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Research article

Effects of food matrix on the prebiotic efficacy of inulin-type fructans: a randomised trial

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Abstract

Recently there is much debate in the scientific community over the impact of the food matrix on prebiotic efficacy of inulin-type fructans. Previous studies suggest that prebiotic selectivity of inulin-type fructans towards bifidobacteria is unaffected by the food matrix. Due to differences in study design, definitive conclusions cannot be drawn from these findings with any degree of certainty. In this randomised trial, we aimed to determine the effects that different food matrices had on the prebiotic efficacy of inulin-type fructans following a standardised 10-day, 4-arm, parallel, randomised protocol with inulin either in pure form or incorporated into shortbread biscuits, milk chocolate or a rice drink. Similar increases in *Bifidobacterium* counts were documented across all four interventions using both fluorescence *in situ* hybridisation (pure inulin: +0.63; shortbread: +0.59; milk chocolate: +0.65 and rice drink: +0.71 (log_{10} cells/g wet faeces) and 16S rRNA sequencing quantitative microbiome profiling data (pure inulin: +1.21 x 10^9; shortbread: +1.47 x 10^9; milk chocolate: +8.59 x 10^8 and rice drink: +1.04 x 10^9 (cells/g wet faeces) (all $P \leq 0.05$). From these results, we can confirm that irrespective of the food matrix, the selectivity of inulin-type fructans towards *Bifidobacterium* is unaffected, yet the compositional make-up of the food matrix may have implications regarding wider changes in the microbiota.

Trial registration: clinicaltrials.gov ID: NCT05581615.

Key words

Prebiotics, food matrix, carbohydrates, inulin-type fructans, gut microbiota

1. Introduction

Diet, being one of the key drivers of fermentation in the gut, can strongly influence the composition and thus the functionality of the gut microbiota. One way to modify the composition and activity of the gut microbiota is via prebiotic functional foods as they provide a safe, affordable and effective dietary approach (Sanders *et al.*, 2019). Oligofructose (OF) and inulin are the most widely researched prebiotics belonging to a class of non-digestible carbohydrates referred to as inulin-type fructans (ITF) (Karimi *et al.*, 2015). ITF are linear polydisperse carbohydrates composed of monomers of fructose linked by β-(2-1) glycosidic (fructosyl-fructose) linkages. A non-reducing α-D-glucose moiety may or may not be present (Roberfroid, 2007) and based on the degree of polymerisation (DP), ITF can be separated into OF (DP 2-9) and inulin (DP $\geq$ 10) (van Loo, 2006).

Due to their structure and the absence of brush border β-fructosidases the majority of ITF reach the colon intact functioning as prebiotics by displaying high selectivity towards certain beneficial microbial groups such as *Bifidobacterium.* This is a key feature of the prebiotic concept along with providing a series of health benefits to the host as summarised in these series of reviews (Ahmed and Rashid, 2019; Gibson *et al.*, 2017; Sanders *et al.*, 2019; Wilson and Whelan, 2017). Furthermore, due to their physicochemical properties ITF can also act as fat and sugar replacers as well as texture modifiers while still providing potentially prebiotic dosages. They are becoming an increasingly common ingredient within the food industry (Shoaib *et al.*, 2016).

The concept that the food matrix may impact on the prebiotic efficacy of ITF has become of increasing interest in recent years. This is in part due to previous research suggesting that food matrices may either hinder or enhance the bioavailability of phenolic compounds, fatty acids and other nutrients (Ribas-Agusti *et al.*, 2018; Thorning *et al.*, 2017). Furthermore, there is evidence that high levels of dietary fibre present within the matrix can influence the absorption of such compounds via the sequestration of ions and formation of complexes (D'Archivio *et al.*, 2010; Palafox-Carlos *et al.*, 2011). This concept also applies to the microbial fermentation of unabsorbed secondary metabolites in the diet and resulting metabolites within the colon (Aguilera, 2019).

Depending on the processing parameters, ITF may or may not be subject to degradation during the production process. Critical processing parameters include pH, with the critical cut-off appearing to be ≤ 4 (Glibowski and Wasko, 2008; Mensink *et al.*, 2015), pasteurisation (often used during fruit juice production) (Klewicki, 2007), heating such as during baking (Poinot *et al.*, 2010; Rodriguez-Garcia *et al.*, 2012) resulting in participation in caramelisation and Maillard reactions (indicated by the level of browning in bread, cakes, biscuits, etc) (Mensink *et al.*, 2015). Degradation could also be caused by high temperature and pressure extrusion (ready-to-eat cereals and snacks) (Duar *et al.*, 2015) and enzymatic hydrolysis via yeasts and bacteria (bread and beer production) (Struyf *et al.*, 2017). Generally, the processing time, temperature, and the DP of ITF used appear to be critical if the potential degradation of ITF is to be avoided. Each aspect needs to be carefully considered in order to optimise product quality while maintaining ITF integrity (Jackson *et al.*, 2022b).

To date, studies have explored the effects of ITF on the gut microbiota in both pure form, as well as several food products such as biscuits, yoghurt, stewed apple, cereal bars, cocoa drinks, and fruit juices as vehicles for ITF supplementation (Azpiroz *et al.*, 2017; Brighenti *et al.*, 1999; Gibson and Roberfroid, 1995; Healey *et al.*, 2018; Kleessen *et al.*, 2007; Ramnani *et al.*, 2010; Rao, 2001; Slavin and Feirtag, 2011). The results of these studies all document that the selectivity of ITF towards *Bifidobacterium* is unaltered as result of the food matrix. However, as a subgroup analysis from So *et al.*, (2018) concluded, fibre interventions delivered through supplementation resulted in significantly higher *Bifidobacterium* spp. compared to placebo/lower fibre controls (SMD: 0.75; 95% CI: 0.52, 0.98; $P \leq 0.00001$, I2 = 83%). No differences were found between food interventions and comparators (SMD: 0.20; 95% CI: −0.36, 0.76; P = 0.49, I2 = 88%), although considerable heterogeneity persisted in both analyses. This emphasizes that definitive conclusions on whether the food matrix matters in the supplementation of ITF cannot be drawn due to differences in study design (crossover vs parallel study design, number of participants, length of the intervention), differences in the implementation of controlled vs non controlled and exclusion diets (excluding or not excluding other fructans), the type and amount of ITF supplemented (inulin vs OF), time point of stool samples collection), combined with the lack of washout periods, differences in reporting changes in microbial numbers (dry vs wet weight of faeces) and analytical techniques used (fluorescence *in situ* hybridization (FISH) vs selective media vs quantitative polymerase chain reaction (qPCR)).

Many of the food products utilised in the studies mentioned above are sources of other potential prebiotics including phenolic acids, β-glucan, arabinoxylans and bovine milk oligosaccharides. Each possesses the potential to alter the fermentation selectivity and have been shown to influence levels of *Lactobacillus*, *Bacteroides*, *Enterococcus*, *Prevotella,* and *F. prausnitzii* (Gomez *et al*., 2016; Kemperman *et al*., 2013; Scott *et al*., 2019; Valeur *et al*., 2016) amongst others. A critical aspect often overlooked by researchers when considering study designs regarding food-based prebiotic supplementation studies. This leads to the question of whether the food matrix matters in the supplementation of ITF? This question is becoming increasingly important to answer given the interest in the addition of ITF into various food products with several manufacturers marketing these products as beneficial for health (Rolim, 2015). Therefore, this study aims to determine the effects that different food matrices may have on the prebiotic efficacy of ITF following a standardised protocol. The hypothesis to be tested is that the food matrix does not impact on the selectivity of ITF towards *Bifidobacterium*.

2. Materials and methods

Subjects and recruitment

Healthy adults, both males and females, were recruited from the Reading area via previous email lists and posting on social media. The inclusion criteria were volunteers aged 18-65, BMI $\geq$ 18.5 and $\leq$ 30 kg/m^2, no evidence of gastrointestinal diseases and following what could be deemed a typical Western European diet. They were free of food allergies and had a stool frequency of at least 3 bowel movements per week. Exclusion criteria were extreme diets (i.e., ketogenic, vegetarian, vegan, intermittent fasting), antibiotic treatment in the four

months preceding the study, anaemia, chronic or acute diseases i.e., (pre)-diabetic. Potential subjects were also excluded if they had undergone surgical resection of any part of the bowel, were current smokers and/or had a history of alcohol or drug misuse. Potential volunteers were excluded if they were pregnant or lactating. Use of laxatives was not permitted 4 weeks prior to beginning of the intervention.

Study design and interventions

The study design was a prospective, non-placebo controlled, parallel-group, randomised trial lasting ten days. Ten days was the chosen intervention length based on the results of previous research demonstrating that the bifidogenic effect of ITF can be seen after approximately seven days of daily intake (Nagy *et al.*, 2022). Prior to commencing the study, eligible subjects were provided with both verbal and written study information and gave their informed consent. Enrolled subjects were asked to undergo a two-week run-in period in which they were required to restrict the use of any probiotics, prebiotics and prebiotic or probiotic containing foods or supplements. After the run-in phase enrolled subjects were randomised using REDCap (see below) into one of four groups (n = 24 per group) stratified by sex using a ratio of approximately 2:1 (female : male): (Group A (16 : 8) – pure inulin), (Group B (18 : 6) – inulin-enriched shortbread), (Group C (16 : 8) – inulin-enriched milk chocolate), and (Group D (18 : 6) – inulin-enriched rice drink).

The ITF used in the was highly soluble inulin (Orafti® HSI, DP 2-60, min. 88% inulin, maximum of 12% glucose, fructose, and sucrose (DM), BENEO-Orafti, Tienen, Belgium) produced from chicory. The interventions used in this study were provided by BENEO. Interventions were chosen based on the outcomes of our literature review reflecting the most common food products that undergo inulin fortification (Jackson *et al.*, 2022a; Jackson *et al.*, 2022b). This not only reflects a wide degree of matrices (baked, semi-solid and liquid), but also those consumed as part of the population's habitual diet (Murakami and Livingstone, 2016). Each portion of pure inulin or enriched food product contained 5 g of ITF and was consumed twice per day resulting in a total daily ITF intake of 10 g. This dosage was chosen based on the amount of ITF that can be successfully fortified into study products without changes in product characteristics. Pure inulin was used as the comparator to determine if the prebiotic efficacy was altered as a result of different food matrices. Details on composition of each study product per 100 g and per daily portion can be found in Table 1.

	Pure inulin		Shortbread		Milk Chocolate		Rice Drink	
Amounts	**per 100 g**	**per 11.4 g daily portion**	**per 100 g**	**per 58 g daily portion**	**per 100 g**	**per 52 g daily portion**	**per 100 mL**	**per daily 300 mL portion**
Energy kJ/kcals	875/216	87.5/21.6	1766/422	1024.28/244.76	2187/523	568.36/271.96	465/111	1534.5/330
Carbohydrates (g)	11	1.1	54.7	31.73	31	16.12	20.4	61.2
of which is sugars (g)	11	1.1	12	6.96	30.4	15.81	11.8	35.4
Fat (g)	Negligible	Negligible	15.9	9.22	36.3	18.88	2.3	6.9
of which is saturates (g)	Negligible	Negligible	7.2	4.18	21.6	11.23	0.8	2.4
Protein (g)	Negligible	Negligible	5.4	3.13	7.2	3.74	0.5	1.5
Fibre (excluding fructans) (g)	0	0	1.36	0.79	2.46	1.28	0.77	2.3
Fibre (including fructans) (g)	88	10	18.6	10.79	21.7	11.28	4.1	12.3
Salt (g)	Negligible	Negligible	1	0.58	0.2	0.104	0	0

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Stool and urine samples were collected at Day 0 and Day 10. Details of sample collection are presented below. No intervention was given until both baseline samples had been provided. Subjects were instructed to consume their assigned pure inulin supplement or food product for the entire 10 days, one portion in the morning and one portion in the evening with no other food or drink and within 15 min of opening. Volunteers were told to not alter their diet or fluid intake during the trial with exception of portion size to make allowances for additional calories consumed as part of the intervention. Volunteers were only considered compliant if consumption for the whole ten-days of the intervention was achieved. In order to assess compliance volunteers were asked to complete an online daily check-in dairy. Changes in habitual dietary intakes at Day 0 and Day 10 were assessed using a modified version of the validated eNutri2019-DE web application specifically designed to capture short-term changes in dietary intake. In-depth details on the eNutri2019-DE web application have been described elsewhere (Franco *et al.*, 2019).

Data were collected and managed using REDCap electronic data capture tools hosted at the University of Reading (Harris *et al.*, 2009). REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources.

Outcomes

Primary outcomes

The primary outcome was differences in *Bifidobacterium* count as measured by fluorescence *in situ* hybridisation flow cytometry (FISH-FLOW).

Secondary outcomes

The secondary outcomes were changes in microbial composition and urinary metabolites as measured 16S rRNA sequencing and ^{1}H-nuclear magnetic resonance (^{1}H-NMR). Details on sample collection, processing and analysis are detailed below.

Bowel habit and GI sensation diaries were completed daily throughout the of the ten-day intervention, in order to assess day-to-day changes in flatulence, intestinal bloating, abdominal pressure, abdominal pain and feeling of fullness (all none, mild, moderate and severe) (Costabile *et al.*, 2008; Ramnani *et al.*, 2010; Walton *et al.*, 2012), stool frequency and consistency according to the Bristol Stool Form Scale (Lewis and Heaton, 1997). Any medication use or adverse events were also recorded.

Sample collection

Faecal samples

Volunteers were provided written and verbal instruction on how to collect stool samples, and with sterile stool sample pots for Day 0 and Day 10 collections. Freshly collected faecal samples were kept in 2.5L Oxoid™ AnaeroJar™ (Oxoid, Hampshire, United Kingdom) with Oxoid™ AnaeroGen™ 2.5L sachets (O_2 ≤0.1%; CO_2: 7-15%). Faecal samples were collected from the volunteer's place of residence within 2 hours of voiding. Samples (1.5 g) for metabolic profiling were stored at -80 °C until the study had been completed. An additional 3 g of the same faecal sample was diluted 1:10 (w:w) in anaerobic phosphate-buffered saline (PBS, 0.1 M; pH 7.4), then homogenised using a stomacher (260 paddle beats/min) for 2 min at room temperature. 20 mL of faecal slurry were then vortexed with 3 mm diameter glass beads for 30 s before being centrifuged at 1,500 x g for 3 min at room temperature. 75 µL were then diluted in 675 µL phosphate buffered saline (PBS mol l^{-1}; pH 7.4) (1:100 dilution), aliquoted in to 1.5 mL Eppendorf tubes and stored at -80 °C until cells could be fixed. Samples were then centrifuged at 11,337 × g for 5 min and the supernatant was decarded. Pellets were then resuspended in 375 µL of 0.1 M PBS and fixed in 4% (w:v) paraformaldehyde (1,125 µL) for 4 h at 4 °C. Fixed cells were centrifuged at 11,337 × g for 5 min at room temperature. Samples were then washed with 1 mL PBS, pellets aspirated and centrifuged at 11,337 × g for 5 min. The washing process was repeated twice more. Samples were re-suspended in 150 µL PBS and stored in ethanol (1:1, v:v) at −20 °C until analysis via fluorescence *in situ* hybridisation – flow cytometry (FISH-FLOW).

Urine samples

Day 0 and Day 10 mid-stream urine samples were collected as the first urine sample after waking in sterilised specimen pots. Urine samples were collected from volunteers at the same time as faecal samples. Urine samples were stored at – 80 °C until analysis by Proton Nuclear Magnetic Resonance spectroscopy (^{1}H-NMR) could be conducted.

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

FISH by flow cytometry was carried out as described by (Grimaldi *et al.*, 2017). Probes used in this study are listed in Table 2. Fluorescence measures were performed by a BD Accuri™ C6 Plus (BD, Erembodegem, Brussels) measuring at 488 nm and 640 nm. Thresholds of 9000 in the forward scatter area (FSC-A) and 3000 in the side scatter area (SSC-A) were placed to discard background noise, a gated area was applied in the main density dot to include 90% of the events. Flow rate was 35 uL/min, with limit of collection set for 100,000 events and analysed with Accuri CFlow Sampler software. Bacterial counts were then calculated through consideration of flow cytometry reading and PBS dilution. The number of $\log_{10}$ cells is presented as per gram of wet fresh faeces.

	Sequence (5' to 3')	Target 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)
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* spp.	(Walker *et al.*, 2005)
Prop853	ATTGCGTTAACTCCGGCAC	*Clostridium* cluster IX	(Walker *et al.*, 2005)
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives	(Suau *et al.*, 2001)

Table 2: Name, sequence, and target group of oligonucleotide probes used for bacterial enumeration

Microbial Profiling

Bacterial DNA extraction

Bacterial DNA was extracted from faecal samples using the QIAamp Fast DNA Stool mini kit (QIAGEN) according to the manufacturer's instructions. Faecal samples were homogenised and allocated into 2 mL screwcap tubes containing 0.6 g 0.1 mm glass beads. Bead beating was run on a fastprep24 instrument (MPBiomedicals); 4 cycles of 45s at speed 4). 200 mL of raw extract were then used for DNA isolation.

DNA isolation, library preparation and 16S rRNA gene 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 2.5ng/mL, using Tris-Buffer and 5 mL were used in 1st Step PCR, together with 5x HOT FIREPol® MultiPlex Mix (Solis BioDyne,

Estonia) and 4uM primer mix (fwd+rev) 515F/806R (Microsynth, Balgach, Switzerland). 1st Step PCR samples were purified with NGS Clean Beads (Labgene, Switzerland). Bead ratio was 1:1:2, Beads were washed with 75% ethanol, airdried and resuspended in Tris buffer. The 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%. Paired-end reads which 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. 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 contained ambiguous bases or were outliers regarding the amplicon size distribution were also discarded. Samples that resulted in less than 5000 merged reads were discarded, to avoid distortion of the statistical analysis. Remaining reads were denoised using the UNOISE algorithm implemented in USEARCH to form Amplicon Sequencing Variants (ASVs) 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 compared 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 consideration numbers 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.*, 2017).

Metabolic profiling using ^{1}H-NMR spectroscopy

For analysis urine samples were thawed, A phosphate buffer (pH 7·4 sodium phosphate with 0.2M disodium phosphate (Na_2HPO_4), 0.04M monosodium phosphate (NaH_2PO_4) in deuterium oxide (99·9 %) was prepared, with 1mM 3-(trimethylsilyl) propionic acid-d_4 sodium salt (TSP) and 3mM sodium azide in the solution. 400 µL of each urine sample were mixed with 200 µL buffer. 550 µL aliquots of supernatant were collected and dispensed into 5 mm NMR tubes.

^{1}H-NMR spectroscopy analysis was carried out using a Bruker Avance DRX 500 MHz NMR spectrometer (Bruker Biospin, Germany). The spectrometer was operated at 500.13 MHz. Urine water spectra were acquired using a standard 1D pulse sequence [recycle delay (RD)-90°-t1-90°-Tm-90°-acquire free induction decay (FID)] with water suppression applied during RD of 2 s, a mixing time Tm of 100ms and a 90° pulse set at 7.70 µs. Per spectrum, a total of 128 scans were carried out with a spectral width of 14.0019 ppm. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening. Acquired spectroscopic data were processed using the TopSpin 3.6.5 software package (Bruker Biospin, Rheinstetten, Germany). Data Processing was undertaken using the nPYc-Toolbox 1.2.7. Further details on the nPYC-Toolbox can be found at https://github.com/phenomecentre/nPYc-Toolbox

Chemometric analysis

Processed spectroscopic data were imported to the SIMCA 13.0 software package (Umetrics AB, Umeå, Sweden) to conduct unsupervised multivariate statistical analysis. Principal components analysis was used to evaluate similarities/differences in urinary metabolite composition between groups. The R^2 and Q^2 variables provided an indication of goodness of fit (R^2) as well as goodness of prediction (Q^2) of the models.

Ethics

The study was given favourable ethical consent by the University of Reading's Research Ethics Committee (36/2020). The trial was registered as a clinical trial (clinicaltrials.gov ID: NCT05581615) and conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent prior to study entry. There were no protocol changes once the trial commenced.

Sample size and statistical analysis

The primary outcome measure was bifidobacterial population as $\log_{10}$ cells/g wet faecal sample as measured by fluorescence *in situ* hybridisation. It was calculated that to detect a difference in *Bifidobacterium* populations between interventions, a total of 92 volunteers was required. This is based on an 80% probability that the study could detect a 0.5 $\log_{10}$ cells/g wet faecal sample difference in colonic bifidobacterial population at a two-sided 0.05 significance level based on the assumption of a standard deviation of 0.7 $\log_{10}$ cells/g wet faecal sample bifidobacteria.

Statistical Package for Social Science version 27 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Changes in bacteriology (FISH-FLOW, RMP and QMP), dietary data and bowel habit data were analysed using a linear marginal model (LMM) in order to assess both repeat measures (changes from baseline) and Day 10 group comparisons. Baseline values were included as a covariate to assess differences between groups. Participant metrics were assessed using a one-way ANOVA. All comparisons were corrected for type 1 errors using a Bonferroni adjustment within each LMM and ANOVA. Results are presented as mean and standard error (SE) unless otherwise stated. All tests were two tailed and *P* values ≤ 0.05 were considered statistically significant.

3. Results

Subject characteristics

110 subjects expressed interest in the trial with 100 potential subjects completing the screening visit. Of these, 14 did not meet the inclusion criteria, 96 eligible subjects were randomized ($n = 24$ per group) and included in the analysis for all primary and secondary outcomes (Figure 1). Baseline characteristics are reported in Table 3.

Figure 1. CONSORT diagram of participant flow through the trial

Table 3 reports the subject data (age, height, weight, and BMI) mean and range segregated by intervention. Average subject age was 37.89 y, weight 68.05 kg, height 169.08 cm and BMI 23.70 (kg/m^2). No significant differences were recorded between any of the groups.

Metric	Pure inulin (*n* =24)	Shortbread (*n* =24)	Milk Chocolate (*n* =24)	Rice Drink (*n* =24)	*P* (b)
Age (y)	39.46 (25-63)	34.46 (20-62)	38.29 (19-64)	39.33 (19-64)	*P* = 0.54
Weight(kg)	69.86 (50-110)	67.76 (51-105)	66.98 (53-86)	67.82 (45-98)	*P* = 0.89
Height (cm)	170.2 (157-193)	168.4 (152.4-189)	170.2 (155-193)	167.5 (147-195)	*P* = 0.73
BMI (kg/m²)	23.89 (18.37-30.37)	23.79 (19.57-30.79)	23.11 (19.71-28.72)	24.03 (18-29.9)	*P* = 0.74

Table 3: Subject data – age, weight, height, and BMI mean and SE segregated by intervention (*n* = 24 per group). *P* values are the results of using a one-way ANOVA to compare differences in categorical data.

Dietary intake

Nutrient data collected at Day 0 and Day 10 of the intervention are presented in Table 4. No significant differences were detected in total energy, protein, carbohydrates, total sugar, starch and PUFAs intakes (Table 4). Analysis of total fat revealed significant differences between interventions at day 10 ($P = 0.026$) with fat intakes in the milk chocolate intervention being significantly different from the rice drink intervention ($P = 0.019$). Repeated measure comparisons showed that total fat intake was significantly greater at Day 10 in the milk chocolate group only ($P = 0.042$). Finally, no significant differences in dietary fibre were detected between interventions at Day 10 (Table 4). Follow-up comparisons revealed that dietary fibre intake was significantly greater at Day 10 within each group (all $P \leq 0.001$) (Table 4).

	Pure Inulin (*n* =24)			Shortbread (*n* =24)			Milk Chocolate (*n* =24)			Rice Drink (*n* =24)			*P* (b)
	Day 0	**Day 10**	***P* (a)**	**Day 0**	**Day 10**	***P* (a)**	**Day 0**	**Day 10**	***P* (a)**	**Day 0**	**Day 10**	***P* (a)**	
Total energy (kcals)	2139 (156.60)	2056 (167.90)	0.58	2127 (149.40)	2302 (180.80)	0.25	2429 (168.20)	2570 (172)	0.35	1990 (135.70)	2083 (129.90)	0.53	0.552
Protein (g)	93.51 (6.98)	96.17 (5.9)	0.69	88.4 (8.07)	89.71 (8.40)	0.84	98.22 (6.76)	97.73 (6.4)	0.94	79.99 (6.91)	76.83 (6.82)	0.64	0.293
Fat (g)	88.04 (8.11)	84.31 (6.82)	0.59	87.48 (8.03)	81.98 (8.72)	0.59	98.92 (9.70)	113.2 (9.24)	**0.042**	83.38 (6.12)	79.27 (6.38)	0.55	**0.026**
PUFA (g)	16.44 (1.43)	15.09 (1.27)	0.38	15.86 (1.74)	15.41 (1.85)	0.77	17.98 (1.92)	18.52 (1.71)	0.72	14.50 (1.38)	14.73 (1.31)	0.88	0.499
CHO (g)	250.30 (18.51)	247.80 (25.59)	0.89	248.60 (22.59)	276.50 (19.77)	0.13	280.70 (18.34)	276.90 (17.33)	0.84	228.20 (16.59)	236.40 (17.06)	0.66	0.599
Starch (g)	130.90 (10.71)	127.50 (15.07)	0.74	133.80 (10.36)	143.30 (11.70)	0.37	147.80 (12.79)	138.20 (11.73)	0.33	122.20 (11.23)	132.50 (11.84)	0.33	0.616
Total sugar (g)	116.80 (10.91)	116.90 (15.36)	0.99	112.80 (13.82)	110.60 (11.11)	0.85	129.80 (12.44)	134.10 (9.48)	0.71	104.50 (10.62)	115.40 (10.18)	0.35	0.748
Fibre (g)	31.04 (2.09)	38.64 (2.11)	**≤ 0.001**	27.06 (2.38)	38.04 (2.51)	**≤ 0.001**	30.23 (2.18)	39.01 (39.01)	**≤ 0.001**	21.69 (21.69)	35.14 (1.76)	**≤ 0.001**	0.902

Table 4: Energy and nutrient intake at baseline (Day 0) and at completion (Day 10) of intervention study in 96 volunteers (*n* = 24 per group). Mean and standard error (SE). (a) *P* values are as a result of planned Day 0 vs Day 10 comparisons (grey columns). **(b)** *P* values are as a result of using Day 0 data as a baseline covariate for between group Day 10 comparisons (orange column). Keyword: CHO = Total carbohydrates; PUFA = Polyunsaturated fatty acids

Bacterial enumeration by FISH

96 subjects provided stool samples at baseline and end of the intervention. Figure 2 and Figure 3 report changes in bacterial counts observed in the four intervention groups between Day 0 and Day 10 of the intervention.

Figure 2A reports the changes seen in total bacteria counts (Eub I-II-III). Analysis revealed no significant differences between interventions at completion ($P = 0.315$). There was an average 0.07 $\log_{10}$ cells/g wet faeces increase in Eub I-II-III counts across all four interventions going from 9.74 to 9.81 (0.07) ± 0.025 (SE) $\log_{10}$ cells/g wet faeces. All values at end of intervention were significantly different compared to respective baseline samples (all $P \leq 0.05$) (Supplemental Data Table 1).

Similarly, regarding Bif164 (*Bifidobacterium* spp.) counts no significant differences were detected between interventions at Day 10 ($P = 0.641$). Repeated measures analysis revealed significant increases in Bif164 counts at Day 10 across all four interventions: average numbers increasing from 8.36 to 9.00 (mean difference 0.64) ± 0.05 (SE)) Log_{10} cells/g ($P \leq 0.001$) (Figure 2B).

Figure 2. Bacterial groups measured by FISH-FLOW (Log_{10} cells/g wet faeces) using probes: (A) total bacteria (Eub338 I-II-III), (B) *Bifidobacterium* spp. (Bif164). Box and whisker plot - min and max with all points. 96 volunteers ($n = 24$ per group). Results that are statistically significant within and between subject (intervention) are displayed by specified P values.

Bacteroides (Bac303) counts are reported in Figure 3A. Increases in Bac303 counts were observed across all four interventions, yet the extent of change varied greatly. Largest increases in numbers of Bac303 were observed in the shortbread intervention increasing from 8.06 to 8.31 (mean difference 0.25 ± 0.04 (SE)) $\log_{10}$ cells/g wet faeces (P = 0.002). Bac303 counts at the end of the interventions (Day 10) were not significantly different between interventions (P = 0.201) (Supplemental Data Table 1).

In contrast, significant differences in Rrec584 (*Roseburia/Eubacterium rectale*) counts were observed between interventions at Day 10 (P = 0.022). Subsequent analysis identified significantly greater increases in Rrec584 counts in the shortbread intervention compared to milk chocolate (P = 0.021). Significant increases from baseline in Rrec584 counts were only detected in the shortbread group going from 8.39-8.61 (mean difference 0.22 ± 0.07 (SE)) $\log_{10}$ cells/g wet faeces (P = 0.005) (Figure 3B).

Additionally, *Faecalibacterium prausnitzii* (Fprau655) (Figure 3C) counts differed significantly between interventions at Day 10 (P = 0.029), with increases in the shortbread intervention being significantly different from milk chocolate (P = 0.048). In Day 0 vs Day 10 comparisons the most noticeable changes in Fprau655 were recorded in both the shortbread and rice drink interventions with increases from 8.73 to 8.93 (0.20 mean difference ± 0.07 (SE)) $\log_{10}$ cells/g wet faeces (shortbread) and 8.77 to 8.84 (0.18 mean difference ± 0.08 (SE)) $\log_{10}$ cells/g wet faeces (rice drink). Both changes were statistically significant compared to respective Day 0 samples - shortbread (P = 0.004) and rice drink (P = 0.012) (Figure 3C).

Finally, no significant differences were observed in changes of numbers of *Clostridium coccoides-Eubacterium rectale* group (Erec458) or *Propionibacterium* (Pro853) either within or between intervention at completion (Supplemental Data Table 1).

Figure 3. Bacterial groups measured by FISH-FLOW (Log_{10} cells/g wet faeces) using probes: (A) most *Bacteroidaceae* and *Prevotellaceae* (Bac303), (B) *Roseburia* (Rrec584) and (C) *Faecalibacterium prausnitzii* (Fprau655). Box and whisker plot - min and max with all points. 96 volunteers (n = 24 per group). Results that are statistically significant within and between subject (intervention) are displayed by specified P values

Microbiota Profiling Analysis

Figure 4 reports 16S rRNA sequencing results for Relative Microbiome Profiling (RMP) along with Quantitative Microbiome Profiling (QMP) for *Bifidobacterium* data across all four interventions.

Figure 4. Relative Microbiome Profiling (RMP) (A) and Quantitative Microbiome Profiling data (QMP) (B) of *Bifidobacterium* 16SrRNA sequencing results. Mean and standard error (SE). 96 volunteers (n = 24 per group). Results that are statistically significant within and between subject (intervention) are displayed by specified P values.

Relative Microbiome Profiling (RMP)

There were no significant differences in phylum level abundances detected between interventions at Day 10 (Supplemental data Table 2) (all $P \geq 0.05$). At phylum level largest changes were documented in *Actinomycetota* (*Actinobacteria*), *post hoc* analysis documenting significant increases across all four interventions at Day 10: shortbread (P = 0.002), milk chocolate, pure inulin and rice drink (all $P \leq 0.001$) (Supplemental Data Table 2). Subsequently, there were also significant decreases detected in *Bacillota* (*Firmicutes*): milk chocolate (P = 0.002), and pure inulin, rice drink and shortbread (all $P \leq 0.001$). These changes coincided with those seen in *Bifidobacterium* at genus level.

Accordingly, no significant differences were detected at genus level in any bacterial group between interventions (all $P \geq 0.05$) (Supplemental Data Table 2). In line with phylum level, largest changes were recorded in *Bifidobacterium* with significant increases being detected across all four interventions averaging an 92% increase above baseline (all $P \leq 0.001$), (Figure 4A). In addition, while no differences were detected between interventions, several differences in bacterial taxa were documented within intervention including decreases in *Blautia* (pure inulin, shortbread and rice drink), *Clostridium* cluster IVXA + IVXB (pure inulin, milk chocolate and rice drink), *Dorea* (shortbread and rice drink), *Lactococcus* (shortbread), *Ruminococcus2* (milk chocolate), *Lachnospiraceae incertae sedi* (pure inulin and shortbread), *Ruminococcus* (pure inulin, shortbread and rice drink), and increases in *Prevotella* (milk chocolate) (Supplemental Data Table 2).

There were no significant differences in any measure of α-diversity detected between interventions at Day 10 (all $P \geq 0.05$). Several within group differences were detected with significant decreases in Shannon index in both the pure inulin (P = 0.003) and rice drink (P = 0.033) interventions. Trends towards reductions in both shortbread (P = 0.061) and milk chocolate interventions (P = 0.073) were noted. There was also a significant decrease in richness (no. of species) in both the pure inulin (P = 0.011) and rice drink interventions (P = 0.026). Simpson index was reduced in the pure inulin intervention (P = 0.011) (Supplemental Data Table 3).

Quantitative Microbiome Profiling (QMP)

Upon quantification of RMP data no significant differences were detected between groups at Day 10 (all $P \geq 0.05$) (Supplemental Data Table 4). As per RMP, largest increases at phylum level were documented in *Actinomycetota:* pure inulin and rice drink (both $P = 0.003$), milk chocolate ($P = 0.015$) and shortbread ($P = 0.001$). Significant decreases in *Bacillota* (*Firmicutes*) were documented in both the pure inulin ($P = 0.016$) and shortbread ($P \leq 0.001$) interventions, but not in the milk chocolate (all $P = 0.612$) or rice drink interventions (all $P = 0.514$).

Largest changes in microbial counts at genus level were detected in *Bifidobacterium*, *post hoc* analysis revealing significant increases across all four interventions: shortbread ($P \leq 0.001$), milk chocolate ($P = 0.036$), pure inulin ($P = 0.004$) and rice drink ($P = 0.011$) (Figure 4B). This mirrors the changes observed in RMP. Additionally, as per RMP there were a number, albeit fewer, changes in bacteria groups detected within each intervention. These included decreases in numbers of *Blautia* (pure inulin and shortbread), *Clostridium* cluster IVXA + IVXB (pure inulin), *Lachnospiraceae incertae sedi* (pure inulin and shortbread), *Collinsella* (pure inulin) and *Ruminococcus* (shortbread). Along with increases in *Prevotella* (milk chocolate) and *Roseburia* (shortbread) (Supplemental Data Table 4).

^{1}H-NMR spectroscopic profiles

Metabolic profiles of urine samples across the four intervention groups were analysed using unsupervised (PCA) methods (first two components), showing separation between the four interventions at completion ($R^2Cum = 0.18$, $Q^2Cum = 0.122$) (Figure 5). We did not observe any differences in ^{1}H-NMR metabolic profiles between interventions as points did not show any clustering or patterns in relation to intervention. As a result, no subsequent downstream analysis was carried out.

Figure 5. Urinary ^{1}H magnetic resonance (^{1}H-NMR) profiles across the four intervention groups. Unsupervised principal components analysis (PCA) scores plot of endpoint urine samples. $R^2Cum = 0.18$, $Q^2Cum = 0.122$. Key: IN = Pure inulin; MC = Milk chocolate; RD = Rice Drink; ST = Shortbread

Bowel habit and function

Changes in gastrointestinal symptoms (flatulence, intestinal bloating, abdominal pressure, abdominal pain and feeling of fullness) were self-recorded daily throughout the 10-day intervention and are reported as averages of Days 0-5 and Days 6-10. Scores of 0, 1, 2, and 3 corresponded to none, mild, moderate, and severe. Changes in stool consistency were measured as per Bristol Stool Form Scale and stool frequency are reported in Figure 6. There were no differences in flatulence, intestinal bloating, abdominal pressure, abdominal pain or feeling of fullness detected between interventions at completion (D6-10) (Supplemental Data

Table 5), although there was a trend towards significant differences in feeling of fullness (P = 0.058). This reflected the level of significance documented between the rice drink and pure inulin interventions at completion (P = 0.058). Repeated measures analysis revealed a significant decrease in feeling of fullness in the pure inulin intervention only (P = 0.002).

Stool consistency was significantly different between interventions (P = 0.017), with values documented in pure inulin being higher than in the rice milk intervention (P = 0.010). These results are in line with post hoc analysis revealing increases in stool consistency ratings were only detected in the pure inulin group (P = 0.009). Finally, there were no changes in stool frequency either within or between interventions although there was a trend towards increases in stool frequency identified in the pure inulin intervention (P = 0.080) (Figure 6 and Supplemental Data Table 5).

Figure 6. Stool consistency (Bristol Stool Form Scale, A) and stool frequency (B) at (Day 0-5) and again at Day 6-10 after intervention in 96 volunteers (n = 24 per group). Results that are statistically significant within and between subject (intervention) are displayed by specified P values.

Discussion

This is the first study to investigate whether the food matrix impacts on the prebiotic efficacy of ITF using a standardised protocol. In total 96 volunteers provided stool samples at baseline and end of the intervention. One of the main pre-requisites of a prebiotic is to stimulate beneficial changes in microbial composition in certain, but not limited number of bacteria (Gibson *et al.*, 2017). ITF prebiotics primarily target bifidobacteria as they possess the necessary glycosidases and transporters needed to degrade fructans and to assimilate low molecular weight carbohydrates (Falony *et al.*, 2009; Riviere *et al.*, 2018). In this study we used both targeted and untargeted analyses to determine the impact of the food matrix on the prebiotic efficacy of ITF.

In this study, we demonstrate, using both targeted and untargeted analysis, that, irrespective of the food matrix, the selectivity of ITF towards bifidobacteria appears to be unaffected. FISH-FLOW determined similar increases in Bif164 counts across all interventions averaging a 0.64 ± 0.10 Log_{10} Cells/g wet faeces at completion. These findings were further validated using untargeted analysis with an average 92% ± 5.43% (SE) and $1.14 \times 10^9 \pm 1.52 \times 10^8$ (SE) *Bifidobacterium* increase in RMP and QMP abundance respectively. This further confirms the selectivity of ITF towards *Bifidobacterium* (Costabile *et al.*, 2010; Gibson and Roberfroid, 1995; Kruse *et al.*, 1999). No significant differences were detected between interventions (all $P \geq 0.05$). These results are in line with those documented by several previous food-based ITF supplementation studies (Gibson *et al.*, 1995; Healey *et al.*, 2018; Marteau *et al.*, 2011; Ramnani *et al.*, 2010; Reimer *et al.*, 2020; Tuohy *et al.*, 2001). This does not, however, match those recorded by (Slavin and Feirtag, 2011) who documented that upon consumption of 20g/day of ITF supplemented into ice cream, no significant differences

583 in *Bifidobacterium* counts were detected. These differences likely result from subjectivity in
584 using plate counts, lack of a washout period and lack of collection of baseline stool samples
585 (Slavin and Feirtag, 2011).

586

587 Upon completion differences between the interventions in microbial load and composition
588 among the differing food matrices were detected. Using targeted FISH-FLOW analysis there
589 were significant increases in Bac303, Rrec584 and Fprau655 detected in the shortbread
590 intervention. In the rice drink intervention significant increases were seen in numbers of
591 FPrau655. The microbial loads (QMP) documented in both *Roseburia* and *Faecalibacterium*
592 *prausnitizii* were similar to those recorded by FLOW-FISH. The levels of *Roseburia* and
593 *Faecalibacterium prausnitizii* at completion of the shortbread intervention using FISH-
594 FLOW were significantly different from milk chocolate at Day 10 (both $P \leq 0.05$), but not
595 from pure inulin or rice milk (both $P \geq 0.05$).

596

597 These results are of interest because several previous food-based supplementation studies by
598 (Gibson *et al.*, 1995; Kleessen *et al.*, 2007; Tuohy *et al.*, 2001) either noted reductions or no
599 changes in numbers of *Bacteroides* upon consumption of ITF-fortified cereal bars and
600 biscuits. In contrast (Brighenti *et al.*, 1999) and (Rao, 2001) recorded 0.49 and 0.69
601 $\log_{10}$ CFU/g faeces dry weight increases in *Bacteroides* upon consumption of ITF containing
602 extruded ready-to-eat cereal and when pure ITF was supplemented into drinks. These
603 discrepancies probably occur due to the higher levels of *Bacteroides* present in the study
604 conducted by (Kleessen *et al.*, 2007; Tuohy *et al.*, 2001). It should be noted that different
605 analytical techniques were used (FISH-FLOW vs selective media) which directly impedes the
606 comparison and evaluation of results across such studies (Jackson *et al.*, 2022b).

607

608 Additionally, it is difficult to compare results of Rrec584 and FPrau655 to previous food-
609 based ITF supplementation studies due to most studies using targeted analysis not reporting
610 changes in both targeted groups. One food-based supplementation study that counted
611 Fprau655 using FISH-FLOW recorded no change in numbers upon consumption of fruit juice
612 drinks containing Jerusalem artichoke inulin (Ramnani *et al.*, 2010). A trend towards an
613 increase in relative abundances of *Faecalibacterium prausnitzii* was detected upon
614 consumption of pure ITF (Healey *et al.*, 2018).

615

616 *Bacteroides* possess a large number of loci responsible for the assimilation of complex
617 carbohydrates including arabinoxylans (Pereira *et al.*, 2021) as well as complex starches
618 (Dobranowski and Stintzi, 2021). Arabinoxylans are components of the wheat flour used in
619 production of the shortbread biscuits in this study. From this, one could speculate that the
620 significantly larger increases seen in *Roseburia* and *Faecalibacterium prausnitzii* in the
621 shortbread intervention resulted from the utilisation of resulting motifs from the breakdown
622 of arabinoxylans by *Bacteroides*. For example, it was previously demonstrated by (Walton *et*
623 *al.*, 2012) that, consumption of *in situ* produced arabinoxylan-oligosaccharides in bread,
624 resulted in significant increases in *Bacteroides*, *Roseburia* and *Faecalibacterium prausnitzii*

(all $P \leq 0.05$). However, it has also been demonstrated that upon consumption of 2 x 44 g bowls of wheat bran arabinoxylan-rich ready-to-eat cereal no changes in *Bacteroides*, *Roseburia* and *Faecalibacterium prausnitzii* could be detected (Maki *et al.*, 2012). Taking this into consideration, increases in both *Roseburia* and *Faecalibacterium prausnitzii* often coincide with increases in *Bifidobacterium* in *in vitro* studies likely as a result of cross-feeding on acetate and lactate (Kim *et al.*, 2020; Riviere *et al.*, 2016). From this, it could be hypothesised that increases in both *Roseburia* and *Faecalibacterium prausnitzii* in the shortbread intervention may have also occurred from both the utilisation of resulting breakdown arabinoxylan motifs by *Bacteroides* along with cross-feeding on acetate and lactate produced by *Bifidobacterium*.

It can be implied that complementary effects may exist from the presence of other bioactive compounds present within the matrices. For example it was demonstrated by (Ramnani *et al.*, 2010) that upon consumption of high polyphenol-containing fruit shots containing Jerusalem artichoke ITF, in addition to an increase of bifidobacteria, significant increases in *Lactobacillus/Enterococcus* group were detected ($P = 0.042$). Finding means to increase numbers of *Bacteroides, Roseburia* and *Faecalibacterium prausnitzii* alongside *Bifidobacterium* may be of clinical importance via the potential to increase butyrate production, given that butyrate plays a vital role as an energy source for colonocytes, in the regulation of tight cell junction integrity, and in the repair of the intestinal mucosa (Canani *et al.*, 2011). *Faecalibacterium prausnitzii* is considered to be a keystone species and has been associated with lowered risks of IBD and ulcerative colitis (Leylabadlo *et al.*, 2020). Overall, from the findings of this study we can conclude that the selectivity of ITF towards bifidobacteria is independent of the food matrix. Yet, the compositional makeup of the matrix may likely have important implications towards stimulating changes in the wider microbiota.

During the trial volunteers did not alter their diet or lifestyle, with exception of consumption of study product and adjustment of portion sizes to compensate for additional calories consumed. On average, fibre intakes were estimated at 27.5 g/day which is slightly below the current UK recommendations of 30 g/day as laid down by SACN (Scientific Advisory Committee on Nutrition, 2015). They do, however, far exceed those of the average population at just 14.9-18 g/day (Gressier and Frost, 2022; Scientific Advisory Committee on Nutrition, 2015).

Significant increases in dietary fibre intakes were detected across all four interventions (Table 4). Between baseline and completion there was an average increase of 10.2g fibre with an average 37.71 g/day of fibre being consumed by completion suggesting that the addition of 10 g/day of inulin into food products could help people reach or even exceed the daily minimum recommendation. Increasing fibre intake is the 1st line of treatment to improve bowel function. In order to assess changes in stool consistency the validated Bristol Stool Form Scale was used. However, despite an additional consumption of 10 g/day ITF significant changes in stool consistency were only detected in the pure inulin intervention at Day 10 ($P = 0.023$).

In our cohort no differences in stool frequency were detected and scores were stable throughout the intervention. Given that, in this study, volunteers started with higher daily stool frequency at baseline and that increases in stool frequency are often seen in subjects with low fibre intakes, the higher baseline fibre intakes seen in this study likely contributed towards a lack of change in stool frequency (Buddington *et al.*, 2017; François *et al.*, 2014; Grider and Piland, 2007; Isakov *et al.*, 2013; Micka *et al.*, 2017; Ramnani *et al.*, 2010; Slavin and Feirtag, 2011).

Gastrointestinal sensations including flatulence, intestinal bloating, abdominal pressure and abdominal pain were rated as none to mild and remained unchanged throughout the course of the intervention. No discomfort was reported and no discontinuation of the study by any volunteers was recorded. The only significant difference was a decrease in feeling of fullness in the pure inulin intervention ($P = 0.002$). This indicates that chicory inulin in both pure form and supplemented into differing matrices is well tolerated, but the food matrix may have implications regarding satiety. It has been documented that matrices higher in lipids and other non-digestible carbohydrates content such as the interventions used in this study can induce/sustain satiety by regulating smooth muscle stretch receptors and delaying gastric emptying (Aguilera, 2019).

Conclusion

In conclusion, we can confirm that irrespective of the food application and matrix, prebiotic ITF are selectively utilized and lead to specific changes in the gut microbiota. *Bifidobacterium* was the only genus consistently impacted by inulin-type fructans, yet the compositional make-up of food matrix may have implications regarding changes in the wider microbiota. For example, differences in several bacterial groups including *Roseburia* and *Faecalibacterium prausnitzii* were documented at the completion between the shortbread and milk chocolate interventions.

Supplementary material

Supplemental Data Table 1. Targeted microbial analysis vis fluorescence *in situ* hybridisation at Day 0 and Day 10 of intervention.

Supplemental Data Table 2. 16S rRNA relative microbial profiling data at Day 0 and Day 10 of intervention

Supplemental Data Table 3. Alpha diversity measures of 16S rRNA sequencing at Day 0 and Day 10 of intervention.

Supplemental Data Table 4. 16S rRNA quantitative microbiome profiling data at Day 0 and Day 10 of intervention.

Supplemental Data Table 5. Gastrointestinal sensation and bowel habit diary data displayed by day and intervention.

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Conflict of Interest

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

Data Sharing

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

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