g, on average: total dietary fibre 46 g: (β-glucan ~50%, arabinoxylan content ~24%); carbohydrates: 25 g; protein: 15 g; total fat: 6.7 g; sugars (fructose, glucose, sucrose, maltose, and lactose): 4 g.

Reagents

Unless otherwise stated all reagents used in this experiment were sourced from (Merck, Gillingham, UK).

Starch removal

β-Glucan-rich barley flour (100 g) was mixed with 500 mL of deionized distilled water. Amylase MT-3 K (3000 µ/mL, Enzyme Supplies Limited, Oxford) was diluted to 10 units per mL and 2 mL was added. The β-glucan sample was then placed in a stirring water bath at 50°C for one hour. A volume of 100 mL aliquots were transferred to 100–500 Da molecular weight cut-off regenerated cellulose dialysis tubing, and dialysis was performed against 1 mol l^{-1} NaCl at 5°C for 15 h, the dialysis fluid was then replaced and performed for two hours. After dialysis, samples were then aliquoted into 250 mL

Sterilin™ jars and frozen at -20°C. Samples were then freeze-dried. After freeze-drying both starch and β-glucan content were quantified using specific assay kits (AOAC Method 996.11 for total starch and AOAC Method 995.16 for mixed linkage β-glucan, Megazyme, Bray, Ireland). Final total starch and β-glucan content were measured at 2.79 g and 76.80 g per 100 g/dry sample, respectively.

In vitro batch culture fermentation

Faecal sample preparation

Ethical approval of collecting faecal samples from healthy volunteers was obtained from the University of Reading Research Ethics Committee. Freshly voided faecal samples were obtained from three healthy adults aged between 18 and 40, who had not taken antibiotics for at least four months prior to the experiment and had no history of gastrointestinal disorders, were not regular consumers of prebiotics or probiotics and who did not follow any restrictive diet. Faecal samples were diluted 1 in 10 (w/v) using 0.1 mol l^{-1} anaerobically prepared phosphate buffered saline (PBS, Oxoid, Hampshire, UK), pH 7.4. Faecal samples were then homogenized in a stomacher (Seward, stomacher 80, Worthing, UK) for 120 s at 260 paddle beats per min. A volume of 15 mL of faecal slurry was immediately used to inoculate each batch culture vessel.

Basal batch culture nutrient medium

To make 1 L of basal nutrient medium 2 g peptone water, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.6H_2O$, 2 g $NaHCO_3$, 0.5 g L–cystine HCl, 2 mL Tween 80, 10 μL vitamin K1, 0.05 g haemin, 0.05 g bile salts, and 4 mL resazurin (pH7) were added into 1 L of deionized water. 135 mL of medium was placed into glass jars and autoclaved at 121°C for 15 min.

pH controlled, stirred batch culture fermentation

For each donor, one independent batch culture was run. For each batch culture vessel, 14 × 300 mL were set up and 135 mL of basal nutrient media was aseptically poured into each vessel. This system was left overnight with oxygen-free nitrogen pumping through the medium at a rate of 15 mL/min with constant agitation throughout the entire course of fermentation. Before adding the faecal slurry, a water bath was used to set the temperature of the basal medium at 37°C, and a pH of between 6.7 and 6.9 was maintained automatically using a pH meter (Electrolab pH controller, Tewksbury, UK) via the addition of 0.5 mol l^{-1} HCl or 0.5 mol l^{-1} NaOH. Stirring of faecal samples was maintained using a magnetic stirrer. For each donor, 14 different substrates were prepared. To 13 of the 14 vessels one of the following substrate(s) were added at 1% (w/v): OF, OFI, ITF-mix, 2'fl, β-glucan, OFI + 2'fl (50/50, 85/15, and 95/5, w/w), OF + 2'fl, ITF-mix + 2'fl, OFI + β-glucan, OF + β-glucan, and ITF-mix + β-glucan (all 50/50, w/w). One vessel was set up as the negative control with no added carbohydrates. All vessels were inoculated with 15 mL of a 10% (w/v) faecal slurry (diluted with PBS). A sample (6 mL) was removed from each substrate vessel after 0, 4, 8, 24, 36, and 48 h incubation to ensure enough sample was taken for bacterial and short-chain fatty acid (SCFA) analyse by fluorescent *in situ* hybridization-flow cytometry (FISH-FLOW), 16S rRNA sequencing and gas chromatography-flame ionization detection (GC-FID), respectively.

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

A 750 μl sample of batch culture fermentation effluent was centrifuged at 1136 × g for 5 min. The supernatant was then discarded, and the pellet was then suspended in 375 μl filtered 0.1 mol l^{-1} PBS solution. Filtered 4% paraformaldehyde (PFA) at 4°C (1125 μl) was added, and samples were stored at 4°C for 4 h. Samples were then washed thoroughly with PBS three times to remove PFA and re-suspended in 150 μl PBS and 150 μl 99% ethanol. Samples were then stored at -20°C until FISH analysis by flow cytometry could be conducted.

The probes used in this study are presented in Table 1.

Fixed samples were removed from the freezer and 75 μl were mixed with 500 μl filtered cold (4°C) 0.1 mol l^{-1} PBS and then centrifuged at 11 337 × g for 3 min. The resulting supernatant was then discarded, and pellets resuspended in 100 μL of TE-FISH (Tris/HCl 1 mol l^{-1} pH 8, EDTA 0.5 mol l^{-1} pH 8, and filtered distilled water with the ratio of 1:1:8) containing lysozyme solution (1 mg/mL of 50 000 U/mg protein). Samples were then incubated for 10 min in the dark at room temperature and centrifuged at 11 337 × g for 3 min. Supernatants were discarded, and pellets were washed with 500 μl filtered cold PBS by aspiration to disperse the pellet. Samples were then centrifuged at 11 337 × g for 3 min and the supernatants were discarded.

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

Following incubation, 125 μl of hybridization buffer was added to each tube and gently vortexed. Samples were then centrifuged at 11 337 × g for 3 min and supernatants were discarded. Pellets were then washed with 175 μl of washing buffer solution and gently vortexed. Samples were incubated at 37°C for 20 min and centrifuged at 11 337 × g for 3 min. Supernatants were discarded and different volumes of filtered cold PBS (300, 600, and 1200 μl) were added based on flow cytometry load. Samples were kept at 4°C in the dark until flow cytometry measurements could be conducted. Bacterial counts were then calculated through consideration of flow cytometry reading and PBS dilution.

SCFA by gas chromatography-flame ionisation detection (GC-FID)

Samples (1.5 mL) of batch culture fluid were collected and centrifuged at 11 337 × g for 10 min. Supernatants were transferred to 1.5 mL Eppendorf tubes and stored at -80°C until analysis could be conducted. Samples were thawed, and extractions were performed according to Richardson et al. (1989) with modifications. Briefly, 1 mL of sample was transferred into a labeled 100 × 16 mm glass tube (International Scientific Supplies Ltd, Bradford, UK) and 50 μl of 2-ethylbutyric acid (0.1 mol l^{-1}, internal standard), 500 μL concentrated HCl and 3 mL diethyl ether were added to each glass tube before vortexing for 1 min. Samples were centrifuged at 2000 × g for 10 min. The resulting diethyl ether (upper) layer of each sample was transferred to clean 100 mL

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

Probe name	Sequence (5' to 3')	Targeted groups	Reference
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	(Wallner et al. 1993)
Eub338‡	GCTGCCTCCCGTAGGAGT	Most bacteria	(Amann et al. 1990)
Eub338II‡	GCAGCCACCCGTAGGTGT	Planctomycetales	(Daims et al. 1999)
Eub338III‡	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	(Daims et al. 1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	(Langendijk et al. 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*	(Harmsen et al. 1999)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz et al. 1996)
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium* coccoides-*Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb)	(Franks et al. 1998)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus	(Walker et al. 2005)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(Harmsen et al. 2000)
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX	(Walker et al. 2005)
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and relatives	(Suau et al. 2001)
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	(Ramsing et al. 1996)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)	(Franks et al. 1998)

screw top glass tubes. Ether extract (400 μl) and 50 μl N-tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) were added into a GC screw-cap vial. Samples were left at room temperature for 72 h to allow samples to completely derivatize.

An Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS 30 m × 0.25 mm column with a 0.25 μm coating (crosslinked (5%-phenyl)-methylpolysiloxane, Hewlett Packard, UK) was used for the analysis of SCFA. The temperatures of the injector and detector were 275°C, with the column temperature programed from 63°C to 190°C at 15°C min^{-1} followed by 190°C for 3 min. Helium was the carrier gas (flow rate 1.7 mL min^{-1}; head pressure 133 KPa). A split ratio of 100:1 was used. Quantification of the samples was achieved by calibration with acetic, propionic, and butyric SCFA in concentrations between 12.5 and 100 mmol l^{-1}. Mean metabolite concentrations were expressed as mmol l^{-1}.

Bacterial DNA extraction

Bacterial DNA was extracted from batch culture sample pellets using the QIAamp Fast DNA Stool mini kit (QIAGEN) according to the manufacturer's instructions. Batch culture sample pellets diluted in 400 ul PBS were placed in 2 mL screwcap tubes containing 0.6 g 0.1mm glass beads. Bead beating was performed on a fastprep24 instrument (MPBiomedicals; 4 cycles of 45 s at speed 4). Raw extract (200 μl) was then used for DNA isolation.

16S rRNA sequencing

Extracted bacterial DNA was subjected to PCR amplification of the V4 region of the 16S rRNA bacterial gene using two-stage Nextera PCR libraries using the primer pair 515F (5'-GTG YCA GCM GCC GCG GTA A -3') and 806R (5'- GGA CTA CNV GGG TWT CTA AT -3').

Raw sample extracts were diluted to 2.5 ng/μl, using Tris-buffer and 5 μL were used in 1st Step PCR, together with 5x HOT FIREPol® MultiPlex Mix (Solis BioDyne, Estonia) and 4 μM Primer Mix (fwd + rev) 515F/806R (Microsynth, Balgach, Switzerland). 1st Step PCR samples were purified with NGS Clean Beads (Labgene, Switzerland). The bead ratio was

1:1:2, the beads were washed with 75% ethanol, air-dried, and resuspended in Tris-buffer. In 2nd step PCR, each sample was individually barcoded, using Nextera XT Index Kit v2 (Illumina, San Diego, California) and 5x HOT FIREPol® MultiPlex Mix (Solis BioDyne, Estonia). 2nd Step PCR samples were purified with NGS Clean Beads (Labgene, Switzerland). The final 2nd Step PCR products were quantified using a Quant-iT™ PicoGreen™ ds DNA Assay Kit (Thermo Fisher Scientific, Waltham, USA). Amplicons were pooled equimolar prior to sequencing. The final pool was quantified using a Quant-iT™ PicoGreen™ ds DNA Assay Kit (Thermo Fisher Scientific, Waltham, USA) and fragment analyzer (Agilent).

Subsequent PCR libraries were sequenced on an Illumina MiSeq platform using a v2 500 (2×250 bp read length). Pools were diluted to 9.2 pM and loaded together with 15% PhiX (Illumina, FC-110–3001) to increase the diversity of the run, resulting in a raw cluster density of 631 and a cluster passed filter rate of 98%. Produced paired-end reads that passed Illumina's chastity filter were subject to de-multiplexing and trimming of Illumina adaptor residuals using Illumina's bcl2fastq software version v2.20.0.422. The quality of the reads was checked with the software FastQC version 0.11.8, and sequencing reads that fell below an average Q-score of 20 or had any uncalled bases (N) were removed from further analysis. The locus specific V4 primers were trimmed from the sequencing reads with the software cutadapt v3.2. Paired-end reads were discarded if the primer could not be trimmed. Trimmed forward and reverse reads of each paired-end read were merged to reform *in silico* the sequenced molecule considering a minimum overlap of 15 bases, using the software USEARCH version 11.0.667. Merged sequences were again quality filtered, allowing a maximum of one expected erroneous base per merged read. Reads that contain ambiguous bases or were outliers regarding the amplicon size distribution were also discarded. Samples that resulted in less than 5000 merged reads were discarded, as to not distort the statistical analysis. The remaining reads were denoized using the UNOISE algorithm implemented in USEARCH to form amplicon sequencing variant (ASV) discarding singletons and chimeras in the process. The resulting ASV abundance table was then filtered for possible barcode bleed-in contaminations using the UNCROSS algorithm. ASV sequences were com-

pared to the reference sequences of the RDP 16S database provided by https://www.drive5.com/usearch/manual/sintax _downloads.html, and taxonomies were predicted considering a minimum confidence threshold of 0.5 using the SINTAX algorithm implemented in USEARCH. The resulting library was then corrected by taking into considering the number of 16S copies and rarefying to an even sampling intensity to reduce bias in diversity metric calculations and quantified as described by (Vandeputte et al. 2017b).

Statistical analysis

Statistical analysis was carried out using GraphPad Software [version 9.2.0 (332) San Diego, California USA]. A two-way repeated measure ANOVA was used to determine significant differences in microbiota populations and concentrations of SCFA between 0 h and subsequent time points. Differences in relative abundance at 0 and 8 h fermentation were determined using a Friedman test. P values were adjusted with a post-hoc Benjami–Hochberg approach. Differences are stated as statically significant at *($Q < 0.05$), **($Q < 0.01$), and ***($Q < 0.001$).

Results

Organic acids

Figures 1, 2, 3, and 4 report the mean values for acetate, propionate, butyrate, and total SCFA recorded throughout the course of fermentation across the 3 independent donors. Individual SCFA values are presented in Supplemental Data Table 2.

Acetate was the most abundant SCFA detected, representing between 52%–73% of the total SCFA produced by the end of fermentation. Acetate concentrations were highest at the end of fermentation (48 h) in all treatment vessels, and they were all significantly higher compared to their respective 0 h (Fig. 1). Differences were detected in the magnitude of increase in acetate production between substrates and were greatest in the OF/2'fl treatment vessel with an average increase of 114.64 ± 8.14 mmol l^{-1} (SE) above baseline. Other notable acetogenic producing substrates included ITF-mix and the combination of ITF-mix/2'fl with an average increase of 106.27 ± 7.50 and 113.83 ± 8.96 (SE) mmol l^{-1} above baseline, respectively. The lowest increases in acetate production were recorded on β-glucan at 86.65 ± 13.62 (SE) mmol l^{-1}. No differences were detected in acetate production between substrates at the end of fermentation.

Propionate accounted for between 19%–33% of the total SCFA produced throughout fermentation with all substrates producing significant increases in propionate concentrations at 48 h compared to 0 h. Substantial differences in increases of propionate were observed between substrates. Most notable propiogenic substrates included β-glucan and combinations of ITF and β-glucan; more specifically vessels containing β-glucan singular and the combination of OF/β-glucan induced the largest average increase in propionate concentrations at 55.71 ± 2.32 (SE) and 52.56 ± 2.32 (SE) mmol l^{-1} above 0 h sampling respectively. Of all substrates tested, the treatment vessel containing sole 2'fl recorded the lowest increases in propionate concentrations at 48 h at 22.25 ± 3.38 (SE) mmol l^{-1} above baseline. At 48 h significant differences were detected in final propionate production between β-glucan singular/β-glucan-ITF combinations and several other

substrates, including OFI, 2'fl, and OFI/2'fl combinations (50/50, 85/15, and 95/5) (Fig. 2 and Supplemental Data Table 1).

Increases in butyrate production were documented in all substrate-containing vessels, yet substantial differences in concentrations of butyrate produced were observed between the substrates tested. The most noticeable increases in butyrate production were observed in β-glucan and the combination of OF/β-glucan containing treatment vessels resulting in an average increase of 26.21 ± 3.69 (SE) mmol l^{-1} butyrate concentration after 48 h accounting for 14.40% of total SCFA produced. Other notable butyrogenic substrates at 48 h included combinations of ITF-mix/β-glucan and OFI/β-glucan. In contrast, combinations of OFI/2'fl (50/50) and 2'fl alone documented the lowest increases of butyrate concentrations at 10.71 and 10.90 mM accounting for only 8.67% and 9.02% of total SCFA produced, respectively. Additionally, similar to propionate, significant differences were detected at 48 h between β-glucan/ITF-β-glucan and OF and 2'fl utilizing treatments (Fig. 3 and Supplemental Data Table 2). These data correlate strongly with changes seen in butyrate-producing bacteria (*Bacteroides*, *Roseburia*, and *F. prausnitzii*) throughout fermentation.

Bacterial enumeration

To determine changes in bacterial populations, twelve 16S-rRNA fluorescent *in situ* hybridization probes were used to identify changes in the numbers of total bacteria and 11 specifically targeted microbial groups. Results of significant bacterial group counts during the batch fermentation of different prebiotics and prebiotic candidates are shown in Figs. 5, 6, 7, 8, 9, and 10. Individual data is presented in Supplemental Data Table 4.

Significant increases in total bacterial counts (Eub338-II, II, and III) were observed with all substrates tested ($Q < 0.05$) with the exception of 2'fl singular, OFI, and all combinations of OFI/2'fl. The highest total bacterial counts across all substrates were observed at 8 h fermentation. A combination of OF/2'fl recorded the highest average increase in total bacteria at 1.0 ± 0.03 (SE) $\log_{10}$ cells/mL above 0 h sampling. The smallest increases in total bacteria at 8 h were recorded in vessels utilizing singular 2'fl and combinations of OFI/2'fl as substrates averaging an increase in total bacteria of 0.60 ± 0.17 (SE) $\log_{10}$ cells/mL compared to 0 h (Fig. 5).

The largest significant changes in bacterial numbers observed throughout the course of fermentation were recorded in *Bifidobacterium* (Bif164) counts. The bifidogenic effect varied across the different prebiotic fermentations. At 8 h fermentation, the combination of ITF-mix/2'fl recorded the highest average increase in Bif164 counts at 1.99 ± 0.02 (SE) $\log_{10}$ cells/mL. Other notable increases in bifidobacteria were detected in vessels utilizing OF, ITF-mix, OF/2'fl, OF/β-glucan, OFI/β-glucan, and ITF-mix/β-glucan as treatments. In contrast, 2'fl singular induced the smallest average increase in *Bifidobacterium* counts at 1.23 ± 0.41 (SE) $\log_{10}$ cells/mL above 0 h sampling. These changes correlated with the levels of acetate recorded in the respective vessels (Fig. 6).

Vessels containing singular β-glucan and combinations of ITF/β-glucan and ITF (OF and ITF-mix)/2'fl as treatments resulted in increased growth of several propionates and butyrate producing bacterial groups, including *Roseburia* spp.
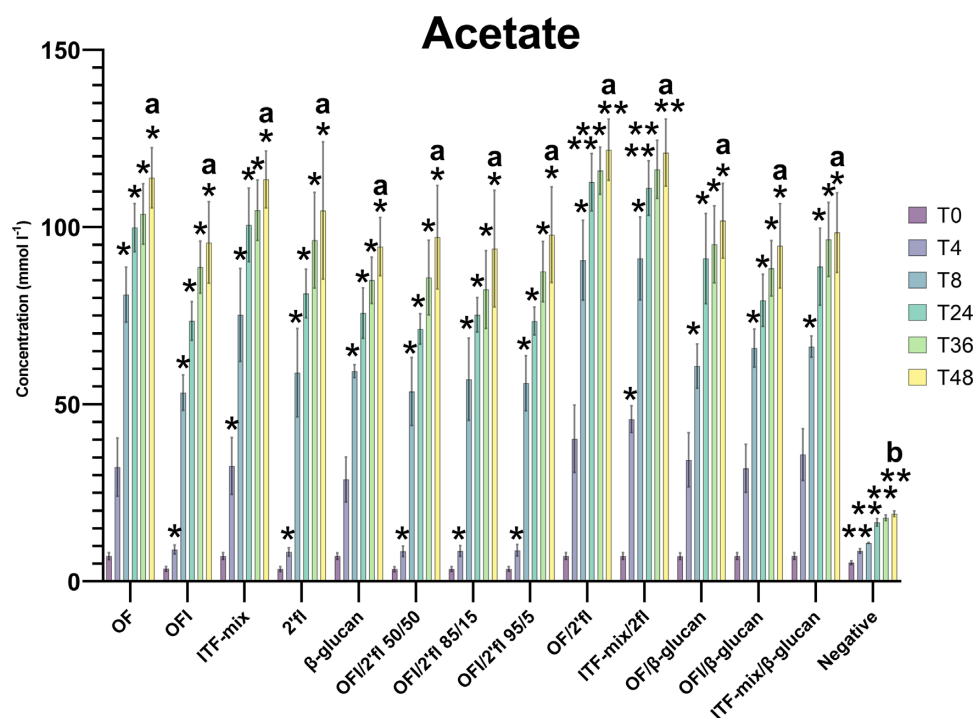
Figure 1. GC-FID analysis of acetate concentrations in the supernatant of effluents collected from vessels 1–14 at 0, 4, 8, 24, 36, and 48 h representing the mean ($n = 3$) of the data. Concentration reported in (mmol l^{-1}) mean and standard error (SE). *($Q < 0.05$), **($Q < 0.01$), and ***($Q < 0.001$) indicate significance compared with 0 h sampling. Significant differences between substrates at 48 h are indicated by differing letters.
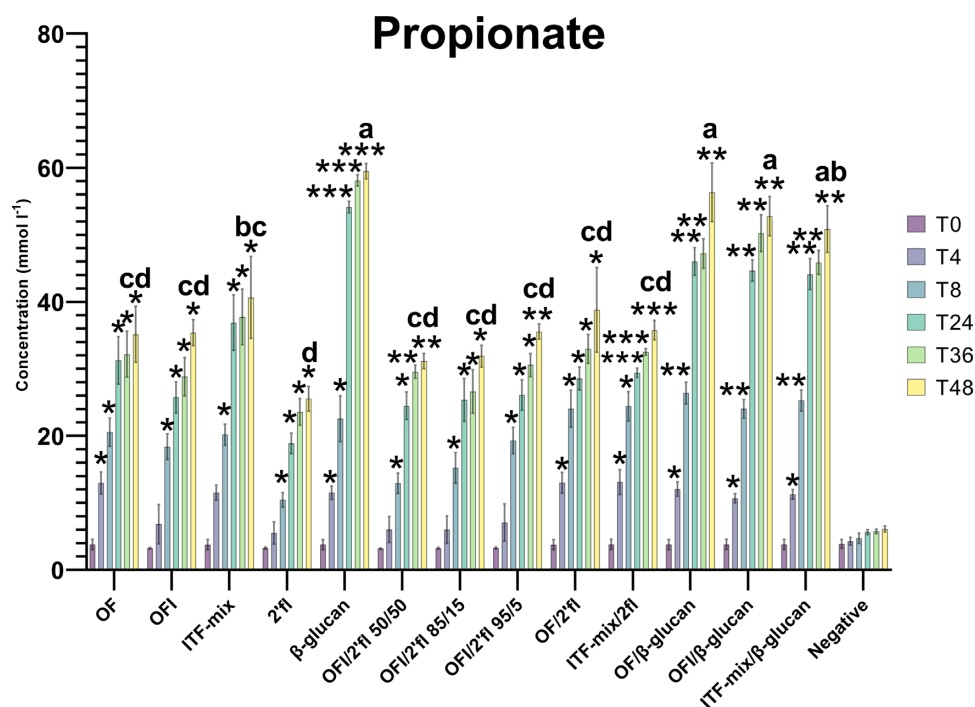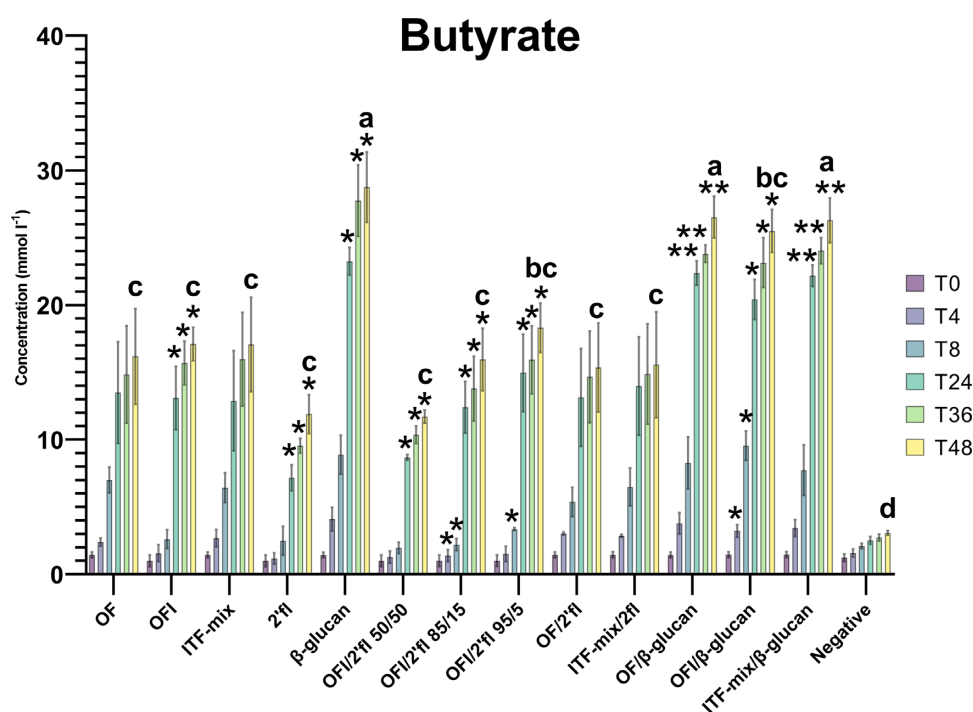

Figure 2. GC-FID analysis of propionate concentrations in the supernatant of effluents collected from vessels 1–14 at 0, 4, 8, 24, 36, and 48 h representing the mean ($n = 3$) of the data. Concentration reported in (mmol l^{-1}) means and SE. *($Q < 0.05$), **($Q < 0.01$), and ***($Q < 0.001$) indicate significance compared with 0 h sampling. Significant differences between substrates at 48 h are indicated by differing letters.

(Rrec584), *Clostridium* cluster IX (Prop853) and *F. prausnitzii* (Fprau655) (Figs. 7, 9, and 10). More specifically, β-glucan alone, OF/2'fl, ITF-mix/2'fl, OF/β-glucan, and ITF-mix/β-glucan treatments induced average increases of 1.19 ± 0.15 log$_{10}$ cells/mL in *Roseburia* spp. and *Clostridium* cluster IX

0.90 ± 0.10 (SE) log$_{10}$ cells/mL at 8 h fermentation (all $Q < 0.05$), respectively. Whereas at 8 h fermentation only OF, ITF-mix, and β-glucan singular recorded significant increases in *F. prausnitzii* at 0.90 ± 0.11 (SE) log$_{10}$ cells/mL (all $Q < 0.05$) (Fig. 4 and 5). Intriguingly, there were also large in-

Figure 3. GC-FID analysis of butyrate concentrations in the supernatant of effluents collected from vessels 1–14 at 0, 4, 8, 24, 36, and 48 h representing the mean ($n = 3$) of the data. Concentration reported in (mmol l^{-1}) means and SE. *($Q < 0.05$), **($Q < 0.01$), and ***($Q < 0.001$) indicate significance compared with 0 h sampling. Significant differences between substrates at 48 h are indicated by differing letters.
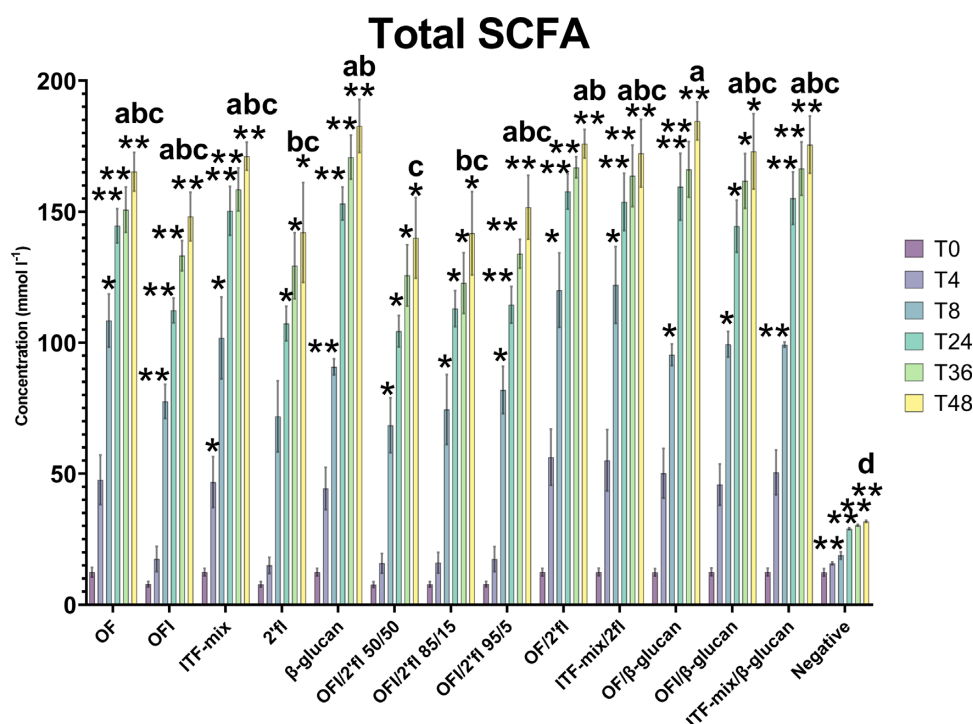

Figure 4. GC-FID analysis of total SCFA concentrations in the supernatant of effluents collected from vessels 1–14 at 0, 4, 8, 24, 36, and 48 h representing the mean ($n = 3$) of the data. Concentration reported in (mmol l^{-1}) means and SE. *($Q < 0.05$), **($Q < 0.01$), and ***($Q < 0.001$) indicate significance compared with 0 h sampling. Significant differences between substrates at 48 h are indicated by differing letters.

creases in *Bacteroides-Prevotella* counts (Bac303) (1.27, 1.31, 1.32, and 1.48 log$_{10}$ cells/mL) observed at 8 h fermentation in β-glucan and ITF/β-glucan containing treatments. However, these were not statistically significant from 0 h sampling (all

$Q > 0.05$) (Supplemental Data Table 3 and 4). The lack of significance likely resulted from the high variability in responses between donors at subsequent time points. These results coincide with the noticeable increases in propionate and
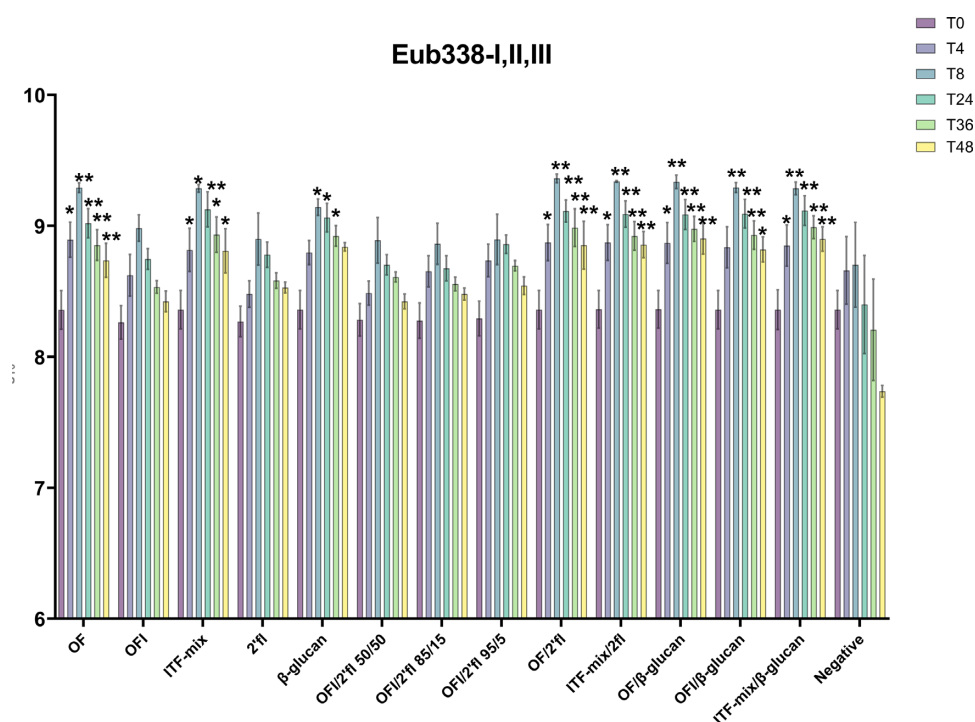
Figure 5. Bacterial groups measured by FISH-FLOW (Log$_{10}$ cells/mL) using probes: total bacteria (Eub338 I-II-III) Mean and SE. *($Q < 0.05$), **($Q < 0.01$), and ***($Q < 0.001$) indicate significance compared with 0 h sampling.
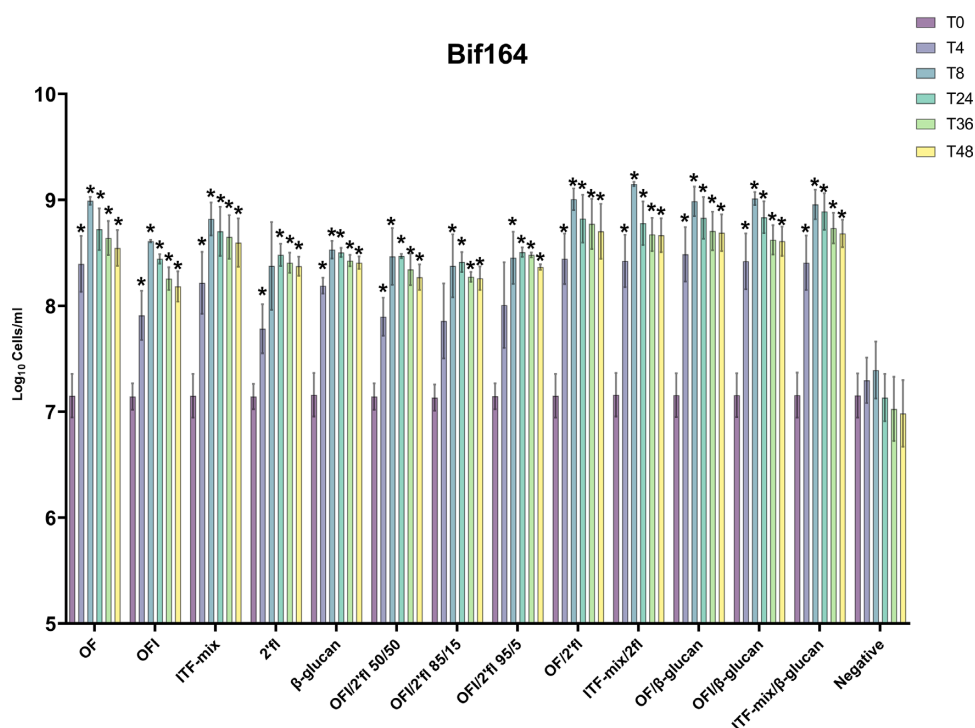
Figure 6. Bacterial groups measured by FISH-FLOW (Log$_{10}$ cells/mL) using probes: *Bifidobacterium* spp. Mean and SE. *($Q < 0.05$), **($Q < 0.01$), and *** ($Q < 0.001$) indicate significance compared with 0 h sampling.

butyrate seen in respective vessels, explicitly those possessing β-glucan as treatments.

There were also significant differences in the numbers of *Lactobacillus/Enterococcus* (Lab158), the *C. coccoides*–*E. rectale* group (Erec482), and the *Atopobium* cluster (Ato291)

detected in several substrates across a number of different time points (Fig. 8 and Supplemental Data 3). Lastly, only transient changes were observed in *Desulfovibrio* spp. (DSV687) and *C. histolyticum* (Chis150) across all substrates tested.

Figure 7. Bacterial groups measured by FISH-FLOW (Log$_{10}$ cells/mL) using probe: Roseburia (Rrec584). Mean and SE. *($Q < 0.05$), **($Q < 0.01$), and *** ($Q < 0.001$) indicate significance compared with 0 h sampling.

Figure 8. Bacterial groups measured by FISH-FLOW (Log$_{10}$ cells/mL) using probe: *Atopobium* cluster (Ato291). Mean and SE. *($Q < 0.05$), **($Q < 0.01$), and ***($Q < 0.001$) indicate significance compared with 0 h sampling.

Quantitative microbiome profiling (QMP)
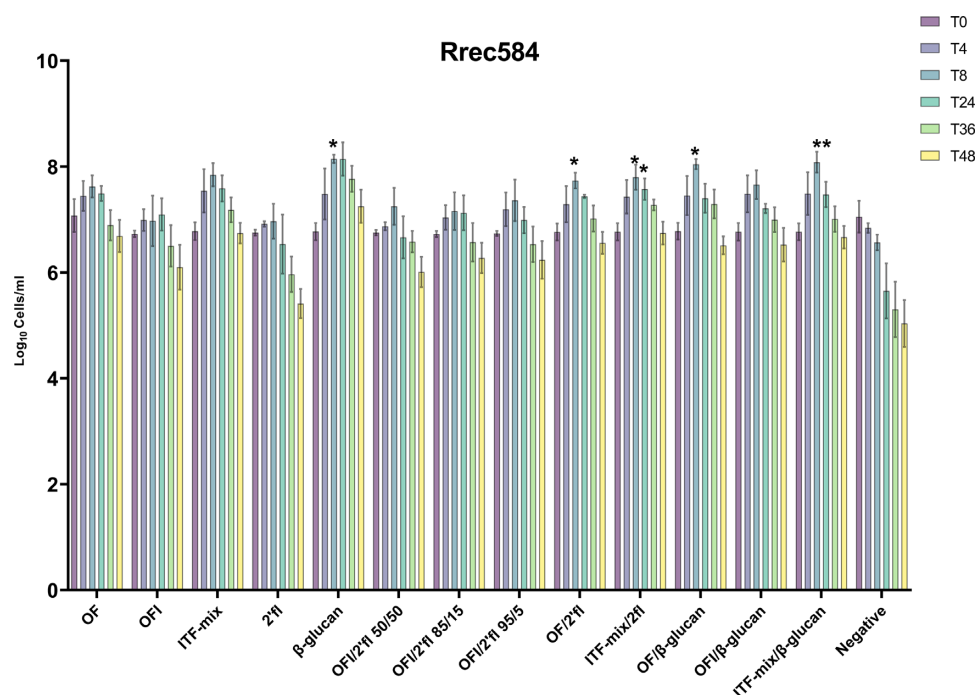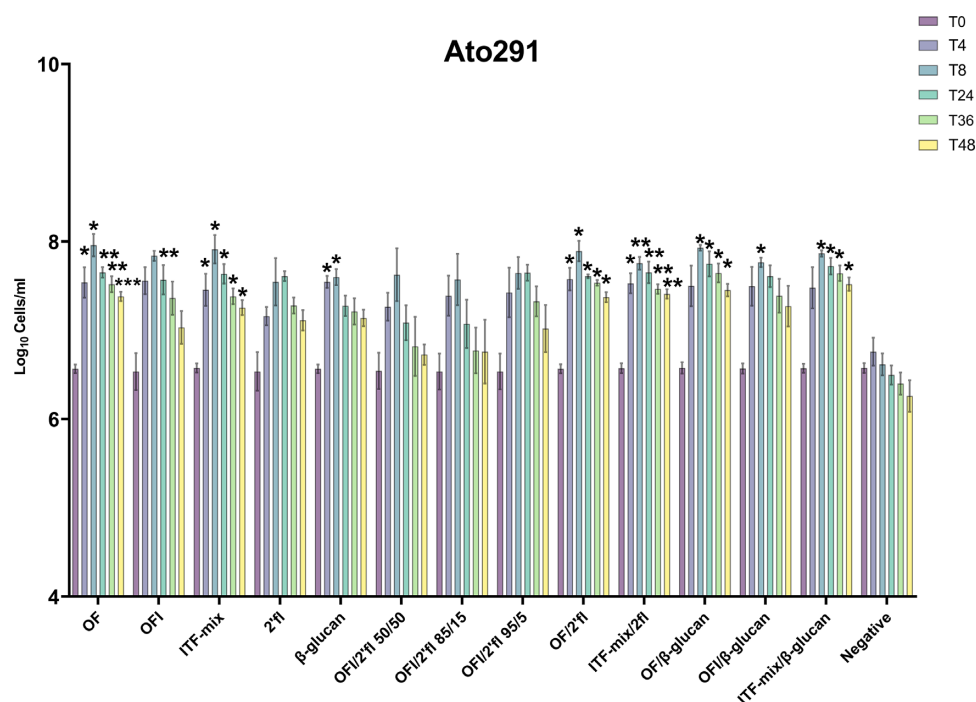
Fig. 11 reports the significant differences in 16S rRNA sequencing results, converted to QMP data, at 0 and 8 h fermentation across all 14 different treatment conditions. Individual *Bifidobacterium* QMP values are presented in Supplemental Data Table 6.

At the genus level, the most notable changes in microbial numbers were seen in *Bifidobacterium*, with significant in-

creases in numbers in several vessels by 8 h. These included OF/2'fl, ITF-mix/2fl, and ITF-mix/β-glucan. Additionally, several treatments displayed trends toward significant increases in relative abundances of *Bifidobacterium*—OFI ($P = 0.29$; $Q = 0.066$), ITF-mix ($P = 0.17$; $Q = 0.060$), OFI/2'fl (95/5) ($P = 0.22$; $Q = 0.063$), and OF/β-glucan ($P = 0.33$; $Q = 0.066$), (Fig. 11 and Supplemental Data table 3). No significant differences were detected in any other genus.

Figure 9. Bacterial groups measured by FISH-FLOW (Log₁₀ cells/mL) using probe: *Clostridium* cluster IX (Prop853). Mean and SE. *($Q < 0.05$), **($Q < 0.01$), and ***($Q < 0.001$) indicate significance compared with 0 h sampling.

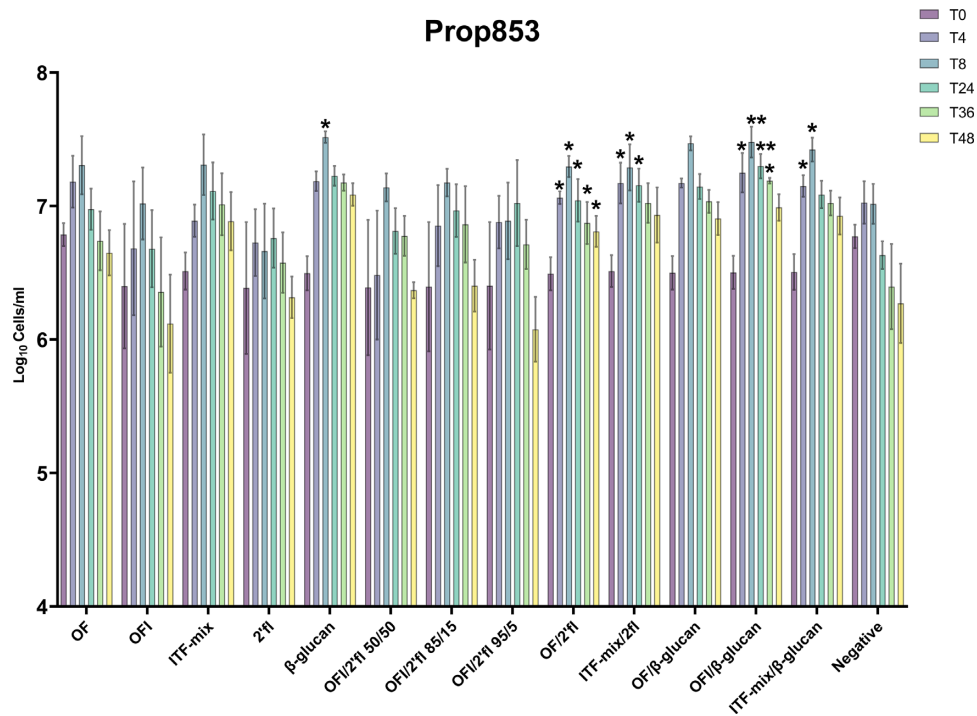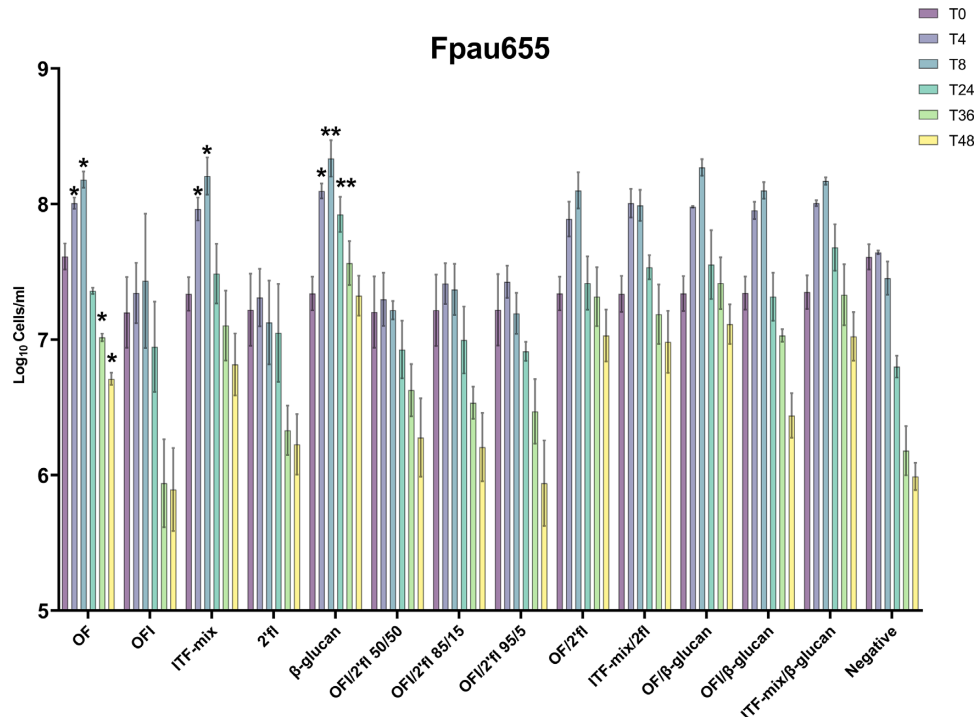**Figure 10.** Bacterial groups measured by FISH-FLOW (Log₁₀ cells/mL) using probe: *F. prausnitzii* (Fprau655). Mean and SE. *($Q < 0.05$), **($Q < 0.01$), and ***($Q < 0.001$) indicate significance compared with 0Ih sampling.

Looking at the data in closer detail, the largest changes in numbers of *Bacteroides* and *Prevotella* were seen on short chain ITF (OF) and ITF-mix either singular or in combination with 2'fl and β-glucan. While in contrast, numbers of *Bacteroides* and *Prevotella* remained unchanged in vessels uti-

lizing singular 2'fl and combinations of OFI and 2'fl as treatments.

Additionally, while all substrates tested singular and in combination resulted in increases of *F. prausnitzii*, there were noticeable increases on OF, ITF-mix, β-glucan, OF/2'fl,
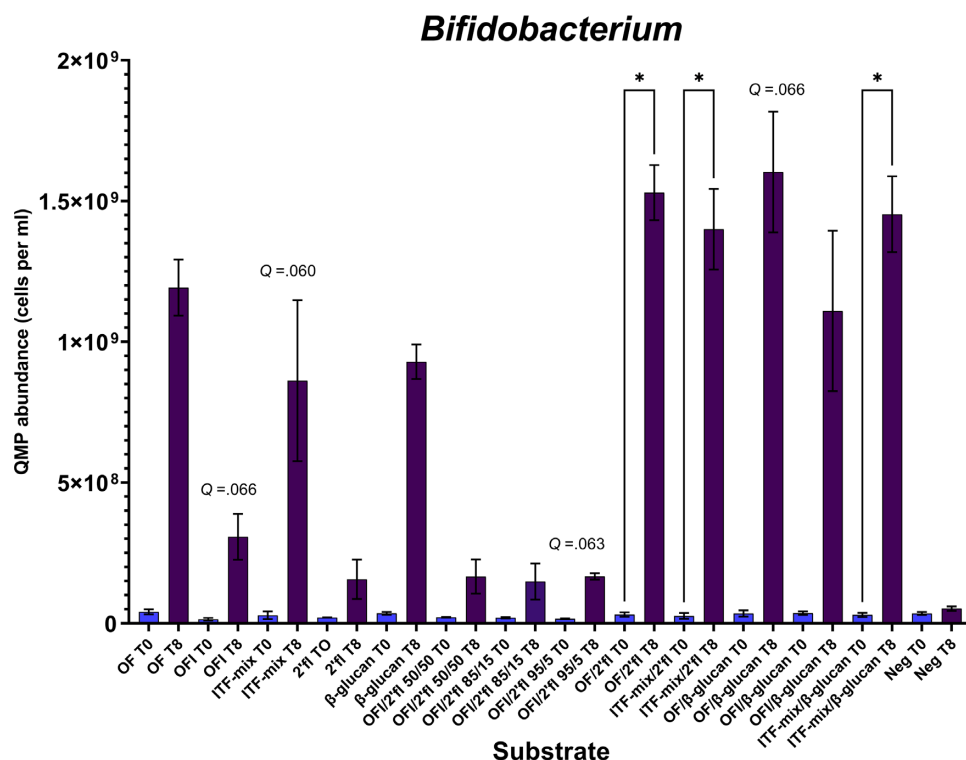
Figure 11. QMP of 16S rRNA analysis mean and SE of batch culture effluents (*Bifidobacterium*) collected at 0 and 8 h fermentation. *($Q < 0.05$), **($Q < 0.01$), and ***($Q < 0.001$) indicate significance compared with 0 h sampling.

OF/β-glucan and ITF-mix/β-glucan treatments with an average increase of $3.3E + 07 \pm 5.1E + 06$ (SE) cells/mL. These trends extended to *Ruminococcaceae, Ruminococcus 2, Lachnospiraceae incertae sedis, Anaerostipes, Collinsella*, and to a lesser extent, *Roseburia*, respectively (Supplemental Data Table 5).

In contrast, there were decreases in the numbers of *Alistipes* at 8 h on all substrates except OF. Similarly, numbers of *Clostridium* cluster IVXA + IVXB decreased across all substrates except ITF-mix, with numbers of *Dorea* decreasing across all substrates by the 8 h fermentation mark.

Finally, numbers of *Lactobacillus* and *Lactococcus* remained virtually unchanged except on β-glucan alone, OFI/β-glucan and ITF-mix/β-glucan treatments, which demonstrated a slight trend toward increases in *Lactobacillus*, respectively.

Discussion

In this present *in vitro* batch culture fermentation study, the experimental design compared the effects that established prebiotics and prebiotic candidates, alone and in combination, had on stimulating changes in microbial load, composition, and resulting SCFA. The results of this novel study indicate a marked variability in the potential of the substrates tested to produce changes in microbial load, composition, and resulting metabolites.

One of the key concepts of a prebiotic is to stimulate selective changes in microbial composition. As such, the majority of prebiotics currently target changes in *Bifidobacterium* due to this bacterium possessing the necessary intracellular and extracellular mechanisms and transporters needed to utilize a wide range of low molecular weight carbohydrates (Riv-

iere et al. 2018). Furthermore, bifidobacteria are associated with a wide array of health benefits, including reducing incidences of non- and antibiotic-associated diarrhoea, reductions in bowel transit time and improved stool frequency (Sanders et al. 2019). Acetate, the main SCFA produced via *Bifidobacterium* fermentation, plays major roles in regulating cholesterol metabolism (Hernandez et al. 2019), mineral and vitamin absorption, along with participating in cross-feeding, allowing for the proliferation of other microbial communities (Rowland et al. 2018).

In this regard, all substrates tested, singular and in combination, were able to stimulate changes in microbial load and composition. More specifically, there were large shifts in microbial populations of *Bifidobacterium* in the majority of substrates tested achieving maximum change after 8 h according to FISH analysis. The largest increases in *Bifidobacterium* were recorded in vessels utilizing ITF, predominantly short-chain OF and the smallest increases in *Bifidobacterium* on singular 2'fl or combinations of OFI/2'fl. The differences in microbial loads of *Bifidobacterium* recorded using FISH were confirmed by the results detected in 16S rRNA sequencing.

The response of *Bifidobacterium* to ITF is unsurprising, especially short-chain oligofructose *in vitro* with several previous studies reporting similar findings (Gibson et al. 1995, Pompei et al. 2008), further confirming the selectivity of ITF toward *Bifidobacterium*. Changes in *Bifidobacterium* by 2'fl seen in this study are similar to those of (Salli et al. 2019) who noted that 2'fl, while comparable, could not quite match the relative changes in bifidobacteria recorded by GOS. These results also correspond with those documented by (Ryan et al. 2021) with 2'fl appearing to be no more efficacious in stimulating the growth of bifidobacteria compared to OF and OF/inulin mixture in healthy adult donors. Similarly, Li et al.

(Li et al. 2012) recorded that during the *in vitro* fermentation of pigs faeces, both LNnT and pooled HMOs appeared to be no more effective than OF in inducing changes in *Bifidobacterium*, *Lactobacillus*, *B. vulgatus*, *Clostridium* cluster XIVa, and in *Clostridium* cluster IV.

Moderate increases were seen in microbial numbers in *Bacteroides/Prevotella* after 8 h on OF, β-glucan and ITF/β-glucan treatments. *Bacteroides/Prevotella* are a predominant member of the human gut microbiota possessing a plethora of polysaccharide degrading enzymes including loci with the potential to degrade ITF and mixed linkage β-glucan (Falony et al. 2009, Dejean et al. 2020). However, the results of this study indicate that *Bifidobacterium* was able to outcompete *Bacteroides/Prevotella* for substrates.

There were significant increases in the numbers of *Roseburia* detected on β-glucan and ITF/β-glucan combinations as well as combinations of OF/2'fl and ITF-mix/2'fl, but not OFI/2'fl combinations. Moreover, *F. prausnitzii* were most enhanced in response to β-glucan, OF and ITF-mix during the first 8 h. The differences recorded in microbial load of both *Roseburia* and *F. prausnitzii* between substrates were confirmed by the trends reported in 16S rRNA (Supplemental Data Table 3). These results are interesting given that several *Roseburia* strains, including *R. intestinalis* L1-82 and M50/1 have previously been demonstrated to grow in the presence of OF with several species known to possess OF utilization genes (Scott et al. 2015, Hillman et al. 2020). While in contrast, several strains of *Roseburia*, including *R. intestinalis inulinivorans* A2-194 and L1-83 do not appear to be able to grow in the presence of OF. The ability of *F. prausnitzii* to utilize ITF and β-glucan is somewhat inconsistent (Harris et al. 2019) as is seen in this study. Yet previous studies (Fehlbaum et al. 2018, Hillman et al. 2020) have noted that *Roseburia* are able to grow in the presence of either/both β-glucan and arabinoxylans. From this, it could be speculated that the growth of *Roseburia* in this study may be in part due to their ability to utilize arabinoxylans (a key component of the barley flour fraction used in this study making up 11% of the coarse fraction) as carbon sources (Harris et al. 2019). Furthermore, several previous studies have noted that increases in both *Roseburia* and *F. prausnitzii* often coincide with increases in *Bifidobacterium* (Riviere et al. 2016, Kim et al. 2020) suggesting that the increases in *F. prausnitzii* and *Roseburia* seen in this study may have resulted from the utilization of β-glucan and arabinoxylan and cross-feeding on the acetate produced by the *Bifidobacterium*.

Similarly, several treatments containing singular β-glucan as well as combinations of ITF/2'fl and ITF/β-glucan also resulted in increases in *Clostridium* cluster IX (Prop853) at 8 h. The results are in line with those recorded by (Hughes et al. 2008) who documented that supplementation with β-glucan fractions resulted in either maintaining or slightly increasing *Clostridium* cluster IX counts, as well as those documented by (Collins et al. 2021), noted that combinations of inulin and arabinoxylans resulted in substantial increases in *Clostridium* cluster IX. *Clostridium* cluster IX are propionate producers within the gut, and several species within this cluster can convert succinate to propionate (Gonzalez-Garcia et al. 2017). Additionally, several other species, including Megasphaera elsdenii and *S. ruminantium* can produce propionate from lactate (Hosseini et al. 2011, Gonzalez-Garcia et al. 2017). There was also a moderate increase in *Bacteroides* recorded in these vessels, and several strains within this genus encode the neces-

sary methylmalonyl-CoA decarboxylase (*mmdA*) gene to utilize the succinate pathway (Reichardt et al. 2014). As well as significant increases in *Bifidobacterium*, this further indicates that cross-feeding within the gut appears to be a critical feature in maintaining a diverse ecosystem (Henson and Phalak 2018). These results coincide with considerable increases in propionate concentration and sustained or reduced ratios of acetate to propionate ratios on β-glucan and ITF/β-glucan treatments at 24 h with similar concentrations maintained until the end of fermentation (Supplemental Data Table 7). These results are similar to those documented previously (Carlson et al. 2017, Fehlbaum et al. 2018). This may potentially have significance to health given that propionate acts as a precursor in gluconeogenesis, improves satiety via stimulation of leptin production in adipocytes, and regulates cholesterol synthesis (Hosseini et al. 2011, Soty et al. 2015).

There were also notable increases in butyrate concentrations in response to both singular β-glucan and ITF/β-glucan treatments but not treatments containing either singular 2'fl or OFI/2'fl, which are similar to those documented previously (Hughes et al. 2008, Fehlbaum et al. 2018, Suligoj et al. 2020). This is likely due to the increases in both *Roseburia* and *Faecalibacterium* as a result of β-glucan degradation and acetate utilization (Louis and Flint 2009, Fehlbaum et al. 2018). Additionally, several butyrate-producing bacteria, including *Anaerostipes* and *Lachnospiraceae incertae sedis* can convert lactate to butyrate via the butyryl-CoA: acetate CoA-transferase route (Louis and Flint 2009, Bui et al. 2019). However, while quantitative abundances of these organisms were higher at 8 h on OF, β-glucan and ITF/β-glucan treatments (Supplemental Data Table 3), lactate concentrations were not measured as part of this study. Thus, it can only be speculated that maintaining higher relative abundances of lactate-producing bacteria resulted in additional increases in butyrate concentrations. Finding means of causing substantial and sustainable increases in butyrate concentrations could be highly beneficial in human health, given that butyrate plays a vital role in acting as an energy source of colonocytes, along with regulation of tight cell junction integrity, repair of the intestinal mucosa, and is often associated with lower levels of IBD (Ryan et al. 2021). These findings suggest that β-glucan may provide a complementary effect compared to ITF supplementation alone.

Finally, increases in Atopobium were seen across a wide array of substrates and time points with the exception of LC-ITF/2'fl combinations. Atopobium is a common genus isolated from human faeces and has been reported to be increased in the presence of both simple and complex carbohydrates, including ITF (Vinke et al. 2017) with the results of this study seemingly supporting this. However, what this means regarding clinical health outcomes is not known, as Atopobium currently has no identified role in human health.

It should be noted that the use of *in vitro* models to identify changes in microbial diversity does not necessarily capture changes in microbial diversity seen *in vivo*. Additionally, high concentration β-glucan substrate was used in its pre-processed state and would not mimic real-life scenarios such as cooking and processing, which may impact prebiotic functionality (Jackson et al. 2022). Additionally, the high fraction β-glucan flour did not undergo upper GI tract predigestion procedures to remove residing proteins prior to fermentation. There is a possibility that the proteins present in the coarse barley fraction may have impacted the results seen in respec-

tive treatment vessels, and the result should be interpreted as such. The strengths of using *in vitro* models, however, include the ability to test novel substrates for potential changes in the microbial composition prior to investigation in human intervention trials as well as minimizing the heterogenicity seen in *in vivo* studies.

Conclusions

Overall, these results imply that combinations of ITF and β-glucan, can provide a complementary effect to ITF via the stimulation of propionate and butyrate producing bacteria namely *Bacteroides*, *Roseburia*, and *Faecalibacterium* along with sustained propionate and butyrate production. The ability of 2'fl to alter microbial composition appears to be extremely limited *in vitro* except in the presence of OF, and therefore combinations of both OF/2'fl and ITF/β-glucan warrant further investigation to determine their *in vivo* effects.

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Ethics statement

This research did not include human subjects, however, faecal samples were sourced from willing volunteers. The use of faecal samples for *in vitro* work has been approved by the University of Reading Research Ethics Committee.

Supplementary data

Supplementary data is available at *JAMBIO* online.

Conflict of interest

We acknowledge that this research was financed by BENEO. ST and JVH are employees of BENEO.

Author contributions

Peter Philip James Jackson (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing), Anisha Wijeyesekera (Project administration, Writing – review & editing), Jessica van Harsselaar (Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Writing – review & editing), Stephan Theis (Conceptualization, Funding acquisition, Writing – review & editing), and Robert Adrian Rastall (Conceptualization, Project administration, Supervision, Writing – review & editing).

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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