University of Reading

An exploration of the prebiotic concept: from food matrix to mood state

Volume I: Effect of food matrix on the prebiotic efficacy of inulin-type fructans

A thesis submitted as a partial fulfillment for the degree of Doctor of Philosophy

Food and Nutritional Sciences

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged

Peter Jackson, 2023

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Table of Contents

General Abstract

Oligofructose and inulin are a class of prebiotics referred to as inulin-type fructans. Due to their physiochemical properties inulin-type fructans can function as fat and sugar replacers while still providing prebiotic dosages. Yet, can be subject to degradation depending on the processing parameters used during production. Additionally, one aspect frequently overlooked when designing prebiotic food-based supplementation studies is the presence of other bioactive compounds within the matrix including polyphenol and arabinoxylans, each of which can alter microbial composition. As a results, there is interest on whether the food matrix matters in the supplementation of inulin-type fructans and effects on microbial composition. To address this question we firstly summarised the literature regarding previous inulin-type fructan food-based supplementation studies. The findings suggesting that the bifidogenic effect of inulin-type fructans is unaltered as a result of the food matrix, but due to differences in study protocols no definitive conclusions can be made based on these findings. To test our working hypothesis that the bifidogenic effect of inulin-type fructans is unaltered as a result of the food matrix we conducted a 10-day 4-arm parallel, randomised, non-placebo-controlled non-inferiority trial following a standardised protocol. Similar increases in bifidobacteria were detected across all four interventions (all $P \leq 0.05$). Significant differences in *Roseburia* and *Faecalibacterium* were detected between shortbread, and milk chocolate interventions (all $P \leq 0.05$). Stool consistency was only significantly higher in the pure inulin intervention ($P \leq 0.05$). No differences in stool frequency or gastrointestinal sensations were detected between interventions with exception of feeling of fullness in pure inulin compared to the rice drink intervention ($P \leq 0.05$). In conclusion, the findings of this thesis suggest that irrespective of the

food matrix the bifidogenic effect of inulin-type fructans is unaltered. Yet, the compositional nature of the food matrix may have implications regarding stimulating changes in the wider microbiota.

List of Tables

List of Figures

List of Appendices

Abbreviations

3'SL	3'sialyllactose
6'SL	6'sialyllactose
6'SLN	6'sialyllactoseamine
ABC	ATP-binding cassette
ANOVA	Analysis of variance
BMI	Body mass index
BMO	Bovine milk oligosaccharides
CFU	Colony forming unit
CLA	Conjugated linoleic acid
DNA	Deoxyribonucleic acid
DP	Degree of Polymerisation
DSL	Disialyllactose
FFQ	Food Frequency Questionnaire
FID	Free induction decay
FISH	Fluorescence *in situ* hybridisation
FLOW	Flow cytometry
FSC-A	Forward scatter area
HD	Highly dispersible
HP	High performance
HPAEC-PAD	High performance anion exchange chromatography pulse amperometric detection

HSI	High soluble inulin
IQ	Instant quality
ITF	Inulin-type Fructans
LC	Long chain
NMR	Nuclear Magnetic Resonance
OF	Oligofructose
PBS	Phosphate buffered saline
PCR	Principal Component Analysis
PUFAs	Polyunsaturated fatty acids
QMP	Quantitative microbiome profiling
REDCap	Research Electronic Data Capture
RMP	Relative microbiome profiling
RPM	Revolutions per minute
SACN	Scientific Advisory Committee on Nutrition
scFOS	Short-chain fructooligosaccharides
SE	Standard error
SSC-A	Side scatter area
TEX	Texture

1.1 Study rationale and hypothesis

The effect of the food matrix on the prebiotic efficacy of inulin-type fructans (ITF) is of much interest amongst the scientific community. Yet, while previous supplementation studies suggest that the bifidogenic effect of the food matrix is unaltered as a result of the matrix, due to differences in study design and analytical techniques these conclusions cannot be made with any degree of certainty. This study aimed to investigate the influence that different food matrices had on the prebiotic efficacy of (ITF). The working hypothesis was that the bifidogenic effect of inulin-type fructans appears to be unaltered by the food matrix, but the presence other potentially prebiotic ingredients and components within the food matrix may alter selectivity towards the wider microbiota. Four different matrices were selected – pure inulin, milk chocolate, rice drink and shortbread biscuits not only reflecting a wide degree of matrices consumed as part of people's regular diets, but also representing some of the most common foods to undergo inulin fortification. In order to understand the effects that the food matrix has on the prebiotic efficacy of ITF a 10-day prospective non-placebo controlled randomised non-inferiority trial was conducted following a standardised protocol.

1.2 Literature review

Food for thought! Inulin-type fructans: Does the food matrix matter?

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Abstract

Food matrices can be described as the final composition of a food product which results from complex interactions between compounds found within different ingredients and the processing parameters used in production. These factors, not only impact on the final structure of a product, but also have the potential to alter both the structural integrity and bioavailability of potentially beneficial compounds present, for example, dietary fibres. As a result, there is growing curiosity amongst the scientific community on whether the food matrix may impact on the prebiotic efficacy of inulin-type fructans. Therefore, the purpose of this review is to explore previous food-based inulin-type fructan supplementation studies to determine whether the food matrix directly impacts on their prebiotic efficacy. Our working hypothesis is that other potentially prebiotic ingredients and components present within the food matrix may alter inulin-type fructans prebiotic effect.

1.2.1 Introduction

As diet is a key driver of gut fermentation and thus can strongly influence the composition and functionality of the gut microbiota, one way to modify the composition of the gut microbiota and potentially improve health outcomes is via the use of prebiotics, as they provide a safe, affordable and effective dietary approach (Sanders *et al.*, 2019). Under the latest definition prebiotics are categorised as a substrate that is selectively utilized by the host microorganisms conferring a health benefit (Gibson *et al.*, 2017). The most supported of all prebiotics are oligofructose (OF) and inulin, which belong to a class of non-digestible carbohydrates referred to as inulin-type fructans (ITF) (Karimi *et al.*, 2015).

ITF were first discovered over two centuries ago and are a natural component of several plant species including Jerusalem artichokes, bananas, garlic, leeks, dandelion and chicory amongst others (Roberfroid *et al.,* 2010). The amount and degree of polymerisation (DP) of ITF present vary significantly between species. For examples, wheat, bananas and onions possess short-chain ITF (max DP < 10). Jerusalem artichokes possess medium-chain ITF (max DP < 40) with globe artichokes and chicory root possessing long-chain ITF (max DP < 100) (Roberfroid *et al.,* 2010). In this regard, with exception of chicory root which possesses approximately 70% inulin on a dry weight basis, most of these fruits and vegetables only possess trace amounts of ITF and as a result isolation of supplemental ITF primarily focuses on chicory root (Mensink *et al.*, 2015).

From a chemical standpoint, in general, ITF are linear polydisperse carbohydrates composed of monomers of fructose linked by β-(2-1) glycosidic linkages. A starting *α*-D-glucose moiety may or may not be present (Roberfroid, 2007). Based on the DP ITF can be separated into oligofructose (OF) (DP 2-9) and inulin (DP 2-60+) (van Loo, 2006) (Figure 1.1).

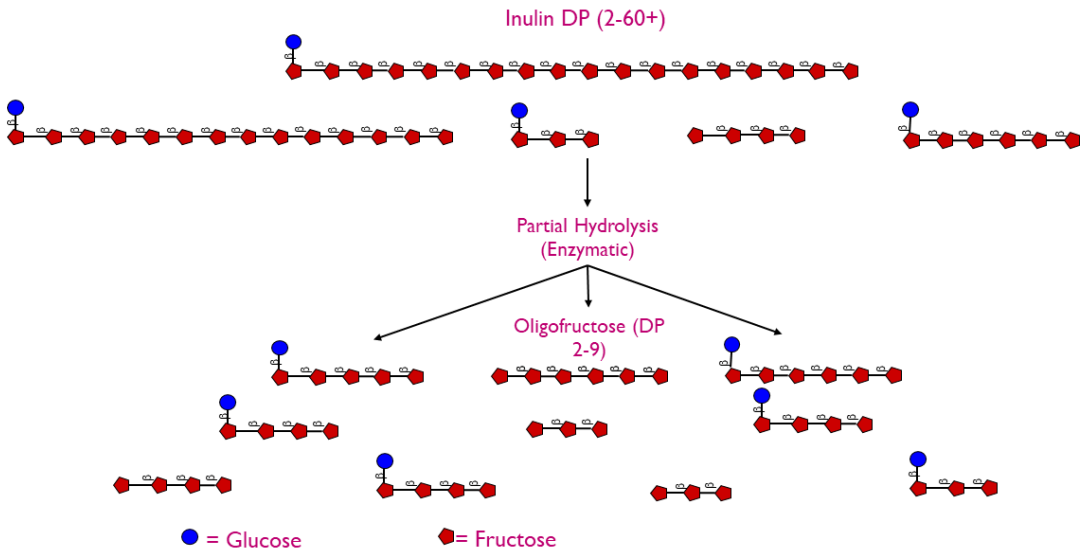

Figure 1.1. Structure of inulin type fructans

The ability of ITF to alter the composition of the gut microbiota and manipulate health parameters has been investigated extensively (Gibson and Roberfroid, 1995; Kleessen *et al.*, 2007; Marteau *et al.*, 2011; Ramnani *et al.*, 2010; Rao, 2001). These benefits include increasing and decreasing the numbers of beneficial and potentially harmful bacteria, for example stimulating the growth of bifidobacteria as they possess the necessary glycosidases to hydrolyse the β-(2-1) glycosidic (fructosyl-fructose) linkages (Falony *et al.*, 2009; Riviere *et al.*, 2018). This saccharolytic fermentation reduces the fermentation of undigested or endogenous proteins (Wang *et al.*, 2019; Wang *et al.*, 2020) and produces beneficial metabolites such as short-chain fatty acids, associated with increased satiety (Morrison and Preston, 2016), modifications in immune function (Delgado *et al.*, 2012) and improvements in bowel transit function (Buddington *et al.*, 2017) amongst others.

The concept that the food matrices may impact on the prebiotic efficacy of ITF has become of increasing interest amongst the scientific community in recent years. This is due in part 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). Yet, while several studies have utilised various food products such as biscuits, yoghurt, stewed apple, cereal bars, cocoa drinks and fruit juices as vehicles for ITF supplementation (Brighenti *et al.*, 1999; Gibson *et al.*, 1995; Kleessen *et al.*, 2007; Ramnani *et al.*, 2010; Rao, 2001; Slavin and Feirtag, 2011) none of these were specifically designed to determine the effects that the food matrices had on the prebiotic efficacy of ITF. Furthermore, the food products used in these studies can also be sources of several other potential prebiotic candidates including phenolic acids, β-glucan, arabinoxylans and bovine milk oligosaccharides. Many of these possess the potential to alter microbial selectivity and is an aspect often overlooked by researchers when considering study designs regarding food-based prebiotic supplementation studies.

The only study to date to consider whether the matrix altered the prebiotic efficacy of ITF (Kleessen *et al.,* 2007). The results suggested that the prebiotic (bifidogenic) efficacy of ITF was unaltered as a result. Therefore, this leads to the question of whether food matrices matter in the supplementation of ITF. This question is becoming increasingly important to answer given the interest in the addition of ITF into various foods products which are supposed to be beneficial for health. Thus, the purpose of the remainder of this review is to explore previous food-based ITF intervention studies aiming to conclude whether the matrix likely impacts on the prebiotic efficacy of ITF. Figure 1.2 presents a theoretical overview of how the food matrix may potentially impact on the prebiotic efficacy of ITF.

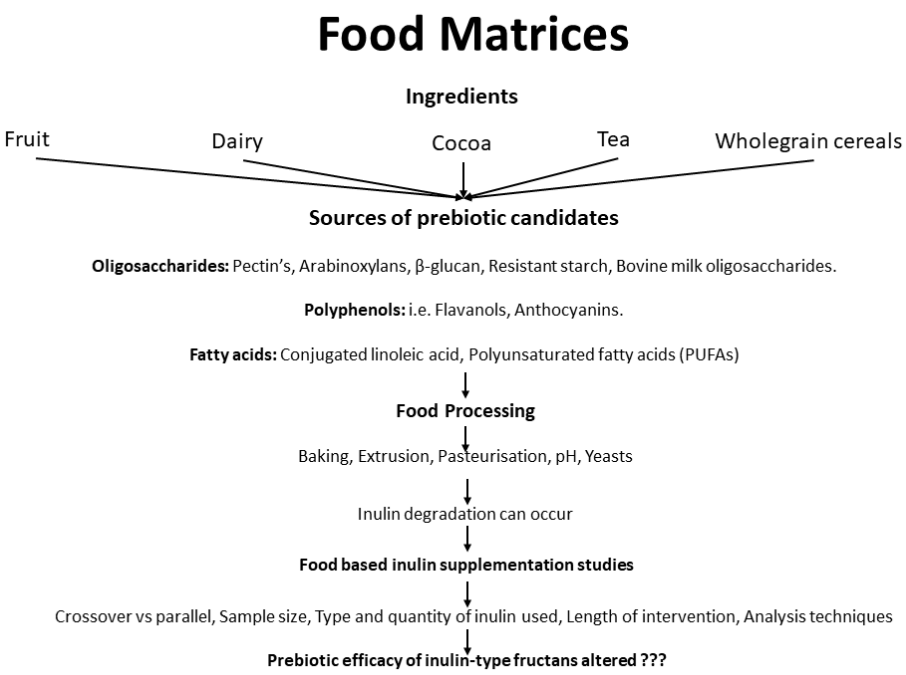

Figure 1.2. Overview on how the food matrix may impact on the prebiotic efficacy of inulin-type fructans

1.2.1.1 The Food Matrix effect: What are food matrices?

The potential importance of the food matrix has received much attention as the composition of the matrix can directly influence the bioavailability of nutrients due to its effect on the digestion process. For example, during digestion the bioavailability of phenolic compounds, mineral and fats in the gastrointestinal tract can be directly influenced by the presence/absence and combination of carbohydrates, proteins, fats, calcium and minerals (Palafox-Carlos, Ayala-Zavala and Gonzalez-Aguilar, 2011; Thorning *et al.*, 2017; Zeng *et al.*, 2016). Given the strong structure-function relationship existing between dietary fibre and its behaviour during digestion, one must comprehend this relationship in order to recognise the potential effects this may have on physiological outcomes (Capuano, 2017). For example, there is evidence existing that the presence of high levels of dietary fibre present within the matrix can directly influence the absorption of the afore mentioned compounds, via the sequestering of ions and binding of phenolic compounds (Capuano, 2017; D'Archivio *et al.*,

2010; Palafox-Carlos, Ayala-Zavala and Gonzalez-Aguilar, 2011). This concept also applying to the microbial fermentation of unabsorbed compounds and the absorption of resulting metabolites within the colon (Aguilera, 2019).

Food matrices at face value can be crudely classified into several basic types: liquid, emulsions, gels, viscoelastic, dense, and porous (Aguilera, 2019) amongst others, and represent a wide variety of different products including yoghurt, cakes, sauces, soups, biscuits, ready-to-eat breakfast cereals and snacks, orange and other fruit juices with several food products consisting of more than one matrix. Additionally, how these food products are produced can not only modify the structure of the matrix (i.e. viscosity, porosity, density etc) but when considered alongside other processing factors including, milling, grinding, pH, pressure and temperature, have the potential to directly influence the physicochemical (particle size, intactness, exposedness) properties of dietary fibres (Duar *et al.*, 2015; Klewicki, 2007; Poinot *et al.*, 2010). Furthermore, one must consider other biological ingredients present within the food matrices, including phenolic compounds, dietary fats and other fermentable dietary carbohydrates that can also directly influence the composition of the gut microbiota. As a result, it is increasingly difficult to predict the behaviour of dietary fibre and their physiological effects when administered in solutions (beverages) or a solid food matrix, even those of the same class and compositional nature (Capuano, 2017). It is for these reasons that food matrices might have effects on the prebiotic efficacy of ITF.

1.2.1.2 Effects of food processing

The physiochemical properties (compositional nature) of ITF are the hallmark of the prebiotic efficacy of ITF. Given that ITF can also function as both fat and sugar replacers as well as texture modifiers and there has been much interest in the addition of ITF into several different

food products. in order to help reduce not only the consumption of both saturated fat and sugar (sucrose) but also increase people's dietary fibre intakes (Shoaib *et al.*, 2016). Yet, ITF can be subject to structural degradation when exposed to specific processing conditions.

One examples of this is low pH with the critical cut-off point appearing ≤ 4 (often seen in fruit juice production) where at below this pH hydrolysis begins to occur due to protonation of the glycosidic bond, potentially resulting in a loss of functional properties (Glibowski and Wasko, 2008; Mensink *et al.*, 2015). The sensitivity of ITF to low pH only heightens with increasing time and temperature. For instance, during pasteurisation it has been demonstrated that between 70-87% of OF was degraded under two-stage processing of an apple and blackcurrant juice drinks (Klewicki, 2007). Yet, the same authors reported that under less extreme pasteurisation parameters (pH 4.2; 95 °C 30 sec), 80% of OF survived the pasteurisation process. A finding similar to those reported by both Duar *et al.* (2015) and (Glibowski *et al.*, 2020) who noted that OF, native and HP inulin were stable at pH 4 and pH 3 in a model and apple juice drink respectively implying when both pH and time are considered little-to-no breakdown of ITF occurs.

In addition to pasteurisation, other heating processes including baking also have the potential to alter the structural integrity of ITF. One illustration of this comes from Poinot *et al.*, (2010) who demonstrated that bread containing 5% ITF darkened 3 mins quicker than bread containing no ITF. Several other studies have reported that the addition of ITF in baked products altered the colour of the final product suggesting that structural degradation of ITF may have occurred (Zahn, Pepke and Rohm, 2010; Rodriguez-Garcia *et al.*, 2012). The potential for ITF to alter the browning of a product is suggested to be down to the ability of ITF to participate in the Maillard and caramelisation reactions due to the presence of reducing groups (Mensink *et al.*, 2015). Under this premise, there are multiple pathways by which ITF

can participate in the Maillard and caramelisation reactions. Firstly, via direct participation to due to availability of free reducing ends and secondly due to presence of invertase/inulinase which can specifically target and hydrolyse the β-(2-1) glycosidic (fructosyl-fructose) linkages where 80-90% of non-ITF/ITF can be degraded (Morreale, Benavent-Gil and Rosell, 2019; Verspreet *et al.*, 2013) producing more reducing groups. However, to what extent the participation of ITFs in either/both the Maillard and caramelisation reactions has on the bifidogenic properties of ITF is not well understood.

Furthermore, it has been reported that when ITF were added into ready-to-eat breakfast cereals at temperatures including 140 °C at a screw speed of 170 RPM, more than 50% OF was degraded. LC-ITF appeared to be unaffected by low temperature (120 °C) extrusion, however, when screw speeds were adjusted to 120 and 170 rpm only 25% and 34% of LC-ITF were recovered. Low levels of inulin were recovered (35%) when temperatures and screw speeds were operated at their most extreme conditions: temperature (170°C) and pressure (170 rpm) respectively (Duar *et al.,* 2015).

Overall, these results imply that the time, temperature, and DP of ITF used during the production process appears to be critical if the potential degradation of ITF is to be avoided, with each of these aspects needing to be carefully considered in order to optimise product quality while maintaining ITF integrity.

1.2.1.3 Ingredient-ingredient interaction – food for thought?

One aspect that is frequently overlooked when designing food-based inulin supplementation studies is the effects that other potential compounds present within the food matrix may have in altering the prebiotic efficacy of ITF. For example in the production of food products,

including baked goods (cakes, biscuits and bread), dairy products (yoghurt and ice cream) and fruit juices (orange and apple) several ingredients including numerous types of wholegrain flours or cereals (wheat, spelt and barley), dairy ingredients including milk and skimmed milk powder, as well as cocoa powder and fresh and/or dried fruit and vegetables are often combined to produce a final product (Brighenti *et al.*, 1999; Kleessen *et al.*, 2007; Menne, Guggenbuhl and Roberfroid, 2000) with each of these ingredients being a potential source of microbially active compounds. Examples of this include cranberries, apricots, lemons and oranges which are known sources of fermentable polysaccharides and polyphenols, including pectins and hesperidin (Sadler *et al.*, 2019; Sanchez-Patan *et al.*, 2015). Pectins have been shown to increase the levels of bifidobacteria, *Lactobacillus*, some strains of *Bacteroides*, *Enterococcus*, *Prevotella,* and *F. prausnitzii*. The ability of pectins to stimulate changes in microbial composition appear to be structure and microbiome composition dependant (Gomez *et al.*, 2016; Larsen *et al.*, 2019). While polyphenols found in black tea have been shown to increase levels of *Bacteroidetes* (Kemperman *et al.*, 2013), the flavanols present in cocoa have been shown to increase levels of both bifidobacteria and lactobacilli *in vivo* (Tzounis *et al.*, 2011). Yet, while the research on the ability of both pectins and polyphenols to have beneficial effects on the composition of the gut microbiota is building, it is still in its infancy with many of the mechanisms of gut microbiota modulation, particularly by polyphenols, not being well understood (Scott *et al.*, 2019).

Wholegrain cereals, including wheat, rye, spelt and barley, are desirable sources of numerous fermentable carbohydrates namely non-ITF, arabinoxylans and β-glucans (Knudsen, 2015; Maccaferri *et al.*, 2012) for which a body of evidence on prebiotic efficacy is rapidly growing. Bifidobacteria and potentially *Bacteroides* and lactobacilli appear to be main bacteria to benefit from the fermentation of these dietary fibres (Costabile *et al.*, 2008; Knudsen, 2015; Scott *et al.*, 2019; Valeur *et al.*, 2016; Walker *et al.*, 2011).

Another common food ingredient is bovine milk (a common constituent of mousses, ice cream and milk drinks) and is a source of bovine milk oligosaccharides (BMO) with 10 of these BMO being identical to those found in human breast milk (Kirmiz *et al.*, 2018; Zivkovic and Barile, 2011). However, compared to human breast milk, the oligosaccharide content of bovine milk is nearly 10-times less at approximately 100 mg/L (Robinson, 2019) with the predominant oligosaccharides being 3'sialyllactose (3'SL), 6-sialyllactose (6'SL), 6'-sialyllactosamine (6'SLN), and disialyllactose (DSL). Together these make up the majority at 60-94 µg/mL to 347-460 µg/mL (Fong, Ma and McJarrow, 2011).

Whey permeates, a by-product of cheese making and skimmed milk powder production, are becoming an increasingly common ingredients used in baked goods, meats, soups and confectionary (Krolczyk *et al.*, 2016; Smith, Metzger and Drake, 2016). As a result, the bovine milk oligosaccharide concentration in whey permeate and skimmed milk powder is likely to be higher than in milk. However, what these increases in BMO mean regarding alterations in microbial composition has yet to be determined. Moreover, dairy products including milk, cheese and yoghurt are also a source of conjugated linoleic acid, an acid produced as a result of microbial fermentation in the rumen (Devillard *et al.*, 2007). Similar to BMO, the conjugated linoleic acid concentration (CLA) in bovine milk is low at 0.55 to 1.53 g/CLA 100g but may be increased as a result of yoghurt and cheese production (Prandini *et al.*, 2007) and has been shown to decrease proportions of *Firmicutes* (P = 0·003) and increase proportions of *Bacteroidetes* (P = 0·027) in mice respectively (Marques *et al.*, 2015).

However, it can be speculated that several of these potential prebiotic candidates present within various food ingredients may potentially interfere with the prebiotic efficacy of ITF. Due to differences in growing conditions and location, time of the year and the species used in production (Huynh *et al.*, 2008; Marcotuli *et al.*, 2016; Robinson, 2019) there can be significant

variations in the concentration of each of these potentially beneficial compounds present within the final product. Thus, unless each ingredient is measured and standardised for levels of potential prebiotic candidates prior to production, the role each of these potentially bioactive compounds play in altering the composition of the gut microbiota may never be fully known.

1.3 Inulin-type fructan food supplementation studies

1.3.1 Baked goods

1.3.1.1 Cereal bars

One of the only studies to date to question whether the food matrices and the processing methods used altered the prebiotic efficacy of ITF was undertaken by (Kleessen *et al.*, 2007). In this study commercial inulin from chicory (Fibruline® Instant) and Jerusalem artichoke inulin were fortified into snack bars produced from purely vegetable ingredients (not identified) at 7·7 g/per bar. A non-inulin containing cereal bar was used as a control. The control bar contained several wholegrain cereals, dried fruits and fruit juice concentrates. The study design was a randomized, double-blind, placebo-controlled study with parallel groups that included forty-five healthy volunteers. After a one-week run-in period, subjects were randomly assigned into one of three groups (control; chicory inulin bar; Jerusalem artichoke inulin bar). Bars were consumed for 3-weeks once per day during week 1, and twice per week during week 2 and 3. Faecal samples were collected after the run-in period had been completed and then again at day 14, 21 and 28. Changes in microbial composition were determined by fluorescence *in situ* hybridization combined with selective media techniques to determine changes in some less abundant microbial groups/species. Differences in fructans

before and after processing were determined via high-performance anion exchange chromatography-pulsed amperometric detection (HPAE-PAD).

The authors reported that after consumption of either the chicory or Jerusalem artichoke inulin bar, total numbers of faecal bacteria remained constant; while there was a steady increase in bifidobacteria by approximately 1·2 $\log_{10}$ CFU/g faeces (wet weight) after 3 weeks and was significantly different from the placebo group ($P \leq 0{\cdot}05$). Both chicory and artichoke bars also reported lower levels of *Bacteroides*/*Prevotella* compared to the placebo group ($P \leq 0{\cdot}05$). Along with an approximately 0·6 $\log_{10}$ CFU/g faeces (wet weight) fewer numbers of *Clostridium coccoides*/*Eubacterium rectale* group at the end of the intervention period (Day 28). Unfortunately, due to the lack of detail over the ingredients of the intervention bars, it is difficult to put these data into the context of the food matrix. Furthermore, no structural differences were detected in either chicory or Jerusalem artichoke inulin before and after processing, confirming their stability during processing, which is the prerequisite for ITF to exert a prebiotic effect.

The addition of ITF into snacks bars was also undertaken by (Reimer *et al.*, 2020). The study design was a single-centre, placebo-controlled, double-blind, crossover study with a 4-wk washout period involving fifty healthy adults. Subjects were randomly assigned to one of two trials: Trial 1—Moderate Dose ITF (7 g/d) snack bar and Control 1 snack bar; or Trial 2—Low Dose ITF (3 g/d) snack bar and Control 2 snack bar with the composition of the Intervention and control block varying between Trial 1 and Trial 2. Subjects were instructed to consume 1 bar/d for 4 wk in each treatment arm. The total duration of the study including the 4wk washout period was 12 weeks. Each subject provided 10 stool samples: 1 at baseline and 1 for every week of the trial with changes in faecal microbiota composition being determined using 16S ribosomal RNA–based approaches. The results of this study indicated that, compared to

the control group, the moderate dose group showed significant differences across multiple microbial taxa with most notable increases being detected in *Bifidobacterium* (mean ± SEM) 5.3% ± 5.9% to 18.7% ± 15.0% over the 4wk period. With the low-dose ITF snack bar significant increases in *Bifidobacterium* were no longer present after correction for multiple comparison (*P* = 0.55). However, targeted analysis with qPCR showed significant increases in relative abundance of *Bifidobacterium* for the low bar dose at week 2 (*P* = 0.027) and a trend toward an increase at week 4 (*P* = 0.056) compared with the control 2 bar.

1.3.1.2 Biscuits

One of the first studies to utilise food products as means of ITF supplementation was undertaken by (Gibson *et al.*, 1995). Under the premise of this study, eight healthy adults were fed an initial control diet for 15 days consuming 15 g/day sucrose daily, followed by another 15-days in which 15 g sugar was replaced with OF, followed by further 15 days on the sucrose control diet. Additionally, four adults went on to complete a further 25-days study, comprising the same control sucrose diet for 10-days, with sucrose then being substituted for 15 g/day inulin for a further 15 days. 5 g of the OF and inulin were consumed as a supplement with the remaining 10 g being incorporated into biscuits. Stool samples were collected three times during the last three days of each dietary period. Changes in total anaerobes, total aerobes, coliforms, Gram-positive cocci, bifidobacteria, *Bacteroides*, fusobacteria, lactobacilli, and clostridia were analysed via selective media techniques. The results of this study indicated that while supplementation had little to no effect on the total viable counts of aerobes or anaerobes, both OF and inulin increased bifidobacterial counts by 0.7-0.9 $\log_{10}$/g faeces (wet weight).

Tuohy *et al.*, (2001) also used biscuits as means of ITF supplementation but with a larger sample size of thirty-one participants in total. OF was not the only dietary fibre used in this study as partially hydrolysed guar gum was utilised alongside OF. Each subject consumed 6.6 and 3.4 g/day of OF and partially hydrolysed guar gum fortified into three biscuits resulting in a total biscuit consumption of up to 37.5 g/day. Volunteers consumed the experimental biscuits for one 21-day period and then the placebo biscuits for a second 21-day period. Changes in faecal microbial composition were analysed by fluorescence *in situ* hybridisation. The authors of this study reported that the consumption of oligofructose and partially hydrolysed guar gum resulted in a 0.487 $\log_{10}$ cfu cells/g faeces increase in *Bifidobacterium* spp., suggesting that the biscuit matrix does not impact on the bifidogenic effect of inulin-type fructans. The authors also noted there were little-to-no changes in numbers of total bacteria, *Bacteroides* spp., *Clostridium* spp. or *Lactobacillus*/*Enterococcus* spp.

1.3.1.3 Extruded ready to eat snacks

Ready-to-eat breakfast cereals and snacks represent a class of matrices referred to as porous and are composed of mixtures of wholegrains or slurries of grains, sugar and water, extruded under high pressure and temperature conditions and with varying degrees of shear (Peressini *et al.*, 2015; Sacchetti, Pittia and Pinnavaia, 2005; Tsokolar-Tsikopoulos, Katsavou and Krokida, 2015). As the extrusion process allows manufacturers to fortify food products with vitamins and minerals, which may be lost in other parts of the production process, it should come as no surprise that the addition of ITF to extruded food products has been extensively studied (Brennan, Monro and Brennan, 2008; Capriles *et al.*, 2009; Tsokolar-Tsikopoulos, Katsavou and Krokida, 2015). Yet to date, only one study has aimed to determine the effects of addition of ITF to extruded ready-to-eat breakfast cereals on the composition of the gut microbiota

(Brighenti *et al.,* 1999). In this study, inulin (Fibruline® Instant) with an average DP of 7 with 30% of inulin present possessing a DP > 30 was, incorporated at 18% (dry weight) into a test cereal, prepared by puffing a dough consisting of rice flour, salt, sucrose, maltodextrin, and water. Participants consumed 50 g of a rice-based ready-to-eat cereal (placebo), then the same cereal containing 18% inulin (test) in substitution of their regular breakfast. After which, they then returned to the regular habitual diet (wash-out). They followed no other dietary restrictions. Stool samples were collected at baseline and on the fourth day of the last week of the intervention period, with changes in microbial composition determined by selective media techniques (total facultative anaerobes on Difco Tryptic Soy agar, bifidobacteria on NPNL-agar, Bacteroidaceae on kanamycin-vancomycin blood, clostridia on sulfite-polymyxin-milk and coliforms on Difco levine-eosine-methylene-blue agar).

The authors noted that although not significant compared to the basal numbers, upon consumption of the placebo there was a 0.49 and 1.47 $\log_{10}$ CFU/g faeces dry weight increase in *Bacteroides* and clostridia. As well as a 0.08 $\log_{10}$ CFU/g faeces decrease in bifidobacteria. The increases seen in *Bacteroides* in this study could have occurred due to the formation of novel carbohydrate complexes as a result of the extrusion process. Given *Bacteroides* possess the widest array of loci able to target dozens of highly complex glycans (Flint *et al.*, 2012). Yet, upon consumption of the inulin test breakfast cereal numbers of bifidobacteria were significantly higher at the end of the test period compared to the placebo ($P \leq 0{\cdot}05$). Numbers *of Bacteroides* decreased returning close to basal levels. This suggesting that bifidobacteria in the presence of inulin are able to outcompete *Bacteroides* for substrates becoming the dominant genus. This further indicated the high level of selectivity of ITF toward bifidobacteria. Yet, the increase in bifidobacteria seen in this study was relatively small at just 0.33 $\log_{10}$ CFU/g faeces dry weight. The smaller than expected response likely occurring from the higher levels of bifidobacteria present in baseline stool samples. Although one must also

consider that the ITF used in this study possessed an average DP of 7 with < 30% of the ITF possessing a DP > 30, it could have undergone a substantial amount of degradation during the extrusion process (Duar *et al.,* 2015; Tsokolar-Tsikopoulos *et al.*, 2015). This may have also contributed towards the low bifidogenic response. However, as the authors did not analyse carbohydrate structure prior to or post extrusion this cannot be determined with any real degree of certainty. This being an area of much needed research if the functional effects of food processing on dietary fibre behaviour during digestion and the gut microbiota are to be fully understood (Capuano, 2017).

1.3.2 Dairy

1.3.2.1 Cheese

To date, only one study has investigated if the addition of ITF to cheese was able to stimulate changes in microbial composition. In this study, the ability of Swiss cheese containing Beneo Orafti® ST and Orafti® P95 to alter the composition of the gut microbiota was investigated using pH-controlled *in vitro* fermentation experiments in which changes to microbial composition were analysed via fluorescence *in situ* hybridisation (Cardarelli *et al.*, 2007).

The results showed petit Swiss cheese containing a mixture of both Orafti® ST and P95 was able to act as substrates for faecal bacteria. However, rates of fermentation appeared to decline after 6 hours. Furthermore, rates of substrate breakdown were lower than expected likely due to the concentration of ITF used. This is because the majority of *in vitro* studies involve the inoculation of faecal samples with substrate concentrations in the region of 1% ITF (w/v) (Saman *et al.*, 2017; Wang *et al.*, 2019). In contrast, the cheeses used in this study contained a substrate concentration equating to 0.25 % (w/v) at most, suggesting there was likely not enough substrate present to sustain microbial growth. Yet these results do

demonstrate the ability of the addition of ITF to cheese to modify the composition of the gut microbiota suggesting ITF-fortified cheese could be the subject of future human intervention ITF-supplementation studies once product formulation has been optimised.

1.3.2.2 Yoghurt

While biscuits, cereal bars, ready-to-eats and Swiss cheese represent more solid and porous matrices, other dairy products such as yoghurt and ice cream have also been used in supplementation studies involving ITF and represent that middle ground between liquid and solid. In one study (Kruse, Kleessen and Blaut, 1999) investigated using inulin as a replacement for dietary fat in 11 healthy adults. The quantity of ITF FIBRULINE® (DP 2-50 (average DP 9) consumed was based on individual energy requirements and resulted in an inulin intake of up to 34 g/day and was incorporated into commercial yoghurt which was consumed for 64-days. Stool samples were collected at day 8, 27 and 62 of the intervention as well as 34-days following the end of the intervention. Changes in microbial composition were measured by fluorescence *in situ* hybridisation. The authors reported that the consumption of ITF-supplemented yoghurt resulted in an approximate 1 $\log_{10}$/g dry faeces increase in bifidobacteria compared with the control group with numbers returning close to baseline 34 days after ceasing inulin consumption. However, the limitations with this study include that levels of ITF consumed by volunteers were based on each individual's energy needs, thus drawing any conclusion on whether specific dosages of ITF are required to impact on changes in microbial composition cannot be drawn. As well as only analysing changes in bifidobacteria.

The only other study to date to utilise yoghurt as means of ITF supplementation was undertaken (Marteau *et al.*, 2011) with the authors using stewed apple and pear as a means of ITF supplementation. The study design was a double-blind placebo controlled trial in which

native chicory inulin supplemented at 15 g/day (2x7.5 g/sachets) was consumed in either yoghurt or stewed apple or pear. Stool samples were collected the day before Day 0 (V1), Day 14 (V2), and Day 28 (V3) with changes in bifidobacteria being analysed by quantitative polymerase chain reaction (qPCR). The results of this study showed that the consumption of inulin in either yoghurt or stewed apple/pear increased faecal bifidobacteria counts by 0.6 $\log_{10}$/g faeces between Day 0 (V1) and (Day 28) V3 ($P \leq 0.01$) and were significantly higher compared to the control ($P \leq 0.001$). Yet, no significant differences were detected in bifidobacteria between the placebo and the inulin intervention at Day 0 (V1) and Day 14 (V2). However, the authors did not monitor changes in any other microbial groups. Along with not stratifying results into participants who consumed yoghurt and those who consumed the stewed apple and pear. Thus, it cannot be determined whether changes in numbers of bifidobacteria or other microbial genera varied between the two different means of supplementation.

1.3.2.3 Ice cream

To date, only one study has aimed to determine the effects that the addition of chicory inulin to low-fat ice cream had on stool weight, transit time and gut microbiota composition (Slavin and Feirtag, 2011). The study design was a double-blind randomised-controlled crossover trial with the control group consuming a low-fat ice cream (no inulin) while the intervention group consumed low-fat ice cream containing 20 g inulin. Ice creams were consumed for 21-days after which ice creams were swapped and consumed for a further 21-days. Stool samples were collected on day 16 and 37 of the trial. From the results, the authors noted that despite consuming 20 g chicory inulin/day, *Bifidobacterium* spp. increases in the intervention phase were not statistically significant compared to the control phase (0.44 $\log_{10}$ CFU/g faeces) (*P*

= 0.33) with only the number of *Lactobacillus* spp. recording a statistically significant increase throughout the course of the intervention ($P \leq 0.05$).

Regarding changes in microbial composition, these results are unusual given the known response of bifidobacteria to ITF (Roberfroid *et al.*, 2010). There are several reasons these results may have occurred. Firstly, the sample size was small ($n = 12$) with it being well established that there is a high degree of variability in gut microbial composition from person-to-person (Ames *et al.*, 2017), hence a greater number of participants may have been needed to achieve statistical significance. Secondly, analysis techniques used to determine changes in microbiota composition may have resulted in the generation of false results likely due to the lower sensitivity of the spread plates being used to determine changes in bacterial counts as well as subjectivity in the scoring of *Bifidobacterium* spp. Thirdly, no baseline stool sample was taken, and finally and critically no washout period was undertaken between treatment periods which likely confounded results due to a lack of a structural re-shift in the composition of the gut microbiota between stage 1 and stage 2 of the intervention (McBurney *et al.*, 2019).

1.3.3 Juices and drinks

Regarding liquid matrices, ITF supplementation studies have been conducted in a diverse variety of different drinks including fruit juices and cocoa drinks. The addition of ITF from Jerusalem artichokes to fruit and vegetable juice shots and their effects on microbial composition was investigated by (Ramnani *et al.*, 2010). The study design was a double-blind, randomised control trial in which Jerusalem artichoke ITF were incorporated into two different fruit juices: pear-carrot-sea buckthorn and plum-pear-beetroot. Each shot contained 2.5 g of Jerusalem artichoke ITF and was consumed twice a day over 3-weeks, followed by a 3-week washout period. Fruit and vegetable juice shots containing ITF were compared against a

water-based control containing blood orange, carrot and raspberry extracts and flavours but no ITF, with changes in faecal bacteria analysed by fluorescence *in situ* hybridisation. The authors noted that after 21 days of ITF juice supplementation, there was a significant increase in bifidobacteria of between 0.5-0.7 $\log_{10}$ cells/g faeces ($P \leq 0{\cdot}0001$) along with a smaller yet still significant increase in *Lactobacillus*/*Enterococcus* groups of 0.2 $\log_{10}$ cells/g faeces recorded in both ITF/fruit juice interventions ($P = 0{\cdot}042$). This potentially implies that the presence of polyphenols in fruit juices may have aided in the selective stimulation of *Lactobacillus*/*Enterococcus*. Given that it has been previously demonstrated, both *in vitro* and *in vivo*, that *Lactobacillus* are predominant polyphenol utilisers within the gut (Hidalgo *et al.*, 2012; Tzounis *et al.*, 2011).

Kolida, Meyer and Gibson, (2007) also investigated the prebiotic efficacy of ITF this time using a powdered cocoa drink as a means of supplementation. In this study, fifteen men and fifteen women consumed a cocoa drink containing either a placebo (maltodextrin) or 5 or 8 g inulin/day for a two-week period. Each treatment period was followed with a one-week washout before the next treatment commenced. Stool samples were collected at the start of the study (baseline) end of each treatment and washout period, with changes in faecal microbial composition analysed via fluorescence *in situ* hybridization. The results indicated that levels of bifidobacteria compared to the control significantly increased with consumption of both 5 g/day and 8 g/day of inulin ($P \leq 0.05$) with no significant differences between the low and high inulin dosage. Additionally, the authors noted there was a slight decrease in *C. perfringens* – *histolyticum* subgroup upon completion of the higher inulin dosage with respect to levels at washout 2 ($P \leq 0.01$). Along with a significant decline in *C. perfringens* – *histolyticum* upon consumption of the low inulin dose with respect to washout period 1 ($P \leq 0.05$).

Beverages were also the preferred method of (Rao, 2001) for the supplementation of ITF. In this small-scale study, eight subjects were recruited: four males and four females. The intervention was split into two distinct 3-week periods. In the first 3-week period, subjects consumed 5g of sucrose a day, and in the second 3-week period subjects consumed 5 g/day of RAFTILOSE® P95 adjusted to the sweetness and colour of sucrose with aspartame at 2.7 g per kg oligofructose. Both sucrose and RAFTILOSE® P95 were dissolved in the subject's beverage of choice. Stool samples were collected before the start in the intervention and at the end of period 1 (sucrose control) and again at day 11 and finally at end of week 3 of period 2 (RAFTILOSE® P95). Changes in microbial composition were analysed using selective media techniques. The results of the study indicating that the consumption of RAFTILOSE® P95 resulted in nearly a 1 Log_{10} CFU/g wet faeces in bifidobacteria. As well as a 0.66 Log_{10} CFU/g wet faeces increase in *Bacteroides* between the end of the control and day 11 of the oligofructose intervention ($P \leq 0.001$ and $P \leq 0.01$). Furthermore, while there was a slight decline in bifidobacteria between days 11 and 21 of the intervention, this was not statically significant ($P \geq 0.05$). However, as the beverages consumed were the preferred choices of the subject in question and were not documented, determining if the type of beverages had a significant impact on the prebiotic effect of oligofructose cannot be undertaken.

In contrast, Azpiroz *et al.,* (2017) did note the drinks that volunteers consumed as means on ITF supplementation with drinks including water, milk, tea, coffee and juice drinks. The study design was a single-centre, placebo-controlled, parallel randomized and double-blind study involving 36 adults. Adults were randomised into one of two groups (intervention and control). The intervention group consumed 8 g/day ITF with the control group consuming 8 g/day maltodextrin. Both ITF and maltodextrin were incorporated into 200 mL of the volunteers' preferred beverage during breakfast and dinner at 4 g per serving. The intervention lasted four weeks where during the first three days of the intervention only half the dose of

ITF/maltodextrin was administered for adaptation. Faecal samples were collected on the two days before their scheduled visit at the end of each study period (baseline and intervention). Changes in total bacteria and bifidobacteria were analysed by real-time PCR. The results of the study showing that the effect of inulin on bifidobacteria was significantly greater compared to that of the placebo (P = 0.011). The limitations of this study are similar to those of (Rao, 2001) as the authors did not diversify results by drink type. Secondly, participants were instructed to consume substantial amounts of dairy (cheese and milk) daily throughout the course of the intervention which may not give a fair reflection of volunteer's typical habitual diet. Finally, the authors only reported changes in bifidobacteria. Thus, it cannot be determined whether changes in other microbial genera occurred. Although the results do suggest that the bifidogenic effect of ITF appears to be unaltered.

1.3.4 Mixed meals

The only study to utilise multiple foods products incorporated with ITF was undertaken by (Menne, Guggenbuhl and Roberfroid, 2000). In this small-scale study eight participants were recruited with the intervention being split into three distinct periods. Period I) A control period in which the volunteers were all given a controlled diet without any addition of OF. Period II) Treatment I which lasted two weeks during which the controlled diet was supplemented with 8 g/d of chicory OF. Finally, Period III) intervention treatment II, a second treatment period of 3 wk, during which the volunteers consumed their usual home-cooked diet to which they added 8 g/d of chicory OF. The ITF used in this study was Raftilose® L60 and was incorporated into orange juice, various desserts (puddings, creams and fruit mousses), cakes and biscuits. Additionally, participants undertook dietary restraint of naturally occurring ITF foods i.e. onions, leeks, bananas and artichokes. Stool samples were collected on the last day of week 2 of the control period; the last day of week 4 of treatment period I and finally at the last day of

week 7 at the end of treatment period II. Changes in microbial composition were analysed via selective media techniques. The results indicated that consumption of 8 g/day OF resulted in a 1 $\log_{10}$ CFU/g faeces increase in bifidobacteria after 2 weeks compared to the control ($P \leq 0.01$) with numbers in bifidobacteria still being 0.8 $\log_{10}$ CFU/g faeces higher at the completion of treatment II than at baseline ($P \leq 0.01$). No changes in total bacteria or other microbial genera were detected. However, as participants consumed a variety of different food products throughout the course of the intervention, often in combination, no conclusions can be drawn on the impact of differing food matrices on changes in the microbiota. Yet, these results do suggest that mixed food models likely have no impact on the prebiotic efficacy of ITF toward bifidobacteria.

A mixed model concept was also employed by (Hiel *et al.*, 2019). This time not involving direct supplementation of IFT into food products, but instead involving a diet rich in ITF-containing vegetables. The study design was single-group involving 26 healthy adults. The trial lasted 33 days. Volunteers were instructed to consume a controlled diet based on ITF-rich vegetables providing an average intake of 15 g ITF/d over 14 days. Test days were organised day 0 – T0 – baseline), Day 14 (T1- end of inulin-rich diet) and Day 33 –T2 – end of return to habitual diet). Stool samples were collected at within 2 days before each test day. Changes in gut microbiota composition were analysed via 16 rRNA sequencing and qPCR. Major increases in microbial composition were reported in *Bifidobacterium* (3.8-fold, $P \leq 0.0001$). along with a decrease in unclassified *Clostridiales* ($P \leq 0.0001$), and a trend toward a decrease in *Oxalobacteraceae* family ($P = 0.052$). The 3-fold increase in *Bifidobacterium* was also confirmed by qPCR ($P \leq 0.0001$). The limitations of this study are a lack of control group, limited number of subjects focusing purely on hydrogen-producing individuals, along with not analysing for presence of other bioactive compounds such polyphenols (flavanols), also naturally high in several of vegetables (artichokes, leeks) (Negro *et al.*, 2012; Ren *et al.*, 2017) used in this study.

Nevertheless, the results of the study do still imply that consuming adequate amounts of inulin-type fructan-rich vegetables are able to beneficially shift gut microbiota composition.

1.3.5 So does the food matrices matter: Jury out?

Overall, while these results of all of these studies seemingly suggest that the bifidogenic effect of ITF is unaltered as a result of the food matrix, there were several confounding factors including crossover vs parallel study design, number of participants and length of the intervention in the studies conducted thus far. Furthermore, 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) and when stool samples were collected combined with the lack of washout periods, differences in how studies report changes in microbial numbers (dry vs wet weight of faeces) and analytical techniques used (FISH vs selective media vs qPCR). Along with several studies only reporting changes in bifidobacteria mean that drawing definitive conclusions based on these findings should not be undertaken with any real degree of certainty (summarised in Table 1).

Reference	Food matrices	Type of inulin	Quantity of inulin	No. of Volunteers	length of intervention	Analysis technique	Outcome
(Kleessen *et al.*, 2007)	Cereal bars	Fibruline® Instant and Jerusalem Artichoke extract	7.7 g/per bar (2 bars per day)	45	7 day run in – 1 bar per day. Then 14 days at 2 bars per day	FISH-FLOW	1.2 log10/ g wet weight increase in bifidobacteria ($P \leq 0.05$); Reduction in numbers of *Bacteroides*/*Prevotella* ($P \leq 0{\cdot}05$) and fewer numbers of *Clostridium histolyticum*/*C. lituseburense*
(Reimer *et al.*, 2020)	Cereal bars	ITF (Type not stated)	Low (3g) and moderate (7g)	48	12 weeks (2 x 4 weeks with 4 week washout)	16S rRNA sequencing and QPCR	Moderate group = increase in bifidobacteria; Increase in bifidobacteria in low dose at week 2 ($P = 0.027$) and a trend toward an increase at week 4 ($P = 0.056$) compared with the Control 2 bar
(Gibson *et al.*, 1995)	Biscuits	OF and Inulin	15 g/day	8 and 4	(30 and 25 days total) (15 days on OF or Inulin)	Selective media techniques	Both OF and inulin increased bifidobacteria counts by 0.7-0.9 $\log_{10}$/g faeces. OF but not inulin resulted in decreases in counts of *Bacteroides* ($P \leq 0.01$), clostridia and fusobacteria ($P \leq 0.05$ and $P \leq 0.01$)

(Tuohy *et al.*, 2001)	Biscuits	OF + (partially hydrolysed guar gum)	6.6 g (OF) and 3.4 g/day (PHGG)	31	42 days in total (21 on OF)	FISH-FLOW	0.487 log_{10} CFU cells/g faeces increase in *Bifidobacterium* spp. No other changes were detected
(Brighenti *et al.*, 1999)	Extruded ready to eat cereal	Fibruline® Instant (av DP < 7, 30% DP > 30)	Inulin was incorporated into dry cereal at 18% dry weight basis	12	12 weeks total (4 weeks: control, 4 weeks intervention and 4 week washout)	Selective media techniques	Increase in bifidobacteria counts by 0.33 log_{10} CFU/g faeces dry weight. Small decrease in total facultative anaerobes
(Marteau *et al.*, 2011)	Yoghurt/ Stewed apple and pear	Fibruline® Instant	2 x 7.5 g sachets	50	28 days	RT-qPCR	Increase in bifidobacteria counts by 0.6 log10/g faeces
(Kruse, Kleessen and Blaut, 1999)	Yoghurt	Fibruline® (DP 2-50 (average DP 9)	Based on individual's energy requirements	11	64 days	FISH-FLOW	1 log_{10}/g dry faeces increase in bifidobacteria
(Slavin and Feirtag, 2011)	Ice cream	chicory inulin (Frutafit)	20g/day	12	21 day intervention, 21 days control	Selective media techniques	No sig increase in *Bifidobacterium* spp. Small decline in of *Clostridium* spp. (P = 0.33). Significant increase in *Lactobacillus* spp. ($P \leq 0.05$)

(Ramnani *et al.*, 2010)	Juice shots	Jerusalem artichoke ITF	2 x 2.5g per day	66	22 day intervention, 21 washout period	FISH-FLOW	Increase in bifidobacteria between 0.5-0.7 log_{10} cells/g faeces. Increase in *Lactobacillus*/*Enterococcus* groups of 0.2 log10 cells/g faeces
(Kolida, Meyer and Gibson, 2007)	Cocoa drink	Inulin (av DP 9–10 Frutafit IQ)	Low (3g) and moderate (7g)	30 (15 men and 15 women)	14 days followed by a 7 day washout period	FISH	0.12 log log10 cells/g faeces increase in bifidobacteria. No significant increases were seen between the low and high dose groups. Decrease in *C. perfringens – histolyticum* subgroup upon completion of the higher inulin dosage. Slight decline in *C. perfringens – histolyticum* subgroup upon consumption of the low inulin dose with respect in washout period 1 ($P \leq 0.05$)
(Rao, 2001)	Beverages – preferred choice of subject	Raftilose® P95	5 g/day	8 (4 males and 4 females)	2 x 3 week period (3 weeks sucrose followed by 3 weeks P95)	Selective media techniques	1 Log_{10} CFU/g wet faeces in bifidobacteria. No differences in total aerobes or coliforms were detected

(Healey *et al.*, 2018)	Hot and cold beverages of participant choice	Orafti® Synergy 1	16 g/day (2 x 8 g doses)	33	2 x 3 week periods (3 weeks Synergy I, 3 weeks placebo)	16S rRNA sequencing and QPCR	*Actinobacteria* relative abundance significantly increased ($P \leq 0{\cdot}001$) and Firmicutes relative abundance significantly decreased ($P = 0{\cdot}007$) and a trend towards a reduction in Proteobacteria relative abundance ($P = 0{\cdot}070$) during the prebiotic intervention phase. Increase in the relative abundance of *Bifidobacterium* ($P \leq 0{\cdot}001$) and a reduction in *Coprococcus* ($P = 0{\cdot}016$), *Dorea* ($P = 0{\cdot}029$), *Ruminococcus* (*Lachnospiraceae* family) ($P = 0{\cdot}007$) and *Oscillospira* relative abundance ($P = 0{\cdot}031$). There was also a trend towards an increase in *Faecalibacterium* relative abundance ($P = 0{\cdot}088$). In low fibre group only increase detected was in *Bifidobacterium* ($P = 0{\cdot}001$). In the high fibre group *Bifidobacterium* ($P \leq 0{\cdot}001$) and *Faecalibacterium* relative abundance ($P = 0{\cdot}010$), and a significant reduction in *Coprococcus* ($P = 0{\cdot}010$), *Dorea* ($P = 0{\cdot}043$) and *Ruminococcus* (*Lachnospiraceae* family) relative abundance ($P = 0{\cdot}032$)

(Azpiroz *et al.*, 2017)	Drinks: water, milk, orange juice,	Orafti® HSI	8 g (2 x 4 g;/day)	36	4 weeks (1st 3 days only half dose of inulin was consumed)	RT-qPCR	Inulin significantly increased the abundance of bifidobacteria (*P* = 0.001)
(Menne, Guggenbuhl and Roberfroid, 2000)	Mixed model concept	Raftilose® L60	8 g/day	8	3 periods: Period one: controlled diet no ITF: Period two: Controlled diet supplemented with ITF. Period three: normal diet supplemented with inulin	Selective media techniques	1 $\log_{10}$ CFU/g faeces increase in bifidobacteria after 2 weeks compared to the control (P ≤ 0.01) with numbers in bifidobacteria still being 0.8 $\log_{10}$ CFU/g faeces higher at the completion of treatment II than at baseline(*P* ≤ 0.01). No changes in total bacteria or other microbial genera were detected
(Hiel *et al.*, 2019)	Mixed model	Diet naturally high in inulin rich vegetables	Mean 15 g/day	26	3 Period: T0 baseline. T1- end of 14 day controlled diet high in inulin-rich vegetables T2 End of return to habitual diet	16S rRNA Sequencing and qPCR	Significant 3.8-fold increases in relative abundances of *Bifidobacterium* (*P* ≤ 0.0001). Decrease in relative abundance of *Clostridiales* (*P* ≤ 0.0001) and a trend towards a decrease in *Oxalobacteraceae* (*P* = 0.052). Significant increase in *Bifidobacterium* by qPCR 3-fold (*P* ≤ 0.0001)

Table 1.1 Summary of food-based inulin-type fructan supplementation studies

1.4 Future direction and call to action

The popularity of prebiotics and their potentially beneficial effects on microbial composition and health outcomes continues to gain momentum. There is increasing motivation by researchers to design food-based interventions which promote favourable shifts in the gut microbiota, however, due to differences in the type of ITF, the food product and analytical method used, along with variances in the populations and primary and secondary outcomes studied, it has become increasingly difficult to make direct comparisons between studies, hindering of the ability to draw appropriate conclusions on whether the food matrices alter the prebiotic efficacy of ITF. As a result, if we are to establish the impact of food matrices on the prebiotic efficacy of ITF, interventions must ensure several criteria are met. These criteria include ensuring that all ITF-containing foods products are standardised to contain the same type and amount of ITF, combined with undertaking identical eligibility criteria, intervention periods, primary and secondary measurements and analytical techniques. Thus, will allow for more reliable interpretation of results and generation of a consensus regarding ITF-food based intervention studies.

Ethics Statement

No animal or human experimentation was conducted in the writing of this review.

Declaration of Competing Interest

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Literature review - part 2

Inulin-type fructans and short-chain fructooligosaccharides—their role within the food industry as fat and sugar replacers and texture modifiers—what needs to be considered!

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Abstract

Inulin and oligofructose are classes of prebiotics belonging to a group of nondigestible carbohydrates referred to as inulin-type fructans. While short-chain fructooligosaccharides are enzymatically synthesized from the hydrolysis and transglycosylation of sucrose. Inulin-type fructans and short-chain fructooligosaccharides act as carbon sources for selective pathways supporting digestive health including altering the composition of the gut microbiota along with improving transit time. Due to their physicochemical properties, inulin-type fructans and short-chain fructooligosaccharides have been widely used in the food industry as partial replacements for both fat and sugar. Yet, levels of replacement need to be carefully considered as it may result in changes to physical and sensory properties that could be detected by consumers. Furthermore, it has been reported depending on the processing parameters used during production that inulin-type fructans and short-chain

fructooligosaccharides may or may not undergo structural alterations. Therefore, this paper reviews the role of inulin-type fructans and short-chain fructooligosaccharides within the food industry as fat and sugar replacers and texture modifiers, their impact on final sensory properties, and to what degree processing parameters are likely to impact their functional properties.

2.1 Introduction

With the incidence of obesity and obesity-related diseases on the increase, the food industry is facing increasing pressure from both governmental and public health bodies to modify food products to reduce both fat and sugar intakes to help combat the burden of disease (Krystyjan *et al*., 2015; Rodriguez-Garcia *et al*., 2013). Both fat and sugar play vital roles in determining the physical, chemical, and, ultimately, sensory properties of several food products enjoyed by consumers, so it has become necessary for manufacturers to find suitable alternatives to both fat and sugar that do not result in drastic changes to a given food products' final rheological and sensory quality.

Inulin and oligofructose (OF) are nondigestible carbohydrates belonging to a group of carbohydrates termed inulin-type fructans (ITF) (Karimi *et al*., 2015). ITF are composed of monomers of fructose joined by β-(2–1) glycosidic (fructosyl-fructose) linkages with varying degrees of polymerisation (DP) (Mensink *et al*., 2015). ITF are predominantly extracted from plants with the DP depending on the source, time of year, and length of post-harvest storage. For example, wheat, bananas, and onions possess short-chain ITF (max DP < 10). Jerusalem artichokes possess medium-chain ITF (max DP < 40) and globe artichokes and chicory root possess long-chain ITF (max DP < 100) (Roberfroid *et al*., 2010) In this regard, with the exception of chicory root, which possesses up to 70% inulin on a dry weight basis, most of

these fruits and vegetables only possess trace amounts of ITF, and as a result, the production of ITF primarily focuses on chicory root (Mensink *et al*., 2015).

The production of ITF from chicory in principle consists of three major steps: (1) extraction via hot water; (2) purification to remove impurities, and finally, (3) spray drying. If required, inulin may undergo enzymatic hydrolysis to alter the DP resulting in the production of OF (Apolinario *et al*., 2014). The length of the fructose chain determines the physiochemical properties of ITF and therefore its uses in food, with differences in physiochemical properties between OF and inulin becoming increasingly apparent at DP > 10 (Roberfroid *et al*., 2010; Shoaib *et al*., 2016). Alternately, short-chain oligofructose (scFOS) can be enzymatically synthesized by catalysing the hydrolysis and transglycosylation of sucrose leading to the formation of 1-kestose (Glu-Fru2), 1-nystose (Glu-Fru3), and 1F-β-fructofuranosylnystose (Glu-Fru4) (Rastall, 2010).

OF and scFOS due to its shorter chain length and greater solubility combined with possessing a sweetness value of 30%–35% that of sucrose means OF and scFOS can be used as a partial replacement for sugar (Villegas *et al*., 2010). While in contrast, long-chain ITF (LC-ITF) due to their greater DP and resulting water-binding properties can form fat-mimicking gels at concentrations >10%–20% providing reduced fat foods with similar textural and sensory characteristics of full-fat versions (Elleuch *et al*., 2011; Karimi *et al*., 2015).

Furthermore, given the resistance of ITF and scFOS to digestion due to the absence of brush boarder β-fructosidases, they can act as excellent bulking agents due to their low calorific content (1–1.5 kcal/g) having the potential to reduce energy intake by 65%–75% compared to digestible carbohydrates (Soukoulis and Fisk, 2016). This also means that ITF and scFOS reach the colon intact, functioning as a prebiotic for beneficial microorganisms within the gut (bifidobacteria are the group most frequently targeted), providing health benefits to the host

summarized as in these series of reviews (Ahmed and Rashid, 2019; Sanders *et al*., 2019; Wilson and Whelan, 2017).

Due to these abilities, ITF and scFOS have been used in the production of a range of different food products including cakes, muffins, bread, ready-to-eat breakfast cereals, cheese, ice cream, yogurt, fruit juices, and even Lyon-style sausages among others with a great deal of success (Bi *et al*., 2016; Di Criscio *et al*., 2010; Klewicki, 2007; Peressini *et al*., 2015; Rodriguez-Garcia *et al*., 2013; Salazar, Garcia and Selgas, 2009). However, it is common for food products to be subjected to multiple different processes during production including baking (Poinot *et al.,* 2010), pH adjustment (Klewicki, 2007), extrusion (Tsokolar-Tsikopoulos, Katsavou and Krokida, 2015) and even high-pressure pasteurization, all of which have the potential to alter the physiochemical properties of ITF.

Given the development of functional and low-fat and sugar food products aiming to improve and support gut and overall health, this area shows no sign of slowing down anytime soon. In addition, the number of people willing to spend a premium on products that they believe to be good for their health is seemingly on the increase (Karelakis *et al.,* 2019; Vicentini, Liberatore and Mastrocola, 2016). Therefore, the purpose of this review is to explore the role of ITF and scFOS within the food industry as fat and sugar replacers and viscosity modifiers, along with identifying how various food processing parameters may potentially alter ITF physiochemical integrity.

2.2 The physiochemical properties and effects of food processing on the structural integrity of inulin-type fructans and short-chain fructooligosaccharides

ITF and scFOS, in molecular terms, can be divided into two subgroups based on their DP with distinct physiochemical properties. The distinction between these two groups has a critical cut-off point of around DP 10 (van Loo, 2006).

2.2.1 Solubility

The solubility of ITF is closely related to DP with solubility decreasing with increasing chain length. In aqueous solution at room temperature, short-chain ITF (DP < 10) and scFOS are highly soluble at around 80% (w/w) (van Loo, 2006). While in contrast, the solubility of medium and LC-ITFs varies greatly dependent on the DP of the molecule in question. On this basis, Orafti® GR (granulated inulin powder - DP > 10) has a solubility of around 10% while Orafti® HP (inulin powder for fat replacement at low temperature - DP > 23) has low solubility and Orafti® HSI (a highly soluble inulin powder) possesses high solubility (Moser and Wouters, 2014). The solubility of ITF is also somewhat dependent on temperature with the solubility of even LC-ITF increasing as temperatures rise. This was demonstrated using LC-ITF (DP > 23) at temperatures ranging from 50 to 90°C where it was possible to achieve a solubility of between 20 and 34% (Cui, Wu and Ding, 2013; Kim *et al.*, 2013). Clearly, while temperature does appear to increase the solubility of LC-ITF, it does not reach that of OF and scFOS and DP is the most critical factor regarding solubility.

2.2.2 pH

The β-(2,1) linkages of ITF and scFOS can be degraded by acid hydrolysis resulting in the production of OF and fructose leading to a reduction in nutritional properties (Glibowski and

Wasko, 2008; Mensink *et al.*, 2015). The hydrolysis of ITF and scFOS is pH dependent with a little-to-no breakdown of ITF at pH > 4, while at pH < 4, ITF are hydrolysed. This is likely due to the protonation of the glycosidic bond (Blecker *et al.*, 2002; Duar *et al.*, 2015; Klewicki, 2007).

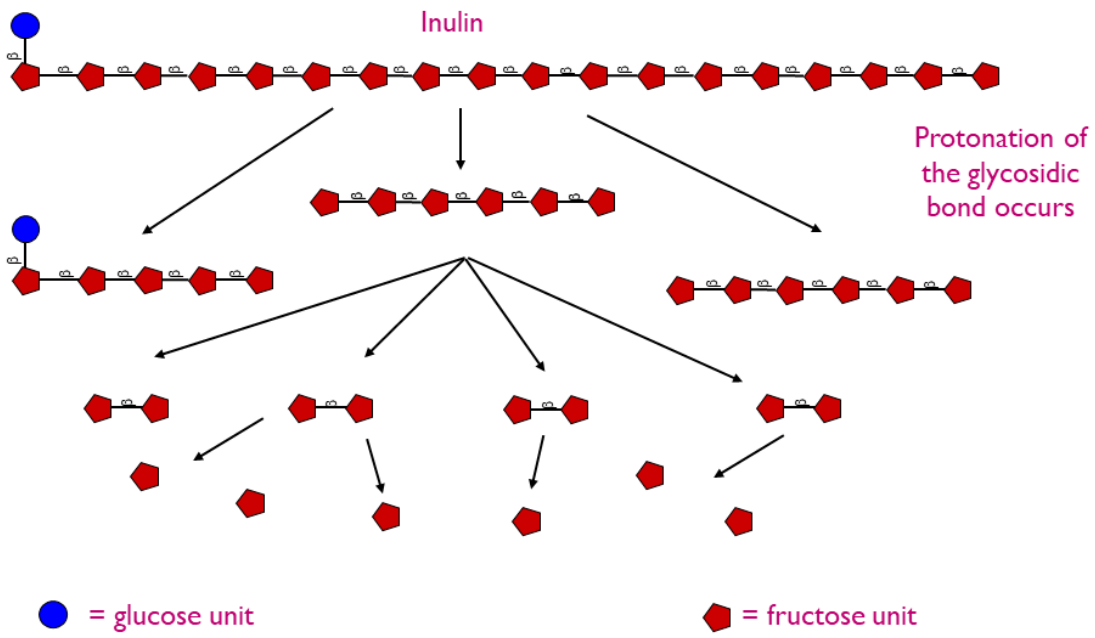

Figure 2.1. Hydrolysis of inulin results in the formation of shorter chains OF, fructose monomers, and a smaller amount of glucose monomers

The hydrolysis of ITF and scFOS follows first-order kinetics with inulin and OF/scFOS reporting similar trends concerning pH, temperature, and molecular weight (Barclay *et al.*, 2012). Yet, rates of hydrolysis do vary between LC-ITF and OF/scFOS due to differences in kinetics. The rates of hydrolysis of LC-ITF are markedly slower than OF and scFOS, to begin with, due to the scarcity of end-chain fructosyl groups (Mensink *et al.*, 2015) along with higher activation energy required to stimulate hydrolytic cleavage implying that hydrolysis of mid-chain glycosidic linkages in long-chain ITF is greater (Barclay *et al.*, 2012). As rates of hydrolysis of mid-chain glycosidic linkages increase, rate of reaction subsequently increases due to the greater number of end-chain fructosyl groups able to participate in the reaction (Blecker *et al.*, 2002).

Yet, it is possible to control the rate at which hydrolysis occurs by altering the conditions or the DP of the ITFs used during production or storage. For example, by keeping the pH > 4 hydrolysis and/or employing LC-ITF, hydrolysis can be significantly reduced even as temperature and time increase up to 100°C for 55 min regardless of the thermal process used (Glibowski and Bukowska, 2011; Matusek *et al.*, 2009).

2.2.3 Thermal processing

ITF and to a lesser extent scFOS are widely used as functional ingredients in several food products, including bread, biscuits, and cakes, where, depending on the process used, they may be subject to thermal degradation. However, while the thermal stability of ITF has been studied, due to several confounding factors results between studies are often hard to compare. For example, in the direct heating of inulin with temperatures ranging from 135 to 195°C for 5–60 min, the most critical times and temperatures for ITF degradation appeared to occur at 165°C for 30 min and 195°C for 15 min, respectively (Bohm *et al.*, 2005). While, in contrast, (Huebner *et al.*, 2008) noted that ITF were functionally stable when heated in solution at 85°C for up to 6 h at neutral pH (pH 7). However, neither of these studies reflects the matrices of a more complex food product.

A more realistic example of the heating of fructans was undertaken by (Whelan *et al.*, 2011) with the authors analysing the fructan content of several supermarket loaves of bread as well as white bread vs. white toast. The authors noted after correction for loss of moisture that white toast recorded a lower fructan content compared to its white bread counterpart (0.28 vs. 0.33 g/slice). However, while these results suggest toasting results in a small loss of fructan content, the amount lost (0.05 g) is possibly not of functional significance.

The formation of brown pigments in toast and baked products occurs as a result of the Maillard reaction, a nonenzymatic reaction between the amino group of amino acids and the carbonyl group of reducing sugars (Lund and Ray, 2017). ITF is a mixture of reducing and nonreducing oligosaccharides, therefore is susceptible to participation in the Maillard reaction (Mensink *et al.*, 2015). Figure 2.2 summarizes the way fructans may participate in the Millard reaction.

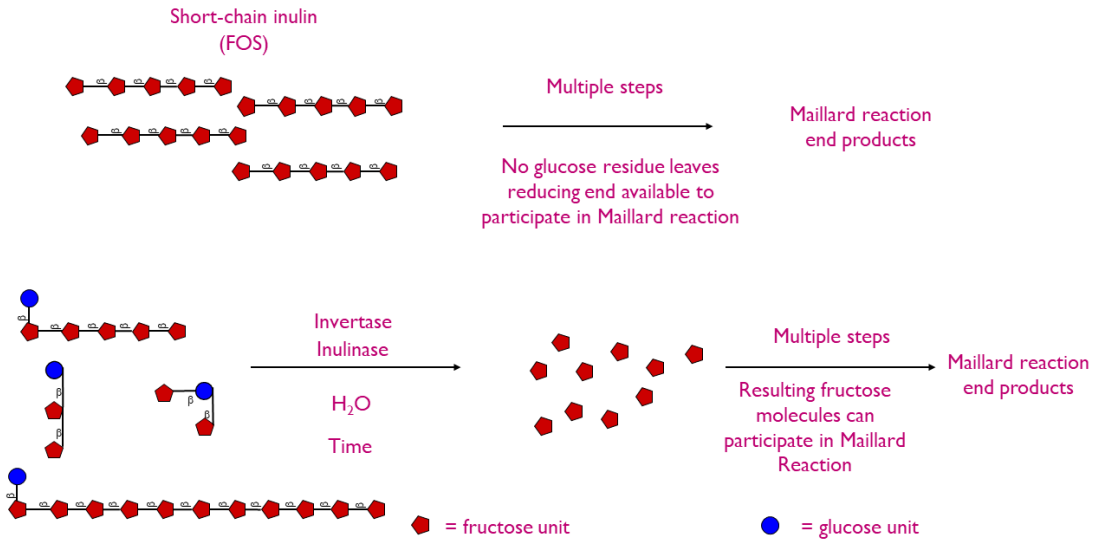

Figure 2.2. Pathways by which inulin-type fructans and scFOS can participate in the Maillard reaction. Path A: Direct participation in the Maillard reaction due to the availability of the reducing end. Pathway B via the enzymatic effects of yeasts, invertase, and inulinase.

The influence of the baking process on the colour of ITF-fortified baked products has been investigated to a substantial degree in bread (Poinot *et al.*, 2010), sponge cakes (Rodriguez-Garcia *et al.*, 2012), muffins (Zahn, Pepke and Rohm, 2010), and shortbread biscuits (Leiva-Valenzuela *et al.*, 2018). The consensus of these studies is that the addition of ITF alters the colour (level of browning) of the final product. Furthermore, Poinot *et al.*, (2010) noted that bread containing 5% ITF darkened 3 min quicker than loaves of bread possessing 0 and 3% ITF. This suggests that reducing ITF do undergo Maillard browning, accelerating the rate of baking. However, as most baked products are only cooked for a short amount of time with the

internal temperature of the food matrix not reaching more than 100°C, the rate of Maillard reaction associated with ITF degradation is likely to be minimal.

2.2.4 Yeast and enzyme degradation

The bread-making process is highly complex involving mixing, bulk fermentation, knocking back, proofing, shaping, and baking (Struyf *et al.*, 2017a; Struyf *et al.*, 2017b). During fermentation, yeasts produce several enzymes, including invertase and inulinase (Struyf *et al.*, 2017b). Invertase primarily not only targets the α-β-(1–2) linkage of sucrose hydrolysing sucrose into its respective monosaccharides, fructose, and glucose but can also target the β-(2–1) glycosidic (fructosyl-fructose) linkages of ITF, scFOS, and wheat-type fructans (Struyf *et al.*, 2017a). On the other hand, inulinase can hydrolyse sucrose but shows higher specificity toward β-(2–1) glycosidic (fructosyl-fructose) linkages of fructans compared to invertase primarily due to the lack of chain-end fructosyl groups (Menezes *et al.*, 2018). Given enough time, up to 80%–90% of non-ITF/ITF and scFOS can be degraded (Morreale, Benavent-Gil and Rosell, 2019; Verspreet *et al.*, 2013) (Figure 2.3). This leaves the reducing fructose and glucose available for both fermentation and the Maillard reaction, probably contributing toward more rapid browning recorded by (Poinot *et al.*, 2010).

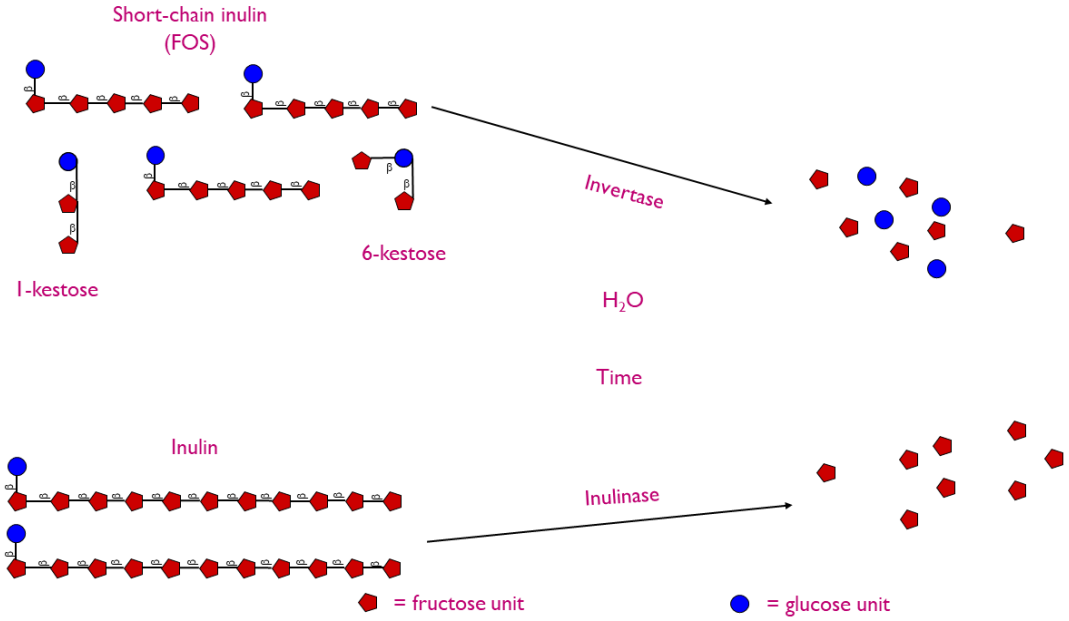

Figure 2.3. The degradation of inulin-type fructans and scFOS via invertase and inulinase, respectively.

The rate of hydrolysis of ITF/scFOS appears to be somewhat dependent on the type of yeast used during production, with commercial and wild bread/beer strains tending to show a higher degree of fructan degradation compared to low/non-invertase-producing yeasts (Fraberger *et al.*, 2018; Gelinas, McKinnon and Gagnon, 2016; Morreale, Benavent-Gil and Rosell, 2019; Verspreet *et al.*, 2013). This suggests that the rate of ITF/non-ITF degradation during bread making may be reduced by using low-invertase-producing yeasts. However, low-invertase-producing yeasts are not readily available to the bread-making industry. A more realistic alternative may be to alter the type of yeast and fructans used, with (Rakha, Aman and Andersson, 2010) indicating that *S. cerevisiae* invertase has a preference for low-DP fructans (< 5). This suggests that the degradation of fructans could be minimized by employing LC-ITF (DP > 10) during the production of bread.

2.2.5 High temperature and pressure extrusion

Extrusion is a process where several foods, including wholegrain and cereal slurries, are subjected to a combination of different temperatures, pressures, times, and shear rates (Offiah, Kontogiorgos and Falade, 2019). This process results in alterations to the physical structure of the whole grain cereals, transforming them into puffed ready-to-eat food products including breakfast cereals and snacks (Peressini and Sensidoni, 2009; Sacchetti, Pittia and Pinnavaia, 2005; Tsokolar-Tsikopoulos, Katsavou and Krokida, 2015).

For a ready-to-eat extruded breakfast cereal or snack to be well perceived, the final product should possess low density and a high degree of porosity and expandability (Bisharat *et al.*, 2013). These properties result in a feeling of lightness and an increased perception of crispness which are considered ideal properties by the consumer (Tsokolar-Tsikopoulos, Katsavou and Krokida, 2015). Extrusion cooking has become an increasingly common technology used in the food processing industry as it allows manufacturers to fortify food products with additives including vitamins, minerals, and dietary fibres which may have been lost in other parts of the production process (Korkerd *et al.*, 2016). Therefore, it should come as no surprise that the addition of ITF and scFOS to extruded food products has been extensively studied (Brennan and Tudorica, 2008; Capriles *et al.*, 2009; Tsokolar-Tsikopoulos, Katsavou and Krokida, 2015).

On this basis, Brennan *et al.* (2008) investigated the effect of the addition of ITF at 5%, 10%, and 15% on the characteristics of ready-to-eat breakfast cereals. The authors noted that increasing the level of ITF increased the level of crispness. However, despite the addition of ITF improving the crispness of ready-to-eat breakfast cereals as ITF, fortification increased from 5%–10%–15% expandability decreased. Yet, even at 10% and 15% ITF fortification

expandability was not statistically different compared to the non-ITF control. This finding is consistent with that reported by Tsokolar-Tsikopoulos, Katsavou and Krokida, (2015) who noted that as ITF fortification increased hardness also increased, while the expansion ratio decreased. As the level of porosity is linked to cell wall rupture (Tsokolar-Tsikopoulos, Katsavou and Krokida, 2015), the addition of ITF to starchy products results in increases in cell wall rupture reducing the expandability of air bubbles, subsequently increasing density and hardness (Anton, Fulcher and Arntfield, 2009). Any shortcoming in expandability can be overcome to a certain degree by increasing temperature and/or screw speed. However, significant adjustments in processing parameters may result in alterations to the functional properties of ITF. This was demonstrated by Duar *et al.*, (2015) who reported that under several different extrusion conditions, including 140°C at a screw speed of 170 rpm more than 50% of scFOS was degraded. Yet, LC-ITF appeared to be unaffected by low-temperature (120°C) extrusion. However, when screw speeds were adjusted to 120 and 170 rpm, only 25% and 34% of LC-ITF were recovered. Furthermore, only low levels of LC-ITF were recovered (35%) when temperature and screw speed were operated at their most extreme conditions: temperature (170°C) and pressure (170 rpm), respectively.

A more realistic way to overcome any losses of ITF/scFOS during the extrusion process may be to add inulin into extruded cereals and snacks via the flavouring process. Under this premise, Capriles *et al.*, (2009) demonstrated that the addition of 13.3% inulin and OF added via the flavouring process to extruded snacks produced a final product with no significant differences in sensory attributes. It seems clear that ITF can provide a realistic avenue for functional fortification in both extruded snacks and ready-to-eat breakfast cereals.

2.3 The role of inulin-type fructans and scfos in the food industry

2.3.1 Inulin-type fructans and short-chain fructooligosaccharides as fat replacers

Fat, particularly saturated fat from animal sources, is a significant contributor to the development of obesity and obesity-related diseases including cardiovascular disease (CVD) (Dickson-Spillmann and Siegrist, 2011; Vasilopoulou *et al.*, 2020). As a result, there have been ever-increasing demands placed on manufacturers to reformulate products to produce low-fat alternatives (La Berge, 2008). Replicating the beneficial properties that fat plays in the final quality of food products is, however, highly complex due to its multifunctional properties. Fat not only contributes to taste, flavour, and caloric intake but it can also inhibit gluten formation providing ideal snap and spread in baked products (Rodriguez-Garcia *et al.*, 2012; Rodriguez-Garcia *et al.*, 2013; Rodriguez-Garcia, Salvador and Hernando, 2014) along with enhancing both the mouthfeel and melting rates of dairy products including cheese, chocolate, and ice cream (Tekin, Sahin and Sumnu, 2017; Wadhwani, McManus and McMahon, 2011). Inulin, in particular LC-ITF, due to their physiochemical properties, can form fat-like gels at >10%–20%. Yet, replacing fat inulin does pose several challenges which are discussed below.
Table 1 summarizes these findings.

Reference	Food product	Type of inulin	Level of fat/fructan replacement/addition	Outcomes/findings
(Akbari *et al.*, 2016)	Ice cream	Fruitfit®TEX, molecular weight 3300, average DP > 20	80% fat to 8% of the final composition	Lower hardness values were detected in ITF fortified low-fat ice cream compared to non-ITF low-fat ice cream, but higher hardness values compared to full-fat control ($P \leq 0.05$) 4% containing low-fat ITF cream produced similar sensory scores compared to full-fat control
(Tiwari *et al.*, 2015)	Ice cream	No details	2%, 4%, and 6%	Melting rates increased significantly ($P \leq 0.05$) with ITF substitution At 2% and 4% ITF-fat replacement, no noticeable differences in sensory characteristics (appearance, flavour, body, and texture were detected). At 6% ITF-fat replacement, sensory characteristics were less acceptable across all test parameters
(Hashemi, Gheisari and Shekarforoush, 2015)	Ice cream	Orafti®HP (High Performance) inulin, average DP > 23	5%	Higher levels of overrun, viscosity, and hardness values compared to the full-fat control No differences in taste, texture, colour, and mouthfeel compared to full-fat control (all $P \leq 0.05$)

(Guven *et al.*, 2005)	Yogurt	Raftiline®HP, average DP > 23	1%, 2%, and 3%	At 1% ITF addition, separation of whey increased. Whey separation was not statically different at 2% and 3% ITF addition compared to full-fat control ($P \geq 0.05$) At 1, 2, and 3% ITF addition, no differences in body or texture were detected ($P \leq 0.05$) At 2% and 3% ITF addition, significant different in term of consistency compared to whole-milk yogurt ($P \leq 0.05$)
(Pimentel, Garcia and Prudencio, 2012)	Yogurt	Raftiline®HP, average DP > 23	2%	Levels of whey separation increased ($P \leq 0.05$) No differences in levels of firmness, cohesiveness, adhesiveness, and gumminess were detected compared to skimmed and full-fat control ($P \geq 0.05$)
(Brennan and Tudorica, 2008)	Yogurt	Frutafit®HD, average DP 8–13 (9)	2%, 4%, and 6%	At 2% ITF addition, no significant differences were detected in firmness, degree of whey separation, creaminess, and mouthfeel compared to both full-fat and skimmed controls ($P \geq 0.05$) At 6% ITF addition, no differences were detected in firmness, creaminess, and mouthfeel scores compared to full-fat and skimmed controls ($P \geq 0.05$) At 6% ITF addition, degree of whey separation was significantly greater compared to both full-fat and skimmed controls ($P \geq 0.05$)
(Kip, Meyer and Jellema, 2006)	Yogurt	Frutafit® HD, average DP ≥ 9	1.5%, 3%, and 4%	As levels of levels of ITF concentrations increased levels of firmness and viscosity increased

(Guggisberg *et al.*, 2009)	**Yogurt**	**Long-chain Inulin (Pacovis, Stetten, Switzerland), average DP >23**	**0%–4%** **(Fat 0.2%–3.5%)**	**The addition of ITF in low-fat yogurt (0.1%, 1%, and 2% fat) at 2% visually reduced syneresis** **Levels of firmness and viscosity were not significantly impacted by different levels of ITF concentrations** **The creaminess was not impacted by either level of fat or inulin (*P* = 0.062 and *P* = 0.065) respectively. Creaminess did trend toward an increase with increasing fat and inulin levels. Creaminess perception was highly influenced by increasing concentrations of inulin and fat**
(Gonzalez, Adhikari and Sancho-Madriz, 2011)	Peach-flavored yogurt drink	Raftilose®P95, average DP < 10	14 g/L (1.4%)	No differences were detected in overall acceptability between whole- and skimmed-milk peach-flavoured yogurt drinks containing ITF (*P* ≥ *0*.05). Whole-milk ITF containing peach-flavoured yoghurt drinks were generally more acceptable than their skimmed milk counterparts
(Borges *et al.*, 2019)	Sheep's milk cheese	Orafti®ST-Gel, average DP < 10	50 g/L	Texture parameters (firmness, adhesiveness, cohesiveness, elasticity, and gumminess) were comparable to that of both whole milk and skimmed controls
(Giri, Kanawjia and Singh, 2017)	Cheese spread	Frutafit®HD, average DP 8–13 (≥ 9)	0%, 4%, 6%, and 8%	At up to 6% ITF addition, spreadability was comparable to the control. At 8% ITF addition spreadability decreased significantly (*P* ≤ 0.05) Compared to control, colour scores for all inulin cheeses were significantly lower (*P* ≤ 0.05) At 8% ITF addition, spreadable cheese recorded significantly higher levels of hardness, coarseness, and spreabability (*P* ≤ 0.05)

(Hennelly *et al.*, 2006)	**Cheese**	**Rafteline®HP, average DP > 23**	**5 g/100 g of inulin gel (54IN1)** **13.75 g/100 g of inulin gel (54IN2), 13.75 g/100 g of inulin solution (54HIN; All moisture content 54 g/100 g)** **13.13 g/100 g of inulin gel with a moisture content of 56 g/100 g was also manufactured (56MC)**	**ITF replacement in 56MC cheese resulted in significantly greater meltability compared to all other ITF-replaced cheeses as well as the 54MC control ($P \leq 0.05$). Hardness values were significantly greater in all ITF-replaced cheeses with the exception of 56MC compared to the 54MC control (all $P \leq 0.05$)**
(Solowiej *et al.*, 2015)	Casein processed cheese	Raftaline®HPX, average DP > 23	1%, 2%, and 3% (1, 2, and 3 g/100 g)	ITF at 1%, 2%, and 3% decreased hardness values compared to anhydrous milk fat control (all $P \leq 0.05$). Cheeses containing 15% or 20% anhydrous milk fat and 2%, and 3% ITF recorded higher melting values ($P \leq 0.05$)
(Miocinovic *et al.*, 2011)	Low-fat unfiltered milk cheese	Inulin (Cosucra, Belgium) Fibruline®—no specific details	1%, 1.5%, 2%, and 3%	Improvement in sensory characteristics with increasing I content, in particular mouthfeel
(Li *et al.*, 2019)	Reduced-fat mozzarella cheese-like product	Inulin Shandong Futian Pharmaceutical Co., Ltd (Qingdao, China)—no details	2% (20 g/kg)	ITF-enriched reduced-fat cheese was considerably less rubbery, less firm, sweeter, and fattier than the non-inulin-containing reduced-fat control mozzarella cheese

(Rodriguez-Garcia *et al.*, 2013)	Shortbread biscuits	Frutafit® HD, average DP 8–13 (9)	10%, 20%, 30%, and 40%	At 20% and 30% fat replacement, panellists could differentiate between the 0 and 20% fat-/ITF-replaced biscuits
(Krystyjan *et al.*, 2015)	Shortbread biscuits	Frutafit® IQ, average DP 8–12	20%, 30%, 40%, and 50%	At 20% and 30% fat replacement, no differences in sensory characteristics compared to the control were detected. At 40 and 50%, a reduction in sensory characteristics was observed
(Rodriguez-Garcia *et al.*, 2012)	Sponge cake	Frutafit® HD, average DP 8–13 (9)	0%, 35%, 50%, 70%, and 100%	At 50% and 70% fat replacement, colour, appearance, taste, texture, and overall acceptability were not statically significant compared to the 0% inulin control ($P \geq 0.05$). Decline in batter viscosity, cake height, increases in chewiness and hardness were detected at 100% fat replacement ($P \leq 0.05$)
(Rodriguez-Garcia, Salvador and Hernando, 2014)	Sponge cake	Frutafit HD®, average DP 8–13 (9)	50%	Loss of batter viscosity and less and more broader bubble distribution. At 50% fat replacement, no noticeable differences in sensory characteristics (appearance, colour, texture, and taste) were detected ($P \geq 0.05$)
(Zahn, Pepke and Rohm, 2010)	Muffins	Color, average DP 10. Fibruline®S20, average DP < 10 Orafti®GR, average DP > 10	50%, 75%, and 100%	Batter flowability increased with an increasing amount of fat, which was substituted Moisture content increased in final muffins—10% for Fibruline® S20 and Orafti®GR, and c. 20% for Fibruline® Instant Muffins replaced with 50% in presented higher surface gloss, greater browning, and sweetness At 75% and 100% inulin replacement, crumb firmness significantly increased as well as glossiness and toughness ($P \leq 0.05$)

(Mendoza *et al.*, 2001)	Dry-fermented sausage	Raftiline®ST, average DP > 10	7.5% and 12.5%	Low- and medium-fat sausages supplemented with ITF at 7.5% scored significantly greater scores for spiciness, tenderness, and softness compared to the full-fat control ($P \leq 0.05$) Low- and medium-fat ITF sausages supplemented with ITF at 7.5% were significantly harder compared to the full-fat control ($P \leq 0.05$)
(Mendez-Zamora *et al.*, 2015)	Frankfurter Sausage	Orafti®GR, average DP > 10	Low fat with 15% inulin; low fat with 7.5% inulin and 7.5% pectin; low fat with 30% inulin; and low fat with 15% inulin and 15% pectin	Addition of ITF at 15% and 30% recorded lower lightness values ($P \leq 0.05$) At 30% fat/ITF replacement, the sausages scored lower in terms of hardness, gumminess, chewiness, flavour, and overall acceptance compared ($P \leq 0.05$)
(Keenan *et al.*, 2014)	Sausage	Orafti® GR and Orafti® HP, average DP > 10 and < 23	18.70% (DP > 23) or 9.35% (DP > 23) and 9.35% (DP > 10)	The addition of ITF at 18.70% (DP > 23) or 9.35% (DP > 23) and 9.35% (DP ≥ 10) reduced the cooking loss (degree of shrinkage) The addition of ITF resulted in slight increases in hardness and chewiness values

(Afshari *et al.*, 2015)	Burgers	Frutafit®TEX, average DP > 23	4% and 8%	Burgers possessing 4% and 8% ITF were significantly harder compared to the non-ITF control ($P \leq 0.05$) Burgers containing 8% ITF possessed significantly higher levels of hardness compared to the 4% ITF burgers ($P \leq 0.05$) No significant differences in terms of overall acceptability were detected between the control and both 4% and 8% ITF-containing burgers
(Salazar, Garcia and Selgas, 2009)	Dry-fermented sausage	Actiligh®950P	2%, 4%, and 6% at 6%, 15%, and 30% backfat	The addition of scFOS to fermented sausages reduced the hardness of dry-fermented sausages The addition of scFOS reduced gumminess, chewiness, and hardness, in particular at 6% supplementation at all levels of back fat
(Caceres *et al.*, 2004)	Hot cooked sausage	Actilight®950P scFOS (GFn, $n \leq 4$) composed of a mix of 1-kestose, nystose, and 1-F-fructofuranosyl nystose	2%, 4%, 6%, 8%, 10%, and 12% without fat reduction 2%, 4%, 6%, 8%, 10%, and 12% with 40% fat reduction	Non-fat reduced and reduced fat sausages supplemented with scFOS recorded higher color values at 12% supplementation ($P \leq 0.05$) The addition of scFOS in non-fat reduced sausages reduced hardness, chewiness, and gumminess ($P \leq 0.05$) Fat-reduced, scFOS-supplemented sausages were able to match the fat-reduced, non-scFOS-supplemented sausage in terms of textural properties Both non-fat and fat-reduced scFOS-supplemented sausages scored higher in terms of juiciness, but lower in terms of tenderness ($P \leq 0.05$)

Table 2.1. Summarized findings of ITF and scFOS used as fat replacers in food products

2.3.2 Inulin-type fructans and short-chain fructooligosaccharides as fat replacers in dairy products

2.3.2.1 Ice cream

Ice cream is a highly complex multiphase food product consisting of fat, sugar, protein, air bubbles, and ice crystals, all dispersed in a semi-frozen solution (Akbari, Eskandari and Davoudi, 2019). On average, ice cream contains between 10 and 16% fat which affects both the physical and sensory properties of the product including smoothness, melting rate, aroma, flavour, aeration, and creaminess (Mahdian and Karazhian, 2013; Tekin, Sahin and Sumnu, 2017). Of all ITF, LC-ITF (DP > 23) represent the most realistic alternative to fat in ice cream due to their excellent gel-forming properties, mimicking the mouthfeel quality found in high-quality dairy-based ice cream desserts (Gonzalez-Tomas, Bayarri and Costell, 2009).

The effects of replacing 80% of fat (8% of the final composition) with ITF (DP > 20) at 2%, 3%, and 4% on the physicochemical properties and sensory attributes of low-fat ice cream were investigated by (Akbari *et al.*, 2016). The authors reported all low-fat ITF-containing ice creams recorded lower hardness values compared to the ITF-free low-fat ice cream but were all still significantly harder compared to the full-fat control ($P \leq 0.05$). Yet, the low-fat ice cream containing 4% ITF recorded similar sensory scores compared to the full-fat control. Additionally, both (Tiwari *et al.*, 2015; Hashemi, Gheisari and Shekarforoush, 2015) noted in ice cream in which fat had been replaced with 2%, 4%, and 5% ITF that no noticeable differences in sensory characteristics with the exception of hardness values were recorded compared to the high-fat control. However, when ITF/fat replacement was increased to 6%, Tiwari *et al.* (2015) recorded noticeable differences in appearance, flavour, body, and texture.

The ability of ITF to alter the hardness, melting rates, and sensory characteristics of low-fat ice cream results from several interacting factors, including altering the stability and composition of the fat crystal network (Muse and Hartel, 2004). LC-ITF water-binding capacity and gel-forming properties modify the rheology of ice cream due to their ability to form microcrystals, which bind water molecules trapping them within the dispersed phase (Aykan, Sezgin and Guzel-Seydim, 2008; Karaca *et al.*, 2009; Tiwari *et al.*, 2015). This likely increase in the amount of unfrozen water found in the dispersed phase not only leads to a reduction in levels of hardness but also the rate of ice crystal formation resulting in depression of the final freezing point (Tiwari *et al.*, 2015).

Furthermore, as the concentration of fat begins to decrease, the level of overrun begins to decline, resulting in a reduction in the number of fat globule clusters present, leading to a decline in the abundance of air bubbles which can be trapped within the matrices. This subsequently increases the rate of heat transfer and the melting rate of ice cream which has been associated with an improvement in sensory properties compared to low-fat non-ITF-containing ice cream (Akbari *et al.*, 2019; Muse and Hartel, 2004). Based on the sensory properties of ice cream, it appears that replacing fat with up to 5% ITF produces no detectable differences in product quality.

2.3.2.2 Yogurt

Yogurt is one of the most highly consumed dairy products worldwide, frequently being consumed multiple times a day, and is a known source of several vitamins and minerals including calcium, iodine, and vitamin B12 (Moore, Horti and Fielding, 2018). Yogurt represents one of the most promising areas of fructan fortification (Shoaib *et al.*, 2016) due to its potential to prevent textural losses from fat reduction while also increasing/decreasing fibre and saturated fat intake simultaneously (Kleniewska *et al.*, 2016). In addition, yogurt

presents the opportunity to produce a symbiotic product potentially providing additional health benefits to the consumer (Moghadam *et al.*, 2019).

All types of ITF have been utilized in the production of yogurt including OF, medium-chain, and LC-ITF (Guggisberg *et al.*, 2009; Guven *et al.*, 2005; Paseephol, Small and Sherkat, 2008). Guven *et al.*, (2005) investigated the effects that the addition of HP-ITF (average DP > 23) at 1%, 2%, and 3% had on the rheological and sensory properties of low-fat yogurt. The authors noted that at 1%, the separation of whey appeared to increase, while at 2% and 3% ITF, the degree of whey separation was not statistically significantly different compared to the full-fat control ($P \geq 0.05$). Furthermore, when fat was reduced and replaced with 1%, 2%, and 3% ITF, no statistical differences in body and texture scores were found, however, only the low-fat yogurt with 1% inulin concentration could match the whole-milk yogurt in terms of consistency. These results are similar to those documented by (Pimentel, Garcia and Prudencio, 2012) who noted that the addition of HP-ITF at 2% improved the textural properties of skimmed-milk yogurt similar to that of the whole-milk yogurt control. In contrast (Brennan and Tudorica, 2008; Kip, Meyer and Jellema, 2006) observed that higher additions of LC-ITFs and ITF at 6% and 3% improved the viscosity and textural aspect (firmness, creaminess, and smoothness) of low-fat yogurt in comparison to the low-fat control.

This is consistent with data reported by (Paseephol, Small and Sherkat, 2008) who noted that low-fat yogurt supplemented with 4% HP-ITF produced rheological behaviour comparable to that of the full-fat control yogurt. However, (Guggisberg *et al.*, 2009) demonstrated that while the addition of ITF to low-fat yogurts (2% and 3.5% fat) improved levels of creaminess when fat was reduced to 0.2% and ITF increased to 4%, it was not possible to produce a yogurt with the same level of consistency and creaminess as the whole-milk sample. Finally, (Gonzalez, Adhikari and Sancho-Madriz, 2011) documented that peach-flavoured drinking yogurt made

with non-fat dried milk and supplemented with OF at 1.4% (14 g/kg) was not significantly different from the control, scoring similar in terms of aroma, colour, and mouthfeel, although whole-milk samples possessing OF were generally more liked than their skimmed-milk counterparts.

The level of firmness and extent to which whey separation occurs in ITF-fortified low-fat yogurt results from the concentration and type of ITF used as well as the levels of milk proteins present. As the concentrations of LC-ITF increase above 4%, the presence of a secondary inulin network may partly hinder the formation of the protein network (Guggisberg *et al.*, 2009), likely increasing the level of syneresis detected. Yet at between 2% and 4%, the addition of LC-ITFs appears to enhance the mouthfeel and consistency of low-fat yogurt by increasing milk gel strength via the formation of electrostatic, hydrogen, and hydrophobic bonds with both whey and casein proteins without altering the milk gel structure. This produces a more viscous final product, offsetting any reduction in milk gel strength seen by decreasing fat concentration (Arango, Trujillo and Castillo, 2013; Krivorotova, Sereikaite and Glibowski, 2017). This suggests that, while higher levels of ITF-supplementation in yogurt may indeed be possible, up to 4% ITF (w/w) replacement for fat appears to be a reasonable target to aim for (Meyer *et al.*, 2011).

2.3.2.3 Cheese

The production of cheese has occurred for centuries and fat plays several functions in the physical, textural, and sensory properties of cheese (Karimi *et al.*, 2015). However, due to increasing demands by consumers for low-fat products, the production of low-fat hard and soft cheese has escalated in recent years (Johansen, Naes and Hersleth, 2011). The manufacture of low-fat cheese is considered a challenge as lowering fat content results in adverse changes in rheological and sensory characteristics (Bi *et al.*, 2016; Sanchez-Macias *et*

al., 2012). These adverse changes include a more dense cheese as well as a chewy and rubbery matrix, poor melting qualities, lack of flavour, and off-putting colour (Diamantino *et al.*, 2014; Rogers *et al.*, 2010). To date, inulin, specifically ITF, has been used in the production of a variety of low-fat cheeses including mozzarella, parmesan, cream, cottage, and spreadable cheeses.

In the production of these cheeses, HP inulin and LC-ITF are the preferred inulin of choice due to their greater DP (> 23) and therefore better gel-forming properties (Arcia, Costell and Tarrega, 2011), and they have been shown to improve the mouthfeel, flavour, and spreadability of low-fat soft and spreadable cheese (Borges *et al.*, 2019; Giri, Kanawjia and Singh, 2017). Additionally, the ability of ITF (Orafti HP®) to replace fat in imitation cheese has also been explored, with (Hennelly *et al.*, 2006) concluding that ITF can replace up to 63% of fat (3.44 g/100 g cheese) in imitation cheese, albeit presenting a slight decrease in the honeycomb structure. Similarly, Solowiej *et al.*, (2015) demonstrated that inulin (Raftaline®HPX) could replace milk fat at 1%–3% in processed cheese analogs while also improving functional properties including increasing meltability and decreasing hardness and adhesiveness. This finding mimicked that found by (Miocinovic *et al.*, 2011), with the results indicating that the addition of inulin (no specific details) at 1.5% improved the texture, as well as the functional properties of low-fat cheese produced from ultrafiltered milk. Furthermore, Li *et al.*, (2019) also studied the addition of inulin (no specific details) to a model reduced-fat mozzarella cheese-like product at 20 g/kg (2%). The authors concluded that despite not being able to exactly match the textural properties of the full-fat control, the inulin-enriched reduced-fat cheese was considered less rubbery, less firm, sweeter, and fattier than the non-inulin-containing reduced-fat control mozzarella cheese. These data suggest that inulin can be used as a partial fat replacer in low-fat soft, spreadable, and imitation cheese without drastic changes in sensory attributes being detected.

2.3.3 Inulin-type fructans and short-chain fructooligosaccharides as fat replacers in baked goods

Baked goods including cakes and biscuits represent a complex system of foams and emulsions. In baked products, fat acts as a leavening agent, inhibiting gluten formation, providing tenderness, and contributing toward spread (Rodriguez-Garcia *et al.*, 2013; Zahn, Pepke and Rohm, 2010) along with adding moistness, occlusion of air bubbles, and providing structural stability (Krystyjan *et al.*, 2015; Matsakidou, Blekas and Paraskevopoulou, 2010). As with ice cream and yogurt, the partial or complete replacement of fat in baked products represents a significant challenge as it can result in significant alterations to the quality of the final product including influencing the perceived levels of crispness, taste, snap, and flavour along with altering shelf life (Blonska, Marzec and Blaszczyk, 2014).

In baked products, several attempts over the years have been undertaken to try and partially replace fat with suitable alternatives including the viscosity modifiers and gelling agents xanthan, guar, and gellan gum (Colla, Costanzo and Gamlath, 2018; Kohajdova and Karovicova, 2009) as well as dietary fibres including ITF and scFOS (Rodriguez-Garcia *et al.*, 2013; Zahn, Pepke and Rohm, 2010). Of all these potential fat replacers, ITF have attracted considerable attention as not only can they act as creaming agents, viscosity modifiers, emulsifying agents, and stabilizers but also provide a much needed source of fibre (Rodriguez-Garcia *et al.*, 2012; Rodriguez-Garcia *et al.*, 2013).

2.3.3.1 Biscuits

The snap, crumbliness, and richness are the principal qualities of biscuits by which they are judged (Blonska, Marzec and Blaszczyk, 2014; Krystyjan *et al.*, 2015; Rodriguez-Garcia *et al.*, 2013). Fat not only acts as a flavour enhancer but also as a lubricant, reducing dough stickiness

and inhibiting gluten formation allowing for optimal biscuit spread to occur during baking, resulting in the production of thinner more delicate biscuits (Laguna *et al.*, 2012; Pareyt *et al.*, 2009).

The ability of fructans, especially ITF, to act as a fat replacer in shortbread biscuits was investigated by (Blonska, Marzec and Blaszczyk, 2014; Krystyjan *et al.*, 2015; Rodriguez-Garcia *et al.*, 2013) with fat/ITF replacement ranging from 9.3% to 50%. At 20 and 30% fat replacement with ITF, Rodriguez-Garcia *et al.*, (2013) and Krystyjan *et al.*, (2015) recorded that, while panellists could differentiate between the 0% and 20% fat-/ITF-replaced biscuit and the full-fat–fat control, they could only describe slight differences between samples. Yet, Krystyjan *et al.* (2015) recorded that when fat/ITF replacement increased to 50%, differences in sensory qualities of shortbread biscuits became increasingly apparent, recording lower scores for taste, colour, aroma, shape, and consistency and higher levels of dough stickiness and hardness. At 50% fat/ITF replacement, the lower levels of fat present in the final dough allow greater accessibility of flour components, such as gluten, to water (Krystyjan *et al.*, 2015; Mamat and Hill, 2014). This is likely to contribute toward the stickier dough and sensory alterations detected by (Krystyjan *et al.*, 2015), as fat/ITF replacement increased to 50%. Nevertheless, this aside, it appears that replacing 20%–30% fat with ITF can deliver a quality short dough biscuit with desirable sensory characteristics.

2.3.3.2 Cakes and muffins

In contrast to biscuits, sponge cake and muffins are judged on their levels of softness, lightness, moistness, and cohesiveness of the sponge (Rodriguez-Garcia *et al.*, 2012; Rodriguez-Garcia, Salvador and Hernando, 2014; Zahn, Pepke and Rohm, 2010). Sponge cakes contain on average between 15% and 30% fat (w/w) and this is critical for trapping air

bubbles, acting as a leavening agent, along with providing moisture and tenderizing the crumb (Matsakidou, Blekas and Paraskevopoulou, 2010).

In sponge cakes and muffins, the replacement of fat with ITF has been investigated extensively (Rodriguez-Garcia *et al.*, 2012; Rodriguez-Garcia, Salvador and Hernando, 2014; Zahn, Pepke and Rohm, 2010), investigated the effects of replacing 50%, 75%, and 100% of margarine (10%–20% of the final batter) in muffins and cakes with various types of ITF-possessing DP ranging from < 10 to > 10. The authors noted that low-fat muffins replaced with 50% of either ITF were comparable to the full-fat control, only presenting slight differences in crumb firmness, final cake volume, and height. However, as the replacement of fat with ITF increased from 75% to 100%, greater differences in these sensory properties became increasingly apparent. Additionally, Rodriguez-Garcia *et al.,* (2012) noted that cakes made with 50% and 70% ITF (average DP 8–13) were not statistically different in terms of colour, appearance, taste, texture, and overall acceptability ($P \geq 0.05$). Yet, significant differences in batter viscosity and decline in cake height were detected along with alterations in sensory attributes, including increased hardness and chewiness values as the replacement of fat with ITF increased to 100% ($P \leq 0.05$).

Regarding the differences detected in batter viscosity and cake height, ITF must be dispersed in water before creaming leading to the presence of additional levels of free water in the matrices, subsequently reducing batter viscosity (Psimouli and Oreopoulou, 2013). A lack of batter viscosity leads to a reduction in the amount of air that can be occluded during the creaming process (Rodriguez-Garcia *et al.*, 2012; Zahn, Pepke and Rohm, 2010) with a subsequent loss during the baking process. This results in a lack of batter expansion likely leading to the losses in cake height detected by both (Zahn *et al.,* 2010) and (Rodriguez-Garcia *et al.*, 2012). Yet, it must be emphasized that reduction in sensory properties only becomes

apparent when fat replacement with ITF exceeds 50%–70%. Fat replacement in cakes and muffins up to 50%–70% (w/w) with ITF can be achieved without significant losses in sensory properties.

2.3.3.3 Inulin-type fructans and short-chain fructooligosaccharides as fat replacers in meat and pate-style salamis

Besides dairy and baked products, both ITF and scFOS have also been used as fat replacers in Frankfurter, Lyon-style, and dry-fermented sausages, as well as beef burgers (Afshari *et al.*, 2015; Keenan *et al.*, 2014; Mendoza *et al.*, 2001). Mendoza *et al.,* (2001) investigated the effects of replacing backfat with varying levels of ITF, on the sensory properties of dry-fermented sausages. The results showed the addition of inulin at 7.5% improved the spiciness, tenderness, and softness of both medium and low-fat ITF-fortified sausages. However, both the medium- and low-fat ITF-fortified sausages could not quite match the juiciness of the conventional, high-fat control.

In another study, Mendez-Zamora *et al.*, (2015) recorded that the replacement of fat with ITF at 30% improved both the yield and colour of Frankfurter-style sausages, however, at 30% fat/ITF replacement, the sausages scored lower in term of hardness, gumminess, chewiness, flavour, and overall acceptance compared to the full-fat Frankfurter control. Yet, when fat was substituted with ITF at 15%, no differences in sensory characteristics were detected. Additionally, Keenan *et al.*, (2014) concluded that the inclusion of ITF in sausages at 18.70% (DP > 23) or 9.35% (DP > 23) and 9.35% (DP > 10) not only reduced the cooking loss (degree of shrinkage) in the sausages but also resulted in textural modifications including slight increases in the levels of hardness and chewiness detected. This finding is similar to those recorded by Afshari *et al.*, (2015) who concluded that low-fat burgers containing ITF at 8% had not only the highest levels of fat retention but also the highest levels of hardness and gumminess.

In addition to inulin-type fructans, scFOS has also been used as fat replacers in dry-fermented sausages (Salazar, Garcia and Selgas, 2009). In this study, scFOS was incorporated at 2%, 4%, and 6% into sausages containing 30%, 15%, and 6% backfat. The authors noted that the addition of scFOS to fermented sausages reduced hardness with dry-fermented sausages at 15% fatback being the most accepted samples. However, the addition of scFOS did appear to result in a loss of colour (lightness) due to increased turbidity likely due to scFOS gel-forming properties.

Furthermore, another study (Caceres *et al.*, 2004) investigated the effects that scFOS had on the sensory characteristics of cooked sausages either with or without fat reduction (40%) with ScFOS being supplemented at 2%, 4%, 6%, 8%, 10%, and 12%. Unsparingly cooked sausage with and without fat reduction and the addition of scFOS specifically at 12% produced higher colour values ($P \leq 0.05$). While the addition of scFOS in non-fat-reduced sausages reduced hardness, chewiness, and gumminess, the fat-reduced scFOS-supplemented sausages were able to match the non-scFOS fat reduced control sausages in terms of textural properties. More interestingly, in terms of sensory characteristics, both non-fat- and fat-reduced and scFOS-supplemented sausages scored higher in terms of juiciness, but lower in terms of tenderness ($P \leq 0.05$). No other differences were detected with the scFOS-supplemented sausages being considered acceptable by the panellists.

The basis of the textural changes caused by fructans in processed meat products is not well understood. It has been speculated that increases in hardness may occur as a result of fructans' ability to promote interactions between various components found within the matrices or the fact that fats are physically softer in comparison to inulin crystals (Cruz *et al.*, 2010; Keenan *et al.*, 2014). Nonetheless, it appears that fructans may indeed be able to

partially replace fat in meat-type products suggesting further work in this area would be highly beneficial to optimize product formulation. This is particularly important given that several of the meat products mentioned may be able to deliver a high enough dose of fructans to stimulate a beneficial response in the gut microbiome.

2.3.4 Inulin-type fructans and short-chain fructooligosaccharides as a sucrose replacer

The main sugar used in the production of cake, biscuits, ice cream, meat products, jam, jellies, and fruit juices among others is sucrose (Clemens *et al*., 2016). Sucrose is primarily known for contributing a sweet taste, tempering bitterness, and acidity, along with acting as a bulking agent (Goldfein and Slavin, 2015). Beyond this, sucrose is also a precursor for flavour compounds and colour via participation in both the Maillard and caramelization reactions (Hwang *et al*., 2011). In addition, it contributes to the brittle texture of candy and smoothness and creaminess of iced desserts via the melting of sucrose into its crystalline and amorphous form and reducing the rate of ice crystal formation during freezing, respectively (Clemens *et al*., 2016; Cook and Hartel, 2010). Furthermore, sucrose is critical in the preservation of jams, jellies, preserved fruits, and even meat due to its water-reducing activity (Goldfein ad Slavin, 2015). The water-binding capacity of sucrose also provides tenderness in baked products via competition, with starch and protein molecules for liquid components in batters and doughs, limiting gluten formation, and raising starch gelatinization temperature (Rodriguez-Garcia, Salvador and Hernando, 2014). Sucrose also stabilizes cake batters and provides lightness by interactions with egg proteins, making the final batter more elastic (Pareyt *et al*., 2009). These interactions allow more air bubbles to be incorporated during creaming, preventing them from escaping during baking, and permitting expansion to occur (Gao *et al*., 2016). Thus, replacing sugar with a suitable alternative must be carefully considered. Table 2 summarizes the findings of ITF and scFOS as sugar replacers.

Reference	Food product	Type of inulin	Level of sugar/fructan replacement	Outcomes/Findings
(Klewicki, 2007)	Apple and blackcurrant juice Milk–peach–mango drink	Sc-FOS was synthesized using enzyme preparation	1.5 g/100 mL	Under two-stage processing (Stage 1: 95°C 30 s and Stage 2: 84°C for 10/20 min), the concentrations of scFOS tetramers and higher oligomers decreased by up to 70%–87% At pH 4.2, 95°C, and 30 s, 80% of scFOS was retained during the pasteurization of a milk–peach–mango drink
(Duar *et al.*, 2015)	Prototype drink	Sc-FOS, average 1-kestose 33.8%, DP 3; nystose 50.1%, DP 4; and fructosyl nystose 11.6%, DP 5 (GTC Nutrition) Orafti® inulin > 92% oligofructose, average DP 3–60	1% w/w	At pH 3, only between 35 and 50% of scFOS survived the pasteurization process At pH 4, scFOS was relatively stable with 80–90% of scFOS in a prototype drink being retained after the pasteurization process Over 90% of LC-ITF survived the pasteurization at pH 3 with all inulin surviving the pasteurization process at pH 4
(Glibowski *et al.*, 2020)	Apple Juice	(HP) Frutafit®TEX, average DP > 23, (NAT) Frutafit®IQ, average DP > 10 OF Orafti®P95, average DP > 4–5	0%, 2%, and 4 g	No significant differences were detected in reducing sugars throughout the course of storage No differences in sensory characteristics were detected between the control and all ITF-containing samples Both native and HP ITF added into apple juice at 2% and 4% were unaffected via pasteurization at 100°C for 1 min at pH 3.68 ± 0.16

(Aidoo, Afoakwa and Dewettinck, 2014)	Chocolate	Rafteline®HP, average DP > 23	100%	Chocolate replaced with 100% ITF at 30% fat content matched the 0% inulin control in terms of hardness, as well as melting properties at 27%, 30%, and 33% fat content
(Konar *et al.*, 2014)	Chocolate	Inulin (no specific details)	6%, 9%, and 12%	As levels of ITF fortification increased, the largest particle size (D90 value) increased, ranging from 39.80–44.49, 54.23–55.71, to 61.50–62.61 cm^3/cm^2 At 12%, ITF replacement as a mean partial size of 20 um was achieved at 3.5 h conching
(Farzanmehr and Abbasi, 2009)	Chocolate	Frutafit® IQ, TEX, CLR, average DP > 5–7, > 23 7–8	41.8 g/100 g	Sugar-replaced ITF chocolate possessed similar levels of sweetness, hardness, and colour compared to the full-sucrose control A significant difference in melting rate and mouth coating was detected between the control and ITF-replaced chocolate ($P \leq 0.05$)
(Gao *et al.*, 2016)	Muffins	Frutafit®IQ, average DP 5–7	50% and 100%	At 50% sugar replacement, muffins possessed similar texture compared to the control. In contrast, muffins made with 100% sugar/ITF replacement recorded significantly higher firmness and lower springiness values ($P \geq 0.05$)

(Rodriguez-Garcia, Salvador and Hernando, 2014)	Cake	Frutafit®HD, average DP 8–13(9)	0%, 20%, 30%, 40%, and 50%	As ITF/sugar replacement increased, batter viscosity decreased ($P \leq 0.05$) At 50% sugar replacement, number of bubbles decreased and the size of the remaining bubbles increased Cakes at 30% and 40% ITF/sugar replacement recorded significantly greater weight loss (%) after baking ($P \leq 0.05$) As sugar replacement increased to > 40%, cake height decreased significantly ($P \leq 0.05$) As sugar replacement increased, hardness decreased significantly ($P \leq 0.05$) Sugar replacement by oligofructose at 30% (C0-30) did not show significant differences ($P \geq 0.05$) from the control cake Cakes made with 50% ITF/sugar replacement recorded lower overall intention to purchase scores compared to control cake

(Tsatsaragkou *et al.*, 2021)	Cake and biscuits	Orafti®HSI, average DP unknown Fibruline® Instant, average DP approx. 10	0% and 30%	Orafti® HSI produced similar batter viscosity compared to the control. Whereas Fibruline® Instant produced significantly higher batter viscosity ($P \geq 0.05$) Sugar-replaced ITF sponge cake retained significantly higher moisture content compared to the sugar control after baking ($P \geq 0.05$) The springiness of ITF-supplemented sponge cakes was significantly lower compared to the control ($P \geq 0.05$) Orafti® HSI sponge cake exhibited significantly lower ($P \leq 0.05$) firmness values compared to the control. However, Fibruline® Instant-supplemented sponge cake reported significantly higher ($P \leq 0.05$) firmness value Several sensory attributes including colour, dry appearance, dry to touch, springiness, and aroma were all significantly impacted by sugar/ITF replacement in cake ($P \leq 0.05$). ITF-supplemented biscuit dough was significantly harder and possessed higher levels of dough stickiness compared to the control ($P \leq 0.05$) No differences in colour were detected between control and ITF/sugar-replaced biscuits ($P \geq 0.05$) Significant differences in the density of crumb were detected between ITF-supplemented biscuits and the control ($P \leq 0.05$) Several sensory attributes including vanilla flavour and crunchiness were scored significantly lower compared to the full-sugar control ($P \leq 0.05$)

Table 2.1. Summarized findings of ITF and scFOS used as sucrose replacers in food products

2.3.4.1 Fruit juices

Fruit Juices and sugar-sweetened fruit juices are highly desirable products among all age ranges due to their pleasant sensory characteristics and health benefits including being a source of several beneficial vitamins and minerals such as vitamin C, as well as potentially other useful compounds such as pectins and phenolic acids (Gomes *et al.*, 2017). However, due to the presence of high levels of sugars and organic acids, fruit juices play a major role in the development of dental caries (Liska, Kelley and Mah, 2019). As well as being a contributor to an increased risk of diabetes (Xi *et al.*, 2014). Thus, to reduce the onset of dental caries and diabetes, replacing sugars with alternatives has become of increasing interest to the fruit juice industry.

The addition of prebiotics including fructans to fruit juices at face value seemingly makes logical sense as they cannot undergo digestion in the GI tract, including in the oral cavity, due to lack of β-fructosidases needed to hydrolyse β-(2–1) glycosidic linkages (Capuano, 2017). Yet, the addition of fructans to fruit juices represents a major technical challenge for manufacturers due not only to low pH but also the temperatures and pressures used during the pasteurization process (Fonteles and Rodrigues, 2018). Several attempts have been undertaken to replace sugar with fructans in fruit juices. (Klewicki, 2007) exposed apple and blackcurrant juice drinks containing scFOS at 1.5% (1.5 g/100 mL) to two-stage processing (Stage 1: 95°C 30 s and Stage 2: 84°C for 10/20 min) noting that concentrations of scFOS tetramers and higher oligomers decreased by up to 70%–87%. Furthermore, it was recorded that a significant portion of the tetrasaccharides and higher scFOS fraction present in the juice were degraded to trisaccharides, subsequently increasing the number of trimers that could be hydrolysed to di- and monosaccharides, respectively.

However, it could be argued that these conditions do not directly reflect those used in modern fruit juice production. The same authors also reported that under less extreme processing conditions, similar to those used in the food industry (pH 4.2; 95°C 30 s), 80% of scFOS was retained during the pasteurization of a milk–peach–mango drink: a finding mirroring those of (Duar *et al.*, 2015) who recorded that at pH 4, scFOS was relatively stable with 80%–90% of scFOS in a prototype drink being retained after the pasteurization process had been completed. Interestingly, Duar *et al.*, (2015) did detect substantial differences in the rates of hydrolysis between scFOS and LC-ITF, with over 90% of LC-ITF surviving the pasteurization at pH 3. These findings are in line with those reported by (Glibowski *et al.*, 2020), who noted that the levels of both native and HP inulin added into apple juice at 2% and 4% were unaffected by pasteurization at 100°C for 1 min at pH 3.68 ± 0.16.

The degradation of fructans in fruit juices results from complex interactions among chemical structure, pH, temperature, and pressure, whereby the higher the temperature, the more elevated the pressure and the lower the DP. Lower pH (pH 4 being the critical limit) also induces the degradation of fructans (Fonteles and Rodrigues, 2018; Klewicki, 2007). Any losses in fructan as a result of pasteurization can be overcome or minimized by either maintaining pH at or around > 4, storing at < 7°C, and/or utilizing LC-ITF in production. Fructans would seem to be a suitable ingredient in acid-based fruit drinks.

2.3.4.2 Chocolate

Chocolate production is a highly complex physical and chemical process where chocolate can be defined as a suspension/dispersion of non-fat and fat ingredients including cocoa powder and sugar in a Newtonian fluid (Konar, 2013; West and Rousseau, 2018). The quality of chocolate is judged on its snap, melting qualities, aroma, flavour, and particle size distribution

with each of the ingredients mentioned above affecting the quality of the final product (Glicerina *et al.*, 2014).

The majority of sugar used in chocolate production comes in the form of sucrose and plays several major roles including tempering bitterness, contributing to the formation of aroma compounds, and affecting flow quality and nucleation (Svanberg *et al.*, 2011; Torres-Moreno *et al.*, 2012), as well as acting as a bulking agent making up between 30 and 50% of the final product (Aidoo *et al.*, 2013; Gutierrez, 2017). During production, mixtures of cocoa, sugar, and fat are refined via a procedure commonly termed conching (Prawira and Barringer, 2009). Conching is a process where shear and heat are applied to two phases: dry and wet over a 16- and 48-h period resulting in the production of one final liquified mass (Konar, 2013). The process of conching is critical in the production of fine quality chocolate as it results in the reduction in particle size directly impacting final texture, viscosity, and flavour (Schumacher *et al.*, 2009; Torres-Moreno *et al.*, 2012).

To obtain fine-textured chocolate, a reduction of 90% of particles to below 20–25 µm must be achieved (Gutierrez, 2017; Oba *et al.*, 2017). When particles do not reach these values, chocolate is frequently described as coarse and gritty, with chocolate possessing particle sizes >35 µm being labelled as unpalatable (Kalic *et al.*, 2018; Schumacher *et al.*, 2009). Particle size is directly related to the specific surface area, with particles seemingly becoming more spherical as specific surface area increases, resulting in the broadening of particle size (Glicerina *et al.*, 2014). The relationship between particle size and specific surface area ultimately influences both yield stress and fracture rate, directly impacting the phase transition state, from solid at room temperature to liquid at body temperature (Glicerina *et al.*, 2014; Konar *et al.*, 2014). This implies that the replacement of sucrose with alternatives in chocolate must be carefully considered to optimize the ratio between the number of coarse

and fine particles to help reduce the potential impact that replacement of sugar may have on final rheological and sensory properties (Konar, 2013).

Konar *et al.*, (2014) investigated the effects of the addition of ITF (no specific details) at 6%, 9%, and, 12% (w/w) on final particle size. The authors noted that, as the level of ITF fortification increased, the largest particle size (D90 value) increased, ranging from 39.80–44.49, 54.23–55.71, to 61.50–62.61 cm^3/cm^2, respectively. Yet, despite the largest particle size increasing, it was possible to produce a chocolate with a mean particle size of 20 µm at 12% ITF fortification even at the lowest conching time (3.5 h). Adding to this, (Farzanmehr and Abbasi, 2009) noted that in milk chocolate, in which sucrose had been replaced with ITF (Frutafit® IQ, TEX, CLR) at 41.8 g/100 g chocolate, the chocolate had similar levels of sweetness, hardness, and colour compared to the full sucrose control. However, the addition of ITF resulted in alterations to the melting characteristic as detected by the sensory panellists, a finding in line with those reported by (Aidoo, Afoakwa and Dewettinck, 2014) who recorded that chocolate replaced with 100% ITF (Rafteline®HP) could replicate a level of hardness comparable to that of the 100% sucrose control when the fat level was around 30%. Furthermore, the same authors noted that at 100% sucrose replacement, the melting properties remained unaltered compared to the full sucrose control even when fat was increased from 27%, 30%, to 33%. Although, as the melting rate was determined by textural analyser, making any comparisons with the results detected by (Farzanmehr and Abbasi, 2009) cannot be undertaken.

Nevertheless, it seems likely that ITF can be successfully used as a partial replacement for sucrose in chocolate without a loss of physical and sensory properties. In order to optimize product quality, however, it is clear that a greater understanding of the functionality of bulking agents is still required.

2.3.4.3 Baked products

Baked products, notably cakes and biscuits, are well renowned for their high sucrose content contributing to their sweetness, snap, crispness, aeration, browning, and shelf life (Pareyt *et al.*, 2009; Rodriguez-Garcia *et al.*, 2013). Sucrose also elevates starch gelatinization and egg white protein denaturing temperature, allowing for the development of a finer-textured cake crumb and greater retention of final moisture content (Gao *et al.*, 2016). ITF, due to its polydisperse nature consisting of both LC-ITF and OF, represent the realistic alternative to sucrose in cakes and biscuits because of their physically different properties. LC-ITF provides water-holding and occluding properties, while OF mimics the qualities of glucose syrup or sucrose providing a source of sweetness (Martins *et al.*, 2019).

The replacement of sucrose in cakes with OF was investigated by Rodriguez-Garcia *et al.,* 2014 ranging from 0 to 50%. The authors noted that sponge cakes replaced with 30% ITF gave similar scores in terms of colour, appearance, texture, taste, and overall acceptability compared to the full-sugar control. However, there was a noticeable loss in cake height, likely resulting from the decline in batter viscosity and a reduction in the number of occluded air bubbles within the final batter. These alterations become increasingly apparent at 50% sucrose/ITF replacement, with noticeable differences in textural (cohesiveness, springiness, and chewiness) and taste characteristics ($P \leq 0.05$). From this, it could be inferred that the degree to which textural and sensory characteristics are affected is highly dependent on the level of sucrose/fructan replacement. On this basis, Rodriguez-Garcia *et al.,* 2014 suggest that the replacement of sucrose with 30% ITF appears to be an optimal level of sucrose replacement before substantial changes in sensory characteristics begin to become increasingly apparent. However, in contrast, Gao *et al.,* (2016) noted that muffins with 50% ITF/sucrose replacement recorded similar values in terms of firmness and springiness

compared to the control, inferring that the level of sucrose replacement with OF may not be the only determining factor.

Another contributing factor is the DP of inulin used during production, with (Tsatsaragkou *et al.*, 2021) documenting that higher DP inulin (Fibruline® Instant) resulted in a more vicious cake batter ($P \leq 0.05$). This leads to a less homogenous cake structure and greater perceived levels of mouth coating and dryness. While the same authors noted in biscuits produced using lower DP inulin (Orafti® HSI) as a sugar replacer that final biscuits were softer and less crunchy compared to full-sugar control.

In addition to the level of sucrose/fructans, replacement, and DP of fructans used during production, variations in results may have also occurred due to differences in ingredient composition, with a key difference being the initial level of fat used in production. In this regard, Rodriguez-Garcia *et al.,* (2014) noted in cakes possessing higher initial levels of fat, where sucrose was replaced with OF, a more viscous batter was able to be obtained up to 30% sucrose/ITF replacement. As a more viscous cake batter allows for more air to be occluded during creaming, this results in a more pronounced rise during baking producing a lighter and more acceptable final cake/muffin (Psimouli and Oreopoulou, 2013; Wilderjans *et al.*, 2013). Yet interestingly, Rodriguez-Garcia *et al.,* (2014) also recorded that sponge cakes in which fat and sugar had been replaced with 50% and 30% OF had good overall acceptability compared to full-fat and sugar control. This suggests that it may be possible to produce an acceptable sponge cake while reducing both fat and sugar simultaneously, but further work in this area is still required in order to optimize product formulation.

2.4 Inulin-type fructans and short-chain fructooligosaccharides as texture modifiers

The textural aspect of food is an extremely critical factor regarding consumer acceptance (Tomadoni *et al.,* 2018). Manufacturers are frequently targeted with reformulating products due to changes in consumer trends and increasing health concerns (Unal and Akalin, 2013). Dairy products including yogurt, spreadable cheese, and custard-based desserts are among the most common foods subjected to reformulation due to their high levels of saturated fat and/or sugar (Akin, Akin and Kirmaci, 2007; Gonzalez-Tomas, Bayarri and Costell, 2009; Karimi *et al*., 2015). However, the removal of fat and/or sugar results in a loss of textural properties, leading to a decline in consumer satisfaction (Gonzalez-Tomas, Bayarri and Costell, 2009).

One way to improve and maintain the texture of reduced-fat and sugar food is via the use of viscosity modifiers and hydrocolloids including xanthan, guar, and gellan gum (Saha and Bhattacharya, 2010). Yet, despite these texture modifiers and hydrocolloids proving to be effective in their ability to modify product rheology, they do not provide any additional nutritional benefits. It is on this basis that fructans, in particular ITF, represent a realistic alternative to viscosity modifiers due to their ability to form viscous and stable gels along with providing a much needed source of fibre (Meyer *et al.*, 2011).

As the addition of ITF increases beyond 10%, changes in product rheology become increasingly apparent with the formation of an extremely thick solid-like gel (Karimi *et al.,* 2015), whereas above 20%, the gels retain a fat-like texture, with increased levels of firmness. The ability of ITF to form gels with fat-like characteristics results from the formation of microcrystals and aggregates, which can occlude a significant amount of water (Tarrega, Torres and Costell, 2011). The crystallization and aggregation of ITFs are highly complex and the rate is

determined by a combination of several factors including the type, concentration, and DP of the ITF, as well as the rate of shear, temperatures used in production, and, critically, the food product in question (Torres, Tarrega and Costell, 2010).

One of the most promising applications of ITF to modify product rheology is the production of milk-based dairy desserts. Tarrega, Torres and Costell, (2011) supplemented both skimmed- and full-fat milk custard with either short-chain (DP 2–10), LC (DP > 23), native inulin (DP 30%–40% < 10 and 60%–70% DP > 10), and a mix of short and LC-ITF, all at 7.5%, looking for changes in rheological properties and microstructure. The results of this study showed that ITF aggregates did not form in the presence of short-chain inulin, whereas the presence of inulin aggregates was detected in both skimmed- and whole-milk custards made using either LC, native, or mixture of ITF, consequently resulting in a more thixotropic, consistent, and elastic final product. Yet, differences in the rate of changes in rheological properties and aggregate formation were seen between different types of ITF (LC-ITF > mix of SC and LC > native) with these effects being more pronounced in skimmed-milk samples than whole-fat milk samples, respectively.

A finding confirmed by both Gonzalez-Tomas, Bayarri and Costell, (2009) and Torres, Tarrega and Costell, (2010) is that the addition of either short-chain, long-chain, or native inulin at 7.5% to milk-based desserts modified the rheology of the final product. However, alterations to final rheology were more pronounced in milk-based desserts made with skimmed milk compared to whole milk. In addition, (Gonzalez-Tomas, Bayarri and Costell, 2009) also tested the effects of the addition of ITF to dairy based-desserts on sensory quality. The authors, noted that while both skimmed-milk and whole-milk samples containing LC-ITF were perceived to have the same levels of creaminess, dairy-based desserts made with LC-ITF were perceived to be rougher. Dairy-based desserts made with the addition of short-chain or native inulin and

skimmed milk were evaluated as less rough and were also associated with higher levels of sweetness, vanilla odour, and flavour.

The differences in the level of thickness detected between whole- and skimmed-milk dairy-based desserts made with ITFs are complex and dependent on the amount of initial fat present and the type of ITF used in production (Gonzalez-Tomas, Bayarri and Costell, 2009). The fat content of skimmed milk vs. full-fat milk is roughly 1.7% vs. 3.5% (McCarthy, Lopetcharat and Drake, 2017). The lower abundance of fat in skimmed milk allows a higher number of larger ITF crystals and aggregates to form resulting in the formation of a more cohesive and stable network due to an increased ability to hold onto water (Torres, Tarrega and Costell, 2010). Levels of roughness detected in dairy-based desserts appear to be highly dependent on the DP of the ITF used in production. It is frequently reported that in dairy-based desserts made with OF or SC-ITF, lower levels of roughness are detected (Bayarri *et al*., 2011; Tarrega, Torres and Costell, 2011; Torres, Tarrega and Costell, 2010) whereas dairy-based desserts made with LC-ITF are perceived to be rougher (Bayarri *et al.,* 2011; Gonzalez-Tomas, Bayarri and Costell, 2009). The differences are likely to occur as a result of an increase in the aggregation and particle size of inulin crystals.

The perceived level of roughness is also dependent on the rate of shear and thermal production methods used. At low shear, the lower level of disruption to the matrices allows the formation of larger crystals and aggregate clusters to occur (Bayarri *et al.,* 2011; Torres, Tarrega and Costell, 2010). While in contrast, at high shear, better dispersion of these aggregates can be achieved. However, it has been reported that even at high shear, compared to a thermally induced gel, both larger particles and broader particle distributions are still detected. Via the repeated application of high shear throughout the production process, a reduction in particle size can facilitate the formation of a finer network (Mensink *et al.,* 2015).

With optimization of the manufacturing process, production of a low-fat dairy-based dessert with acceptable or improved rheology and sensory properties using either or a mixture of both short- and long-chain ITF at 7.5% (w/w) can be achieved.

2.5 Future work and concluding remarks

It is clear from this review that both ITF and scFOS can act successfully as both fat and sugar replacer as well as a viscosity modifier in several different food products ranging from ice cream to bread, cake, shortbread biscuits, fruit juices, and meat. Yet, the degree to which fat and sugar can be successfully replaced with ITF and scFOS is highly dependent on the product formulation and the methods used during production. Therefore, future work should focus on refining both processing parameters and product formulation to optimize the level of supplementation to ensure consumer quality is met. Furthermore, while several studies have suggested that degradation of ITF and scFOS can occur during production, it remains unclear whether these alterations are substantial enough to alter their prebiotic efficacy, and thus should also be the focus of future work.

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Conflict of interest

None to declare.

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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 control 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

3.1 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 ≥ 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; Wilson and Whelan, 2017; Sanders *et al.*, 2019). 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, Ayala-Zavala and Gonzalez-Aguilar, 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.*, 2022).

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*.

3.2 Materials and methods

3.2.1 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 ≥ 18.5 and ≤ 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.

3.2.2 Study design and interventions

The study was designed 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® HIS, 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, Wijeyesekera and Rastall, 2022; Jackson *et al.*, 2022). 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 3.1.

Measurement	Pure inulin		Shortbread		Milk Chocolate		Rice Drink	
	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

Table 3.1. Compositional breakdown of study products per 100 g and per daily portion.

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.

3.3 Outcomes

3.3.1 Primary outcomes

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

3.3.2 Secondary outcomes

3.3.2.1 Bacterial composition and urinary metabolites

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.

3.3.3 Sample collection

3.3.3.1 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).

3.3.3.2 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.

3.3.4 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 3.2. 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.

Probe	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 3.2. Name, sequence, and target group of oligonucleotide probes used for bacterial enumeration

3.3.5 Microbial Profiling

3.3.5.1 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. .

3.3.5.2 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).

3.3.6 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-Toolbox7

3.3.6.1 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.

3.3.7 Ethics

The study was given favourable ethical consent by the University of Reading's Research Ethics Committee (36/2020) (Appendix 3.1). The trial was registered as a clinical trial (**ClinicalTrials.gov:** 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.

3.3.8 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.

3.4 Results

3.4.1 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 3.1). Baseline characteristics are reported in Table 3.3.

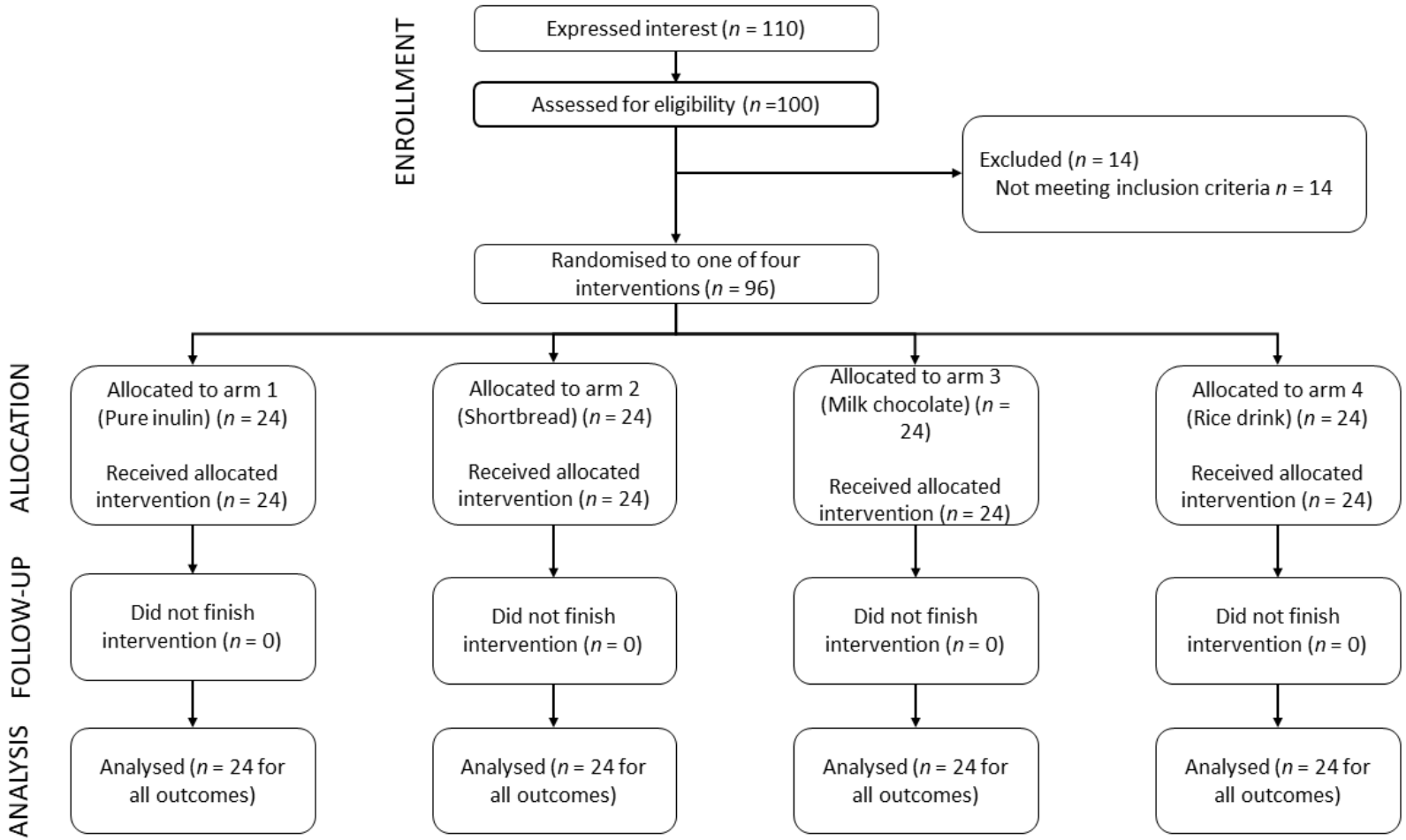

Figure 3.1. CONSORT diagram of participant flow through the trial

Table 3.3 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/m^2)	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 3.3. 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.

3.4.2 Dietary intake

Nutrient data collected at Day 0 and Day 10 of the intervention are presented in Table 3.4.

No significant differences were detected in total energy, protein, carbohydrates, total sugar, starch and PUFAs intakes (Table 3.4). 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 3.4). Follow-up comparisons revealed that dietary fibre intake was significantly greater at Day 10 within each group (all $P \leq 0.001$) (Table 3.4).

	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 3.4. 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

3.4.3 Bacterial enumeration by FISH

96 subjects provided stool samples at baseline and end of the intervention. Figure 3.2 and Figure 3.3 report changes in bacterial counts observed in the four intervention groups between Day 0 and Day 10 of the intervention.

Figure 3.2A 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 $P \leq 0.05$) (Appendix 3.2).

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 3.2B).

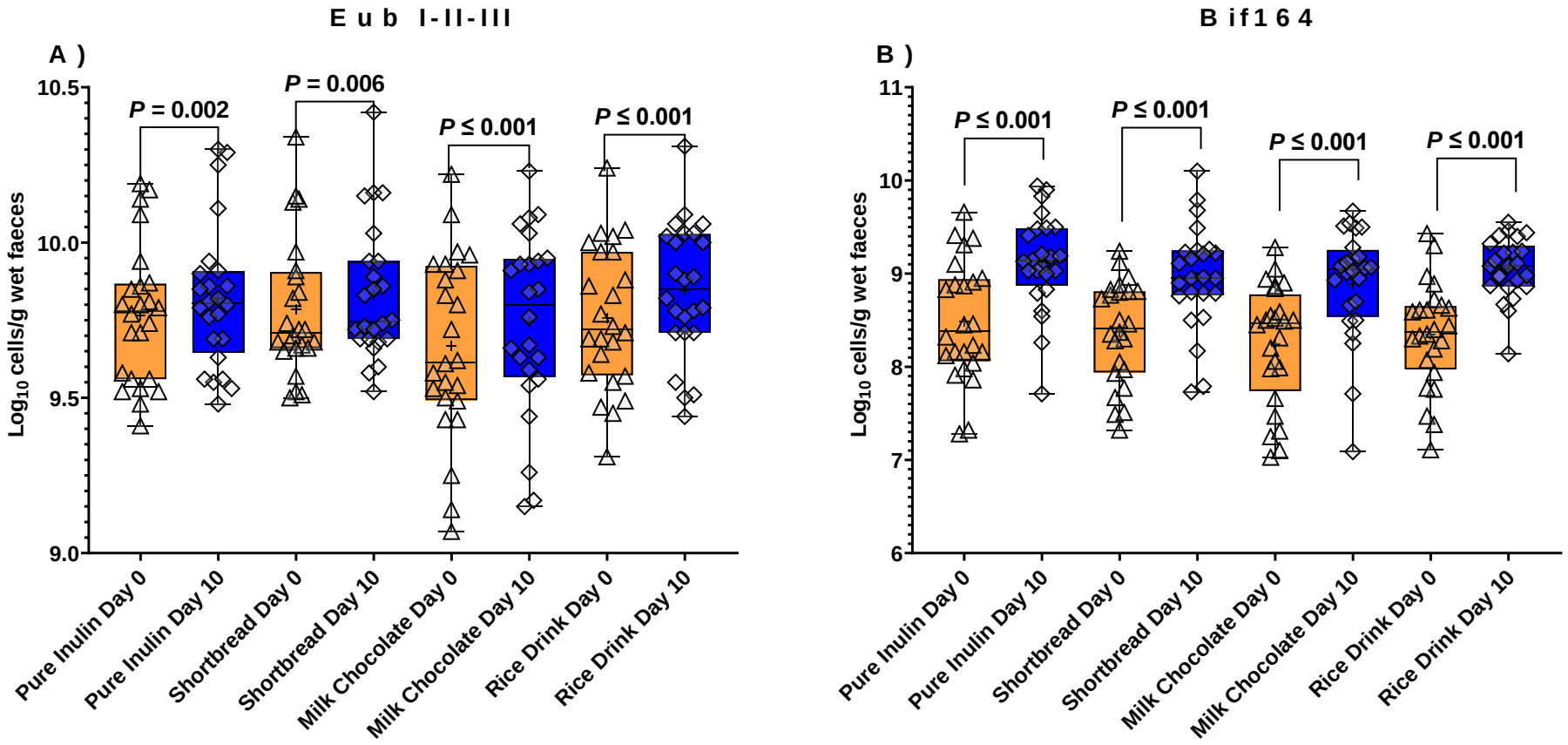

Figure 3.2. Bacterial groups measured by FISH-FLOW (Log_{10} cells/g wet faeces) using probes: **(A)** total bacteria (Eub338 I-II-III), **(B)** *Bifidobacterium* spp. (Bif164). Box and whisker plot - min and max with all points. 96 volunteers (*n* = 24 per group). Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values.

Bacteroides (Bac303) counts are reported in Figure 3.3A. 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) (Appendix 3.2). 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 3.3B).

Additionally, *Faecalibacterium prausnitzii* (Fprau655) (Figure 3.3C) 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 3.3C).

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 (Appendix 3.2).

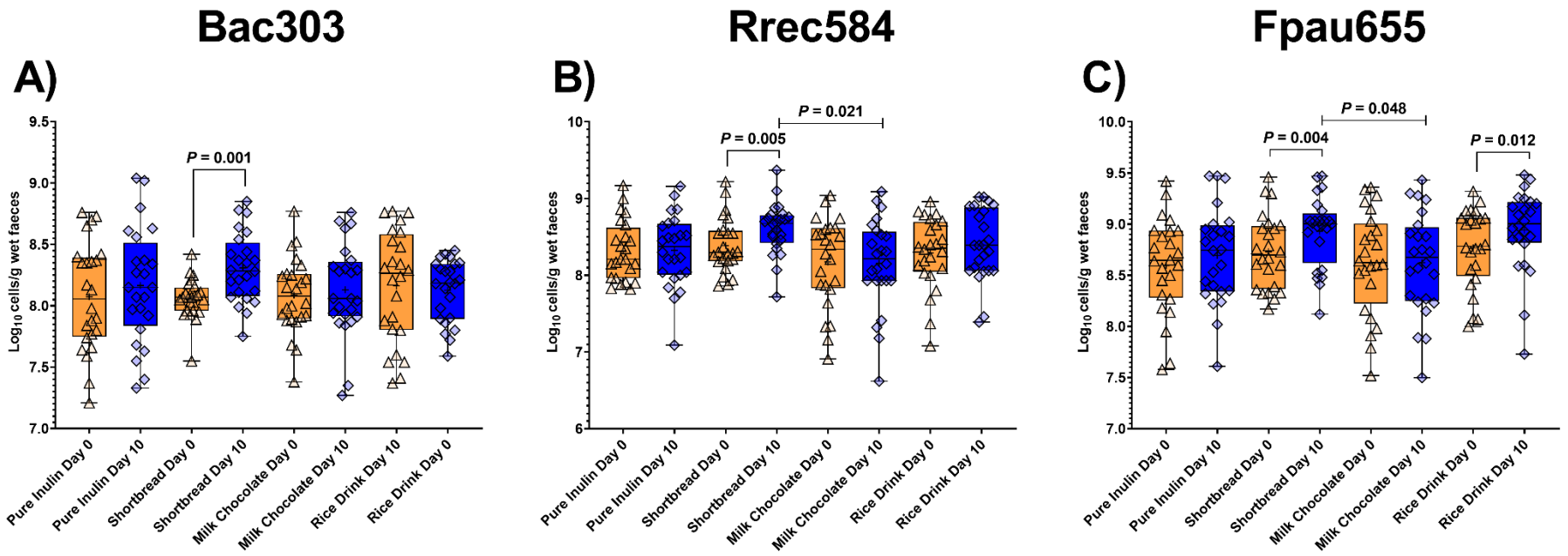

Figure 3.3. Bacterial groups measured by FISH-FLOW (Log_{10} cells/g wet faeces) using probes: **(A)** most *Bacteroidaceae* and *Prevotellaceae* (Bac303), **(B)** *Roseburia* (Rrec584) and **(C)** *Faecalibacterium prausnitzii* (Fprau655). Box and whisker plot - min and max with all points. 96 volunteers (*n* = 24 per group). Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values.

3.4.4 Microbiota Profiling Analysis

Figure 3.4 reports 16S rRNA sequencing results for Relative Microbiome Profiling (RMP) along with Quantitative Microbiome Profiling (QMP) for *Bifidobacterium* data across all four interventions.

3.4.4.1 Relative Microbiome Profiling (RMP)

There were no significant differences in phylum level abundances detected between interventions at Day 10 (Appendix 3.3) (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 $P \leq 0.001$) (Appendix 3.3). 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 $P \geq 0.05$) (Appendix 3.3). 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 3.4A). 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) (Appendix 3.3).

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 ($P = 0.011$) (Appendix 3.4).

3.4.4.2 Quantitative Microbiome Profiling (QMP)

Upon quantification of RMP data no significant differences were detected between groups at Day 10 (all $P \geq 0.05$) (Appendix 3.5). 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 3.4B). 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) (Appendix 3.5).

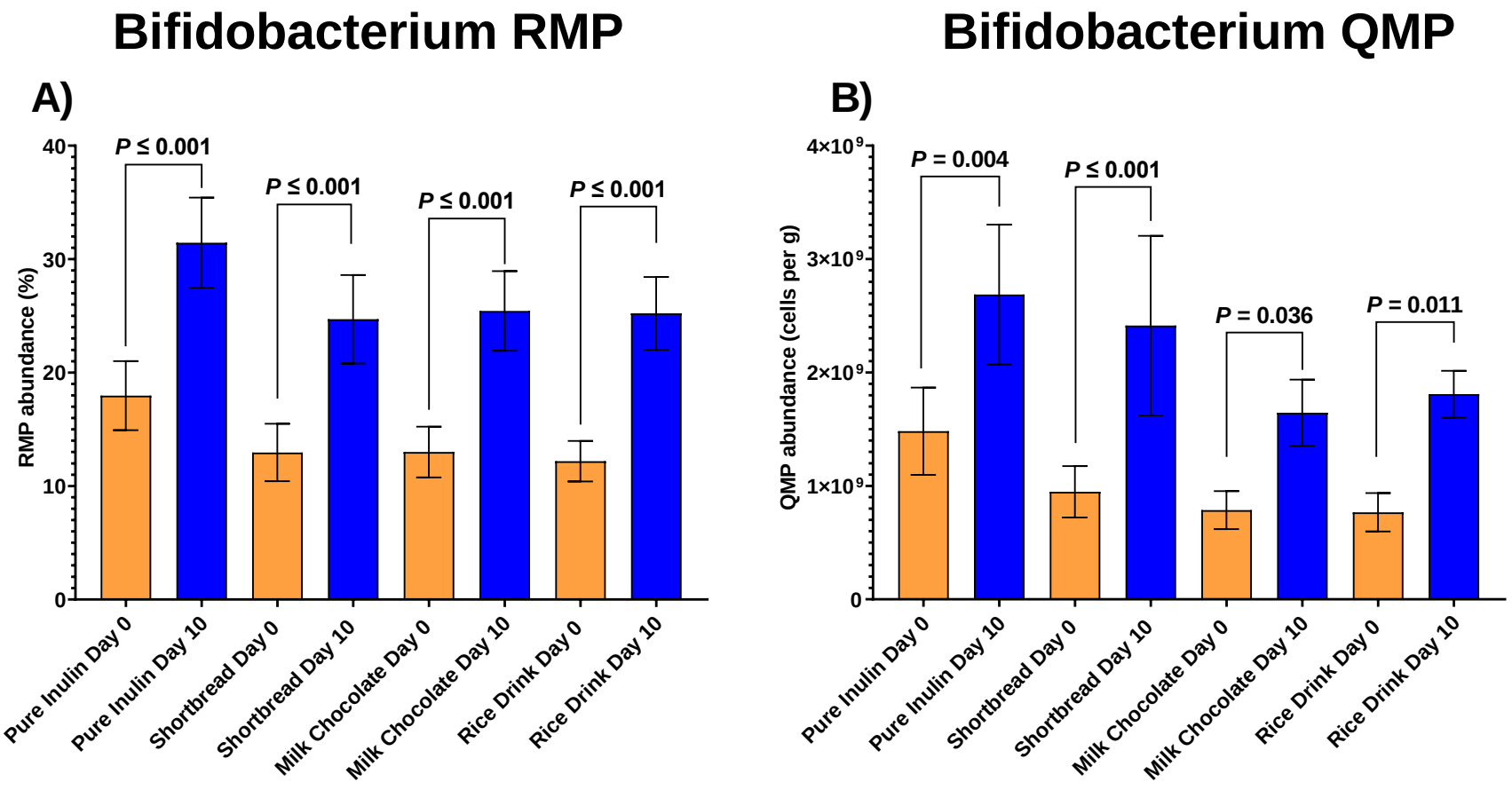

Figure 3.4. Relative Microbiome Profiling (RMP) (A) and Quantitative Microbiome Profiling data (QMP) (B) of *Bifidobacterium* 16SrRNA sequencing results. Mean and standard error (SE). 96 volunteers (*n* = 24 per group). Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values.

3.4.5 ^{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 3.5). 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.

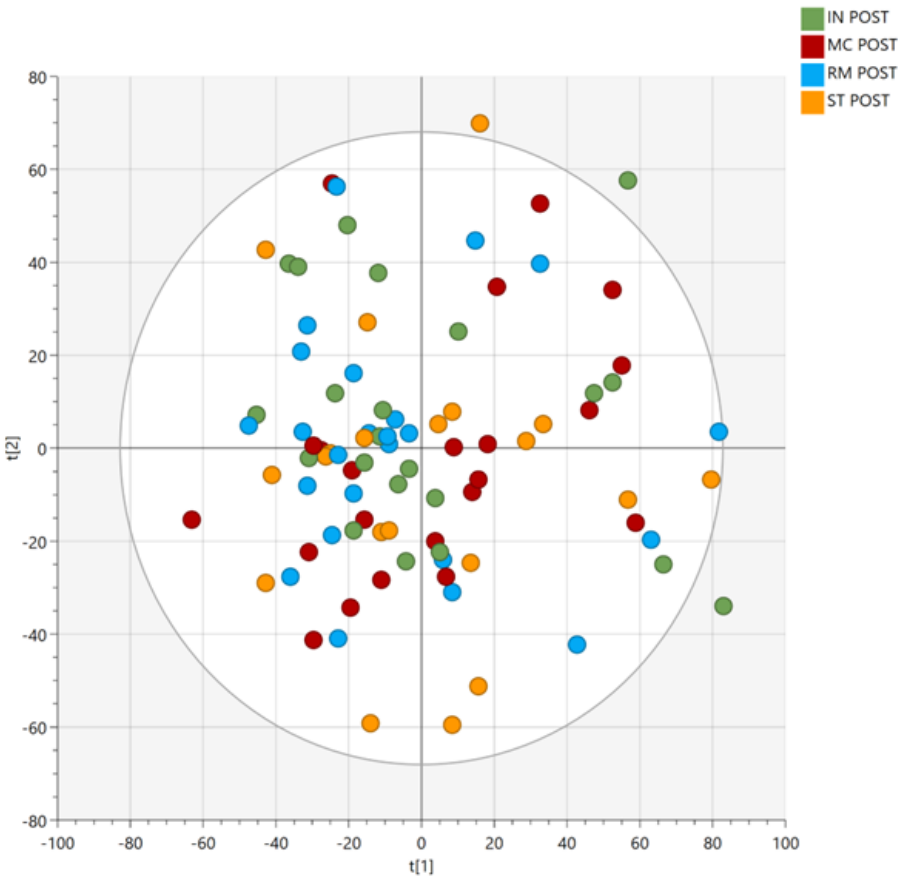

Figure 3.5. Urinary ^{1}H magnetic resonance (^{1}H-NMR) profiles across the four intervention groups. Unsupervised principal components analysis (PCA) scores plot of endpoint urine samples. R^2Cum = 0.18, Q^2Cum = 0.122. Key: IN = Pure inulin; MC = Milk chocolate; RD = Rice Drink; ST = Shortbread

3.4.6 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 3.6. There were no differences in flatulence, intestinal bloating, abdominal pressure, abdominal pain or feeling of fullness detected between interventions at completion (D6-10) (Appendix 3.6), 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 ($P = 0.001$). 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 3.6 and Appendix 3.6).

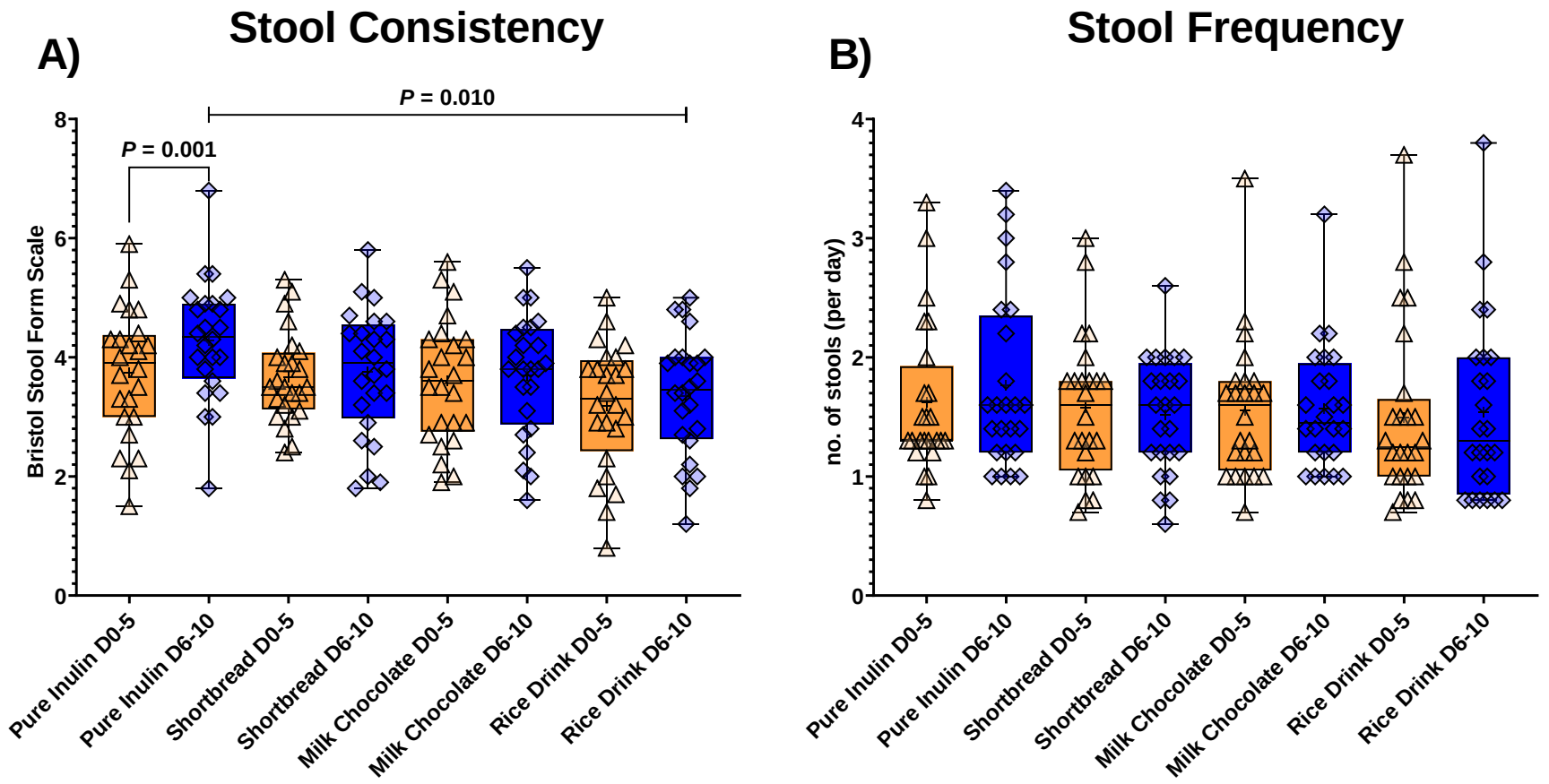

Figure 3.6. Stool consistency (Bristol Stool Form Scale, A) and stool frequency (B) at (Day 0-5) and again at Day 6-10 after intervention in 96 volunteers (*n* = 24 per group). Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values.

3.5 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, Kleessen and Blaut, 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 in

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

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

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

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

Bacteroides possess a large number of loci responsible for the assimilation of complex carbohydrates including arabinoxylans (Pereira *et al.*, 2021) as well as complex starches (Dobranowski and Stintzi, 2021). Arabinoxylans are components of the wheat flour used in production of the shortbread biscuits in this study. From this, one could speculate that the significantly larger increases seen in *Roseburia* and *Faecalibacterium prausnitzii* in the shortbread intervention resulted from the utilisation of resulting motifs from the breakdown of arabinoxylans by *Bacteroides*. For example, it was previously demonstrated by (Walton *et al.*, 2012) that, consumption of *in situ* produced arabinoxylan-oligosaccharides in bread, 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, *g*iven 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 3.4). 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).

3.6 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.

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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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General Discussion

In recent times there is much debate on whether the food matrix matters in the supplementation with prebiotics and the effect on the microbial response of the gut microbiota (Gibson *et al.*, 2017). Of all the current established prebiotics ITF are the most highly researched both *in vitro* and *in vivo* (Costabile *et al.*, 2010; Kolida, Meyer and Gibson, 2007; Gibson and Roberfroid, 1995). Due to the lack of brush border β-fructosidases in the small intestine, combined with bifidobacteria possessing a wide array of glycosyl hydrolyses and ATP-binding cassette (ABC) transport systems, bifidobacteria can efficiently degrade, assimilate and utilise a wide array of low molecular weight carbohydrates including ITF.

Due to their physicochemical properties, ITF can act as both fat and sugar replacers as well as texture modifiers in a wide variety of food products including cakes, biscuits, ice cream, cereals and even meat products while still potentially providing prebiotic dosages (Jackson *et al.*, 2022). Yet, as documented in Chapter 2, these physiochemical properties, depending on the processing parameters used during production, ITF can be subject to degradation, potentially resulting in a loss of prebiotic properties (Jackson, Wijeyesekera and Rastall, 2022). This suggests that ITF use in food products needs to be carefully considered in order to optimise product quality while maintaining ITF integrity.

To date, several different matrices have been supplemented with ITF including biscuits (Tuohy *et al.*, 2001), ice cream (Slavin and Feirtag, 2011), cereal bars (Kleessen *et al.*, 2007) and drinks (Rao, 2001) amongst others. The results of these studies seemingly suggest that prebiotic efficacy of bifidobacteria was unaltered. As previously discussed in Chapter 1, due to

differences in methodological design and analytical techniques, drawing any definitive conclusions based on these findings is difficult. Another aspect that is frequently overlooked, not only in prebiotic but also in probiotic food-based studies, is the additional presence of several bioactive ingredients present within the matrix including polyphenols, polyunsaturated fatty acids (PUFAs), and other fermentable carbohydrates including β-glucan and arabinoxylans (Swann *et al.*, 2020). Each of which have the potential to be selectivity utilised by the gut microbiota. Yet, due to differences in an individual's day-to-day habitual diet, the consequences are still debated. As a result, this study investigated the effects that the food matrix had on the prebiotic efficacy of inulin-type fructans following a standardised protocol. The hypothesis of this study was that the food matrix does not impact on prebiotic efficacy of inulin-type fructans with specific regard to *Bifidobacterium*.

In Chapter 3, we monitored changes in both microbial load and composition using targeted (FISH-FLOW) and untargeted (16S rRNA sequencing) analyses across a 10-day intervention with inulin consumed in either pure form or supplemented into shortbread, milk chocolate and a rice drink. This was intended to reflect the wide array of food matrices consumed as part of habitual diets on a regular basis (Gressier and Frost, 2022; Public Health England, 2021). Targeted analysis via FISH-FLOW revealed significant increases in bifidobacterial counts across all four different matrices with no significant differences detected between interventions at either baseline or on completion. These results were further confirmed by 16S rRNA sequencing with both RMP and QMP abundances also documenting similar increases. These results indicated that the food matrix does not appear to impact of the selectivity effect of ITF towards bifidobacteria and are in line with changes reported in bifidobacterial populations in several previous food-based ITF supplementation studies (Kleessen *et al.*, 2007; Gibson *et al.*, 1995; Tuohy *et al.*, 2001; Marteau *et al.*, 2011).

At completion of the study, significant differences in both *Roseburia* and *Faecalibacterium* counts were detected between the shortbread and milk chocolate intervention. The trends seen using 16s rRNA sequencing reflecting those seen using targeted FISH analysis. A food product is not just one ingredient, but a combination of several ingredients together in order to produce a final desired product (Brighenti *et al.*, 1999; Kleessen *et al.*, 2007; Menne, Guggenbuhl and Roberfroid, 2000). In this regard, several ingredients including wheat flour and rice starch were combined with ITF in order to produce the shortbread biscuits. Wheat flour is a source of several fermentable carbohydrates including the major non-starch polysaccharide arabinoxylan. From this, one could hypothesise that the changes seen in both *Roseburia* and *Faecalibacterium* could have occurred via the direct utilisation of arabinoxylans. It was recently documented that *Roseburia* possess the necessary loci to assimilate and grow in the presence of arabinoxylans (Hillman *et al.*, 2020; Cao and Li, 2022). However, the ability of *Roseburia* to utilise arabinoxylans appears to be highly strain specific with not all *Roseburia* spp. being able to grow on these polysaccharides (Scott *et al.*, 2015).

While not statistically significant from other interventions, large increases in *Bacteroides* were also detected within the shortbread intervention using targeted FISH-FLOW analysis. Of all bacteria present within the gut *Bacteroides* possess the largest number of loci for assimilating a wide array of complex carbohydrates including arabinoxylans (Pereira *et al.*, 2021). It could be speculated that increases in *Roseburia* counts could have resulted from the utilisation of arabinoxylan motifs resulting from the breakdown of arabinoxylans by *Bacteroides*. Yet, as Maki et al., (2012) 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* were detected.

On this basis, increases in *Roseburia* and *Faecalibacterium prausnitzii* often coincide with changes in *Bifidobacterium in vitro* as a result of cross-feeding on acetate and lactate. We did not measure SCFA in this study as faecal samples are not a good indicator of colonic SCFA production, as approximately 95% of SCFA are absorbed into systemic circulation (Ramnani *et al.*, 2010). It is, however, likely that significant increases in acetate and lactate occurred as a result of increases in bifidobacteria. These metabolites probably contributed towards the increases in *Roseburia* and *Faecalibacterium prausnitzii* in the shortbread intervention. Taking this into consideration the greater increases in *Roseburia* and *Faecalibacterium prausnitzii* in the shortbread intervention likely resulted from the direct utilisation of arabinoxylan and arabinoxylan degradation products, as well as acetate and lactate produced via *Bifidobacterium*.

One potential benefit of supplementation into different food matrices seen in this study is the provision of an additional source of dietary fibre. Recent reports suggest that the average UK fibre intake is between 14-18 g/day (Gressier and Frost, 2022). This being significantly below the recommended 30 g/day (Scientific Advisory Committee on Nutrition, 2015). Increasing dietary fibre intakes has been shown to have beneficial effects on a number of health outcomes and in maintaining or improving colonic transit time and bowel frequency (Micka *et al.*, 2017; Vandeputte *et al.*, 2017a). Yet, in this trial we saw no improvements in bowel frequency and only saw changes in stool consistency in the pure inulin intervention. This is understandable given that the effects of ITF on improvements in bowel frequency and consistency is often studied in constipated patients and often correlates with low habitual dietary fibre intake (Buddington *et al.*, 2017; Glibowski *et al.*, 2020; Micka *et al.*, 2017). Our study cohort bowel frequency was already high to start with, combined with above average dietary fibre intakes. Nevertheless, supplementation with ITF resulted not only in significant

increases in dietary fibre but also had no adverse effects on either bowel function or gastrointestinal symptoms and were tolerated by all individuals.

In conclusion this study found that, irrespective of the food matrix, the prebiotic selectivity of ITF towards bifidobacteria appears to be unaffected with similar increases in load and composition being detected across all four interventions. The compositional of the food matrix does, however, stimulate changes in the wider microbiota, with increases in *Bacteroides*, *Roseburia*, and *Faecalibacterium prausnitzii* being detected in shortbread and rice drink interventions but not pure inulin or milk chocolate interventions. The changes documented in *Roseburia*, and *Faecalibacterium prausnitzii* in the shortbread intervention being statically significant from milk chocolate at completion (all $P \leq 0.05$). Supplementation of ITF into differing food matrices also appears to be a viable way of increasing dietary fibre intakes allowing individuals to meet and exceed the minimum the 30 g/day as recommended by SACN without increasing any noticeable impacts on gastrointestinal sensation.

Future perspectives and limitations

In chapter 3, increases in numbers of both *Roseburia* and *Faecalibacterium* were significantly higher in the shortbread and rice drink interventions using targeted analysis. The changes seen using QMP, while following similar trends, did not reach the same levels of significance. QMP is still a relatively new technique that was developed in order to overcome several of the limitations associated with RMP (Vandeputte *et al.*, 2017b). However, one of the limitations of QMP is that it is not possible to log the resulting microbial load data due to the impacts that the library preparation and rarefaction process have on excluding bacteria that would otherwise be captured via targeted analysis. Yet, QMP is able to identify changes in bacteria

that would otherwise be missed by simply relying on RMP or targeted data alone, giving a fairer reflection on the final composition of the microbiota.

A modified version of the validated eNutri food frequency questionnaire (FFQ) designed to capture shorter term changes in dietary response was used in order to collect dietary intake throughout the course of the intervention. One of the limitations of this method of dietary assessment is it relies heavily on accurate recall and documentation of portions sizes from volunteers. This can often result in either the over or underestimation of nutrient intake and may have resulted in over estimation of dietary fibre intakes measured in this study (Resnicow *et al.*, 2000), as fibre intakes in this study were significantly above those documented in the current UK populations (Gressier and Frost, 2022). However, out of all dietary assessments, FFQ, and specifically the validated eNutri app used in this study, provides a wide variety of prompts including pictures and questions in order to obtain as accurate dietary accurate intake as possible (Franco *et al.*, 2022).

Nuclear magnetic resonance (NMR) was used to analyse and identify potential differences between interventions in urinary metabolites. No differences were found at completion of the intervention as no clustering between interventions occurred. This is likely due to the use of healthy adults who did not follow any restrictive diets which would have required much higher population sample sizes to detect any meaningful differences. Additionally, while the bifidogenic effect of inulin-type fructans can be seen in as little as 7 days it can take considerably longer in order to establish longer term effects on the gut microbiota (Leeming *et al.*, 2019).

The food products chosen in this study, while representing a wide degree of food matrices in solid, liquid semi-solid form, are relatively simple in comparison to several ITF-containing food products currently on the market. Thus, future work on the effects of the food matrix on prebiotic efficacy should focus on longer term longitudinal studies in more complex matrices. This would allow us to identify if increasing complexity in the food matrix results in differences in microbial response.

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Appendices

Appendix 3.1 Research ethics application

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Professor Bob Rastall
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Dear Bob, Anisha and Peter

I am pleased to inform you that Professor Richard Frazier has given a favourable opinion for conduct for your study, 'Randomised four-arm trial investigating the effects of different food matrices on the prebiotic efficacy of inulin-type fructans from chicory root' via the in-School exceptions route. This email constitutes your permission to proceed with the studies as described in your application. The following study number has been assigned to your study and you should quote this number in any correspondence you undertake about your studies.

STUDY Number – 36/2020

If you feel that you need to make changes to the way your studies are run, please let us know at the earliest opportunity and we can advise you of whether a formal amendment to your proposal is required or not.
I wish you the best of luck with the projects and finish by reminding you of the need for safe custody of project data at all times (a service that Barbara Parr, copied in, can provide if you require it).

Kind regards
Professor Julie Lovegrove
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Consent Form for Inulin-food matrices study

Please initial boxes

1. I confirm that I have read and understand the Participant Information Sheet dated _____for the above study, which was explained by _____. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason. ☐

3. I authorise the investigator to inform my General Practitioner if any abnormal results are found in relation to my screening results. I will be informed when this happens. ☐

4. I have received a copy of this Consent Form and of the accompanying Participant Information Sheet. ☐

5. I understand that this study has been reviewed by the University of Reading Research Ethics Committee and has been given a favourable ethical opinion for conduct. ☐

6. I have had explained to me that consent for my contact details and personal information to be added to the Hugh Sinclair Unit of Human Nutrition Volunteer Database is entirely voluntary. ☐

7. I understand that the data collected from me in this study will be preserved and will be made available to other authenticated researchers, but only if they agree to maintain the confidentiality of the information provided to them. ☐

Accordingly, I consent as indicated below:

- I consent to my contact details being stored on the Nutrition Unit Volunteer Database. ☐ Yes ☐ No

- I consent to my screening information (including date of birth, height, weight, dietary habits, smoking status, long-term use of medication and pre and probiotics, being stored on the Nutrition Unit Volunteer Database. ☐ Yes ☐ No

Participant details

Name of Participant: _____ Date of Birth: _____

Signature: _____ Date: _____

Address of Participant:

(*Please add if you consent to be part of the Hugh Sinclair Unit of Human Nutrition Volunteer Database*)

_____

_____

Telephone number: _____

General Practitioner (GP) details

Name: _____

Address: _____

Telephone: _____

Witnessed by

Name of researcher taking consent: _____

Signature: _____ Date: _____

PARTICIPANT INFORMATION SHEET

Randomized four-arm trial investigating the effects that the food matrices has on the prebiotic efficacy of ITF from chicory root

You are being invited to take part in a research study. Before you decide if you want to take part it is important that you understand what is involved. Please read the following information and discuss with others if you wish. Please ask us if there is anything you do not understand or if you would like any additional information. Take time to decide whether or not you wish to take part. This human study has been subject to ethical review, according to the procedures specified by the School of Chemistry Food and Pharmacy Research Ethics Committee and has been given a favourable ethical opinion for conduct.

Aim

The aim of this study is to evaluate how the composition of various food products containing inulin-type fructans alters it microbial effects. 92 volunteers will take part in this study who will be randomised into 4 different groups. One group will consume inulin on its own just dissolved in water, teas or coffee. Whereas in the other groups inulin has been fortified into one of three different food products (shortbread cookie, chocolate rice milk or milk chocolate). In addition, a further 10 volunteers will be recruited as reserves. Before you decide whether to take part in the study, please read the following information carefully. If you want to know anything about the study, which is not written here, please ask the investigator.

What are prebiotics?

- Prebiotics are non-digestible fibre that exert bacterial changes in the intestine and bring about health benefits
- They are used in foods to increase beneficial gut bacterial numbers
- They have been found to improve the intestinal bacterial composition of the general population
- They are safe for human consumption
- They have been consumed by humans for hundreds of years

Proposed benefits of prebiotics

- Reduce the number/activities of disease-causing bacteria
- Influence satiety
- Improve immune response
- Improved gut transit time

- Reduce risk of gastrointestinal illness such as travellers' diarrhoea, irritable bowel syndrome, infections
- Increase absorption of minerals and vitamin synthesis

Why is this study being carried out?

Inulin-type fructans are one of the most highly recognised prebiotics where there is a growing trend in the food industry of incorporating these functional carbohydrates into food products in order to not only reduce both fat and sugar intakes but to also stimulate a positive effect on the composition of the gut microbiota primarily in increasing numbers of bifidobacteria. Yet to date, little information exists on whether incorporating inulin-type fructans into different food products alters its prebiotic efficacy. Therefore, the aim of this study is to investigate if the incorporation of inulin-type fructans into food products possessing various compositions alters its prebiotic efficacy.

Inclusion criteria/exclusion criteria

*Inclusion criteria***-** If the following applies to you, you will be considered for participation in the trial:

- *Volunteer is healthy at the time of pre-examination*
- *Volunteer is aged ≥ 18 to ≤ 65 years at the time of pre-examination*
- *Volunteer's BMI is ≥ 18.5 and ≤ 29.9*
- *Volunteer follows an average Western European diet*
- *Volunteer has a stool frequency of at least 3 bowel movements per week*
- *Volunteer is able and willing to comply with the study instructions*
- *Volunteer is suitable for participation in the study according to the investigator/study personnel*
- *Written informed consent is given by volunteer*

Exclusion criteria **-** If the following applies to you, you will be unable to participate in the trial:

- *No command of any local language*
- *Gastrointestinal disorders including IBS, IBD, etc. or other conditions that might affect the gut environment*
- *Food allergies or intolerances*
- *Vegetarians, vegans, and/or extreme diets including high protein/fibre, ketogenic, intermittent fasting and/or carnivore*
- *Use of prebiotics or probiotics (in food products or as supplements) in the last 4 weeks prior to, or during the study period.*
- *Use of laxatives and labelled pre‐and probiotics in the previous 4 weeks before the beginning of intervention*
- *Volunteers currently involved or will be involved in another clinical or food study*
- *Clinically significant diabetes*
- *History of drug (pharmaceutical or recreational) or alcohol abuse*
- *If you have received bowel preparation for investigative procedures in the 4 weeks prior to the study.*
- *If you have undergone surgical resection of any part of the bowel.*
- *If participants are pregnant or are lactating*

What will I be asked to do?

- All participants will be asked to fill out a health screening questionnaire and inclusion/exclusion criteria will be reviewed for volunteer eligibility
- Informed consent from yourself will be required
- On giving consent and passing initial screening, participants' height and weight will be measured
- Once the study begins, participants will be randomly allocated into 1 of 4 groups each assigned a different food product (chocolate rice milk, milk chocolate bar or shortbread cookie) or inulin (sachet) with each portion containing 5 g inulin. Participants will be asked to consume their assigned product twice per day resulting in a total inulin intake of 10g/day.

Volunteers will be asked to consume their assigned product at the same time each day (ideally once in the morning and once in the evening) and not with other food or drink. The length of the intervention is 10 days

- Participants will provide two stool samples: one on day 0 and one on day 10 of the study to identify changes in gut microbiota composition
- Urine Samples will also be provided at day 0 and at day 10 looking for changes in urinary metabolites
- Volunteers will be given containers and specimen pots to take home for initial and final faecal and urine collection. No treatment will be issued until initial stool and urine sample has been provided
- **Maintenance of normal dietary patterns throughout the study is essential** and participants will be required to complete food and drink logs throughout the study via a web-based app
- Any adverse medical events which occur during the trial (e.g. headache, gut symptoms) should be recorded in a diary along with medication taken
- All incidence of respiratory infections, colds or other illness occurring during participation on the study should also be reported
- Daily stool habit should be recorded in a diary
- Please note that participants will be removed from the study if they develop acute gastrointestinal illness (e.g. food poisoning) or intolerance to the supplement/food product or if they do not comply to above stated restrictions

Are there any risks?

The risk of this study is the risk of allergic (adverse) reaction(s) from ingredients found within each of the food products in question. Potential participants will be screened for allergies and any participant found to be at risk of allergic reactions regardless of severity will be excluded from participation in this study.

Inulin is a fermentable dietary fibre and is used world-wide as a food ingredient and as a supplement. Inulin does not pose any risk to participants and has been safely used in our own research and that of others for several years.

Confidentiality

Confidentiality will be maintained by allocating volunteers an identification code, which will be used to identify all samples and data obtained. Volunteer's names will not be used in any reports or publications. All data generated from the study will be held securely within a password

protected file, only the study investigators will have access to. A record of the names of the volunteers will not be held on the same file.

Information matching volunteer names with identification codes will be kept in a locked filing cabinet, the investigators will only use identification codes. The only times data will be matched with volunteer names is for those volunteers that request to have their personal results discussed with them or if they wish to leave the study and want their data to be destroyed. A request for individual results to be discussed will include a review of all sample results for each volunteer. A list of the names and addresses of the subjects in this project will be compiled, this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed. The data collected from me in this study will be preserved and will be made available to other authenticated researchers, but only if they agree to maintain the confidentiality of the information provided to them.

Dietary restrictions during testing

Participants must not consume pre- or probiotic supplements or food products containing them during the study. Participants must not pro- or prebiotic supplements or food products for a minimum of 4 weeks prior to starting the intervention.

Examples of these food products are:

- Danone Actimel yoghurt drink
- Yakult milk drink
- Danone Activia yoghurt
- Kellogg's Rice Krispies multigrain
- Kellogg's all-bran and all-bran prebiotic oaty clusters
- Goodbelly Oat Flakes with Banana & Blueberry
- Weetabix
- Muller Vitality Yoghurt/Drinks

If a participant is unsure if a food product should or should not be consumed during this study they may contact the investigator Peter Jackson at any time on either p.p.j.jackson@pgr.reading.ac.uk or by mobile 000000000 (Phone number currently being processed) or via Food Matrix Effect on Teams

General Information

- You will receive £20 gift voucher for completing the trial and a £10 gift voucher if you are a reserve volunteer who does not receive a treatment. Volunteers that drop out will have their payment pro-rated to cover the part of the study completed.
- Stool sample pots will be provided and advice on how to take stool samples at home will be given
- Analysis of faecal samples will occur at the University of Reading
- You will be provided with enough food product/inulin to last the duration of the study.
- If at any time you wish to withdraw from the study, you are completely free to do so without giving a reasonk
  The information collected will be used for research purposes only. All information will be confidential, and individuals' names will not be used in any reports resulting from this work.
- Once the study has been completed you can request your results
- All unused samples will be destroyed after the completion of the study and sample analysis.

Contact details for further questions, or in the event of a complaint

The University has appropriate insurance and is well used to carrying out these types of trials.

In the event of a complaint, please contact the Principal Investigator, Professor Bob Rastall: r.a.rastall@reading.ac.uk
If he cannot resolve the issue to your satisfaction, the complaint will be taken up with the Head of Department of Food and Nutritional Sciences, Prof. Richard frazier, r.a.frazier@reading.ac.uk.

The investigators thank you for taking time to read this. If you have any queries, please feel free to contact:

Peter Jackson p.p.j.jackson@pgr.reading.ac.uk

Team's account: Food Matrix Effect

Study Timeline

Dates	Stage of Study	Treatment
	V0: Pre-screening visit	• Study briefing: consent will be taken • Medical and lifestyle questionnaire will be taken • Explaining and dispensing of dietary app information • Dispense sample pots for Day-0 faecal and urine collection
Day 0	V1: Start of study	• Provide baseline stool sample • Provide baseline urine samples • Baseline height and weight taken • Dispense bowel habit diaries and web-app food diary information • Dispense food product/inulin sachets. • Dispense sample pots for Day-10 faecal and urine collection • During the 10-day study each food product/inulin sachet will be consumed twice daily. • Food and bowel habit diaries to be completed
Day 10	V2: End of study	• Bowel habit diary to be submitted • Final body weight taken • Confirm food diaries have been submitted. • Provide final faecal and urine samples • Return any unused food/inulin products

Inulin/food matrices study

Medical and Lifestyle Questionnaire

Name:		Title:
Address:		Date of Birth: Age:
Daytime Telephone:	Evening Telephone:	Best time to call:
Weight (kg):	Height (m):	BMI (kg/m^2):
E-mail: Do you use emails on a regular basis? YES/NO		

How did you hear about the study? _____

Please circle as appropriate

<u>Medical questions</u>

1. Have you been diagnosed as having any of the following?

a) High blood cholesterol YES/NO

b) High blood pressure YES/NO

c) Thyroid disorder YES/NO

d) Diabetes or other endocrine disorders YES/NO

e) Heart problems, stroke or any vascular disease in the past 12 months YES/NO

f) Inflammatory diseases (e.g. rheumatoid arthritis) YES/NO

h) Renal, gastrointestinal, respiratory or liver disease? YES/NO

i) Cancer YES/NO

j) Blood disorders YES/NO

k) Haemochromatosis YES/NO

Have you been diagnosed as suffering from any other illness?

YES/NO

If 'YES', please give details

2. Within the past 3 months, have you taken any medication (prescription or non-prescription)?
   YES/NO
   If 'YES', what are they and for what reasons?

3. Have you had any surgery within the past 3 months or do you have surgery planned? YES/NO
   If 'YES', please give details

4. Have you ever suffered from a pulmonary embolism, deep vein thrombosis, blood clots or had a blood transfusion?
   YES/NO
   If 'YES', please give details

5. Do you have a pacemaker?
   YES/NO

6. (Women only) Are you premenopausal, perimenopausal or postmenopausal? *Please circle as appropriate*

If you are premenopausal:

a) Are you using contraception? YES/NO
   If 'YES', please give details (including the name of the contraceptive pill or device)

Do you have regular menstrual cycles?
YES/NO

b) Are you pregnant, lactating or planning a pregnancy in the next year?
YES/NO

Lifestyle questions

7. Are you currently taking part in or within the last 3 months been involved in a clinical trial or a research study?
YES/NO
If 'YES', please give details:

8. Have you been screened or contacted recently about a study?
YES/NO
If 'YES', please give details

9. Do you follow any specific diets (e.g. ketogenic, vegan, vegetarian, carnivore, intermittent fasting)
YES/NO
If 'YES', which diet do you follow.

10. Do you have any food allergies (e.g. gluten, dairy, nuts, soya) or intolerances (e.g. lactose)?
YES/NO
If 'YES', what are they?

11. Do you use any of the following:
    a) Dietary supplements, e.g. fish oils, evening primrose oil, vitamins or minerals? (such as iron or calcium)
       YES/NO
    b) Probiotics, e.g. Actimel, Yakult, Activia yoghurts or capsules?
       YES/NO
    c) Cholesterol-lowering products, e.g. Flora Pro-Activ or Benecol?
       YES/NO
    d) Prebiotics, e.g. inulin, Bimuno?
       YES/NO

If 'YES' to any, please give details

12. Are you following or planning to start a restricted diet, e.g. to lose weight?
    YES/NO
    If 'YES', would you be willing to postpone this until after your final study visit?
    YES/NO

13. Do you drink alcohol? YES/NO
    If 'YES', approximately how many units do you drink per week? _____ *Units*

    One unit of alcohol is half a pint of beer/lager, a single pub measure of spirits e.g. gin/vodka, or a small glass of wine (125 ml).

14. Do you exercise more than three times a week, including walking?
    YES/NO
    If 'YES', please specify the type of exercise, frequency and intensity

15. Do you smoke?
    YES/NO *If 'YES, please give details*

This is the end of the questionnaire - thank you for your time.

All information provided will remain confidential at all times.

Volunteer Diary

Volunteer No. _____

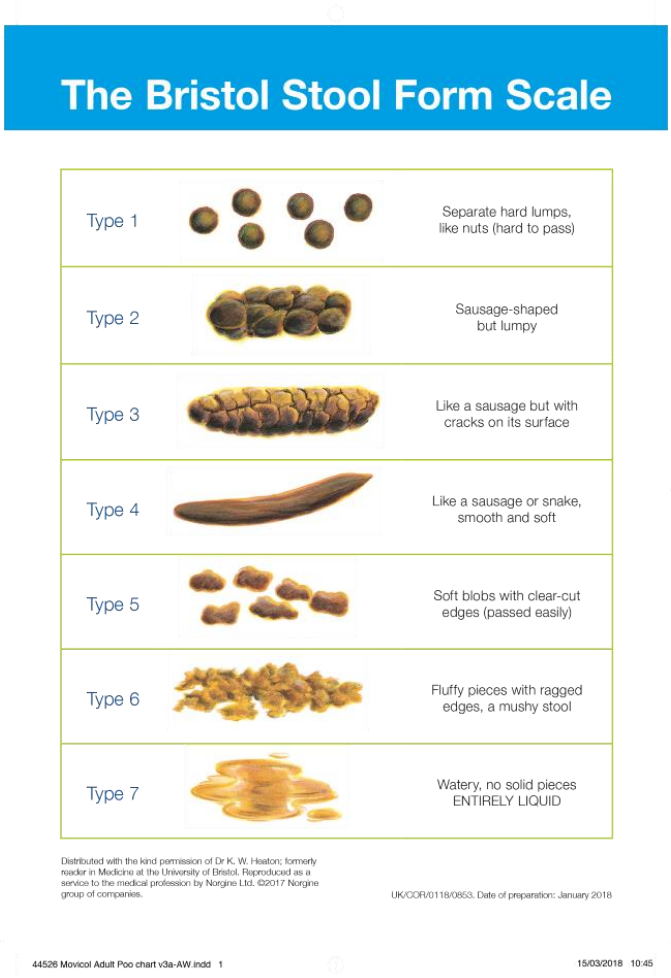
Period No. _____ Day No. to Day No.

Please fill in the diary carefully and completely for each day. If you are unsure how to answer, please give the best information you can. Please return completed diary to Peter Jackson on your next visit.

To be filled in by investigator only!

Date started at:

Next visit at:



The Bristol Stool Form Scale provides a scale relating to stool consistency, please use this chart to rate your stool consistency 1-7 (solid – liquid) in your daily diary.

E.g. a rating of 4 – used in the diary example would relate to “like a sausage or snake, smooth and soft”)

Volunteer number:

Study day	Date d/m	Number of stools (if 0, please include)	Stool consistency as per Bristol chart (page 2)			Study product consumption		Flatulence	Intestinal bloating	Abdominal pressure	Abdominal pain	Feeling of fullness	Comments
						Breakfast	Dinner						
e.g	15/01	2	3	4	X	Yes	Yes	1	1	1	1	1	
1													
2													
3													
4													
5													
6													
7													
8													
9													
10													
11													
12													

Form for Adverse Events

Study name and REC ref number	
Volunteer number	
Principal Investigator	
Study Researcher	

Description of AE	**Category of AE ***	**Date of start**	**Date of end**	**Grading ****	**Date/ time reported**	**Measures taken including medical/ nurse advice/study withdrawal**
				Intensity: Frequency: Relation to study product:		

NB: This form must be completed on the day of the adverse event and sent to all research nurses and unit managers at time of event. This will enable logging of the adverse event and follow up with the volunteer by a nurse.

Form sent to unit managers and nurses: YES / NO completed by: _____ Date: _____

To be completed by a nurse

Followed up by (name): _____ Date: _____

Outcome:

Category of AE*: **1**.Cannula related AE (pain, erythema or swelling)

2a. Upper respiratory **2b**. Lower respiratory

3. Allergy- skin reactions **4**. Gastro – intestinal reactions **5.** Other

Grading**: **Intensity:** light=1; moderate=2; severe=3

Frequency: rare=1; frequent=2; often=3; non applicable=4

Relation to study product: unrelated=1; unlikely=2; probable=3, definitely related = 4

Food Product ingredients list

Milk chocolate

Ingredients
Cocoa butter
Orafti® HSI
Whole milk powder
Sucrose (Table sugar)
Cocoa liquor
Emulsifier (Soy lecithin)
Flavouring

Rice Milk

Ingredients
Cocoa butter
Milk powder
Orafti HIS®
Sugar (sucrose)
Cocoa liquor
Emulsifier (Soy lecithin)
Flavouring

Lincoln Biscuit

Ingredients
Biscuit flour
Orafti® HSI
Vegetable fat
Icing sugar
Water
Remyline AX FG P (rice starch)
Salt
Lecithin
Sodium bicarbonate
SAPP28 (Sodium Acid Pyrophosphate)
Ammonium bicarbonate
Vanilla flavour

How to store and consume inulin/inulin-food product

Inulin sachet storage and consumption sheet

Storage

- Inulin sachets should be stored in their original packaging until time of consumption away from direct sunlight and areas of high moisture

Consumption:

- One sachet: two times per day
- Inulin should not be consumed with any other food or drink products
- Inulin should ideally be consumed at the same time each day:
    - One sachet in the morning and one sachet in the evening
    - Inulin should be consumed 15-60 minutes before a meal, ideally breakfast and dinner. If consumption is forgotten at breakfast inulin should be consumed 15-60 minutes before lunch
- Inulin should be dissolved in cold/room water prior to consumption
- Each sachet of inulin once dissolved must be consumed in a single sitting and within 15 mins of opening

Shortbread cookies storage and consumption sheet

Storage

- Shortbread cookies should be stored in original packaging until time of consumption

- Shortbread biscuits should be stored away from direct sunlight and areas of high moisture and extreme temperature fluctuations

Consumption

- One packet (4 cookies): two times per day
- Shortbread cookies should not be consumed with any other food or drink products
- Shortbread cookies should ideally be consumed at the same time each day:
    - One packet in the morning and one packet in the evening
    - Shortbread cookies should be consumed 15-60 minutes before a meal, ideally breakfast and dinner. If consumption is forgotten before breakfast shortbread cookies should be consumed 15-60 minutes before lunch instead
- Each portion (1 packet) of shortbread cookies must be consumed in a single sitting and within 15 mins of opening

Chocolate rice milk drink storage and consumption sheet

Storage

- Chocolate rice milk drink should be stored in original packaging until time of consumption
- UHT rice milk drink should be stored either in the fridge at 4 °C or kept at room temperature away from areas of direct sunlight and extreme temperature fluctuations

Consumption

- One bottle (300 mL): split into two 150 mL doses
- Chocolate rice milk should not be consumed with any other food or drink products
- Chocolate rice milk should ideally be consumed at the same time each day:
    - One bottle in the morning and one bottle in the evening
    - Chocolate rice milk drink should be consumed 15-60 minutes before a meal, ideally breakfast and dinner. If consumption is forgotten before breakfast chocolate rice milk drink should be consumed 15-60 minutes before lunch instead
- Each portion (150 mL) of chocolate rice milk drink must be consumed in one sitting and within 15 mins of opening

Note

- Chocolate rice milk should be shaken prior to consumption

Milk chocolate bar storage and consumption sheet

Storage

- Milk chocolate should be stored in original packaging until time of consumption

- Milk chocolate should be stored away from direct sunlight, areas of high moisture and extreme temperature fluctuations

Consumption

- 1 bar: 2 times per day
- Milk chocolate should not be consumed with any other food or drink products
- Milk chocolate should ideally be consumed at the same time each day:
    - One bar in the morning and one bar in the evening
    - Milk Chocolate bar should be consumed 15-60 minutes before a meal, ideally breakfast and dinner. If consumption is forgotten before breakfast milk chocolate bar should be consumed 15-60 minutes before lunch instead
- Each portion (1 bar) of milk chocolate must be consumed in one sitting and within 15 mins of opening

Recruitment Poster

University of Reading

Hugh Sinclair Unit of Human Nutrition

Volunteers Required

Help us investigate how different food compositions alter inulin-type fructans functional properties?

We are looking for healthy non-smoking adults:

- Aged 18-65 who follow a typical western style diet
- Willing to consume milk chocolate, chocolate rice milk, shortbread cookies or study nutrient sachet, twice per day for 10 days
- Able to attend 3 study visits at the Hugh Sinclair Unit of Human Nutrition and willing to provide 2 stool and urine samples.

You will be reimbursed for your time

To find out more contact: Peter Jackson
: 00000000000
: p.p.j.jackson@pgr.reading.ac.uk
Or via teams @ Food Matrix Effect

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Investigator context details

Principal Investigator

Professor Bob Rastall

Harry Nursten 2-55

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Team's account

Food Matrix Effect:

Appendix 3.2 Mean bacterial FLOW-FISH populations across the all four interventions at Day 0 and Day 10

	Intervention Group												
	Pure inulin (*n* =24)			Shortbread (*n* =24)			Milk Chocolate (*n* =24)			Rice Drink (*n* =24)			*P* (b)
Probe	**Day 0**	**Day 10**	***P* (a)**	**Day 0**	**Day 10**	***P* (a)**	**Day 0**	**Day 10**	***P* (a)**	**Day 0**	**Day 10**	***P* (a)**	
Eub I-II-III	9.79 (.004)	9.83 (0.04)	**0.002**	9.78 (0.05)	9.82 (0.05)	**0.006**	9.62 (0.06)	9.69 (0.06)	≤ 0**.001**	9.73 (0.04)	9.82 (0.04)	≤ 0**.001**	0.315
Bif164	8.49 (0.13)	9.12 (0.11)	≤ 0**.001**	8.37 (0.11)	8.96 (0.11)	≤ 0**.001**	8.24 (0.13)	8.89 (0.12)	≤ 0**.001**	8.34 (0.12)	9.05 (0.07)	≤ 0**.001**	0.641
Bac303	8.07 (0.09)	8.17 (0.10)	0.243	8.06 (0.03)	8.31 (0.04)	**0.001**	8.09 (0.06)	8.13 (0.08)	0.57	8.13 (0.05)	8.16 (0.09)	0.64	0.201
Erec482	9.20 (0.07)	9.13 (0.08)	0.582	9.18 (0.09)	9.27 (0.07)	0.25	9.11 (0.08)	9.2 (0.07)	0.35	9.18 (0.08)	9.23 (0.08)	0.53	0.291
Rrec584	8.32 (0.08)	8.33 (0.10)	0.923	8.39 (0.07)	8.61 (0.07)	**0.005**	8.18 (0.12)	8.16 (0.12)	0.82	8.29 (0.10)	8.4 (0.09)	0.17	**0.022**
Pro853	8.25 (0.08)	8.32 (0.07)	0.50	8.38 (0.09)	8.39 (0.09)	0.94	8.18 (0.13)	8.11 (0.11)	0.50	8.26 (0.11)	8.36 (0.12)	0.32	0.272
Fprau655	8.6 (0.10)	8.69 (0.10)	0.58	8.73 (0.07)	8.93 (0.07)	**0.004**	8.62 (0.10)	8.62 (0.10)	0.35	8.77 (0.08)	8.94 (0.08)	**0.01**	**0.029**

FLOW-FISH results for probes total bacteria (Eub338 I-II-III), *Bifidobacterium* spp. (Bif164), most *Bacteroidacae* and *Prevotellaceae* (Bac303)*, Clostridium coccoides-Eubacterium rectale* group (Erec452), *Roseburia* (Rrec584), *Clostridial* cluster IX (Prop853) and *Faecalibacterium prausnitzii* (Fprau655). Reported as log10 Cells/gram wet faeces. 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 Day 10 group comparisons utilising Day 0 values as a baseline covariate (orange column)

Appendix 3.3 Relative microbiome profiling (RMP) abundances recorded across all four interventions at Day 0 and Day 10

Phylum	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)	
***Actinomycetota* (*Actinobacteria*)**	23.13 (2.84)	34.55 (4.05)	≤ 0**.001**	17.57 (2.63)	27.72 (3.84)	≤ 0**.001**	18.51 (2.37)	29.47 (3.39)	≤ 0**.001**	16.16 (1.71)	28.92 (3.37)	≤ 0**.001**	0.82
***Bacteroidota* (*Bacteroidetes*)**	3.07 (0.97)	4.80 (1.57)	0.96	1.97 (0.70)	2.80 (0.76)	0.42	2.53 (0.56)	3.29 (0.98)	0.46	1.97 (0.37)	2.47 (0.62)	0.34	0.62
***Pseudomonadota* (*Proteobacteria*)**	2.88 (1.42)	6.56 (3.48)	0.16	1.32 (0.48)	1.09 (0.36)	0.93	1.53 (0.74)	3.21 (1.74)	0.52	4.43 (1.94)	5.50 (2.78)	0.68	0.45
***Verrucomicrobiota* (*Verrucomicrobia*)**	2.69 (1.19)	2.79 (0.90)	≤ 0**.001**	1.35 (0.53)	1.06 (0.31)	0.83	4.38 (1.27)	5.06 (1.99)	0.63	3.45 (1.31)	7.23 (3.26)	**0.01**	0.09
Euryarchaeota	0.89 (0.33)	0.90 7(0.43)	0.97	1.63 (0.61)	0.84 (0.40)	**0.02**	1.05 (0.51)	0.32 (0.17)	**0.03**	1.30 (0.51)	1.37 (0.57)	0.83	0.09
Cyanobacteria	0.01 (0.01)	0.001 (0.001)	0.61	0.03 (0.01)	0.02 (0.02)	0.84	0.04 (0.04)	0.02 (0.02)	0.38	0.02 (0.01)	0.05 (0.04)	0.37	0.54
***Mycoplasmatota* (*Tenericutes*)**	0.25 (0.08)	0.24 (0.12)	0.99	1.08 (0.76)	1.26 (1.06)	0.60	1.05 (0.62)	0.09 (0.05)	**0.01**	0.44 (0.16)	0.38 (0.15)	0.86	0.11
***Bacillota* (*Firmicutes*)**	67.09 (3.19)	50.20 (3.55)	≤ 0**.001**	72.97 (3.16)	59.05 (4.70)	≤ 0**.001**	73.94 (2.14)	62.55 (3.26)	**0.002**	71.30 (3.26)	56.26 (4.03)	≤ 0**.001**	0.40

Relative Microbiome Profiling 16S rRNA (phylum level) from samples collected at Day 0 and Day 10 of the intervention phase. 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 Day 10 group comparisons using Day 0 values as a baseline covariate (orange column)

Genus	Intervention											
	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)	
Bifidobacterium	17.96 (3.04)	31.45 (3.97)	**≤ 0.001**	12.96 (2.53)	24.7 (3.90)	**≤ 0.001**	13.00 (2.24)	25.43 (3.52)	**≤ 0.001**	12.19 (1.79)	25.22 (3.21)	**≤ 0.001**	0.92
Bacteroides	1.59 (0.57)	3.62 (1.34)	0.18	1.20 (0.55)	1.78 (0.55)	0.44	1.48 (0.39)	1.97 (0.71)	0.52	1.06 (0.20)	1.37 (0.40)	0.69	0.28
Prevotella	0.16 (0.06)	0.22 (0.08)	0.70	0.26 (0.09)	0.25 (0.07)	0.96	0.41 (0.12)	0.73 (0.34)	**0.04**	0.32 (0.09)	0.45 (0.16)	0.40	0.64
Alistipes	1.12 (073)	0.48 (0.16)	0.10	0.43 (0.18)	0.42 (0.14)	0.97	0.49 (0.12)	0.55 (0.15)	0.87	0.4 (0.08)	0.37 (0.10)	0.95	0.82
Roseburia	2.96 (0.80)	2.10 (0.41)	0.29	2.61 (0.63)	3.94 (0.92)	0.10	2.80 (0.63)	2.57 (0.42)	0.77	2.48 (0.44)	3.91 (1.32)	0.08	0.64
Clostridium cluster IVXA + IVXB	0.77 (0.11)	0.44 (0.07)	**0.04**	0.76 (0.08)	0.65 (0.09)	0.46	1.07 (0.29)	0.54 (0.12)	**0.001**	0.85 (0.12)	0.52 (0.09)	**0.04**	0.31
Faecalibacterium prausnitzii and relatives	3.74 (1.10)	3.73 (1.20)	0.99	2.27 (0.74)	4.11 (1.30)	0.14	7.76 (2.44)	6.67 (1.89)	0.38	2.62 (0.76)	3.72 (1.31)	0.38	0.37
Ruminococcaceae (excluding Fprau)	12.04 (1.66)	8.59 (1.27)	**0.02**	14.02 (1.88)	10.73 (1.55)	**0.03**	13.89 (1.12)	11.21 (0.94)	0.08	18.01 (2.37)	11.86 (1.45)	**≤ 0.001**	0.21
Ruminococcus 2	2.06 (0.40)	1.86 (0.52)	0.66	2.83 (0.61)	2.41 (0.63)	0.35	3.20 (0.39)	2.19 (0.42)	**0.03**	2.68 (0.32)	1.90 (0.27)	0.09	0.41

Relative Microbiome Profiling 16S rRNA (genus level) from samples collected at Day 0 and Day 10 of the intervention phase. 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 Day 10 group comparisons using D0 values as a baseline covariate (orange column)

Genus (continued)	Intervention												
	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)	
Coprococcus	1.45 (0.29)	1.02 (0.20)	0.15	1.51 (0.22)	1.30 (0.25)	0.49	1.29 (0.19)	1.15 (0.18)	0.65	1.99 (0.35)	1.67 (0.27)	0.28	0.42
Blautia	16.33 (2.03)	9.36 (1.73)	**≤ 0.001**	17.54 (1.82)	10.92 (1.57)	**0.001**	18.37 (2.28)	14.85 (1.97)	0.08	16.21 (1.95)	11.70 (1.57)	**0.03**	0.24
Lactobacillus/ Enterococcus	1.24 (0.51)	1.03 (0.70)	0.89	3.24 (1.25)	5.4 (2.13)	0.16	1.19 (0.51)	3.64 (1.23)	0.11	2.54 (1.18)	1.38 (0.77)	0.45	0.09
Lactococcus	0.18 (0.10)	0.15 (0.06)	0.66	0.17 (0.09)	0.01 (0.01)	**0.04**	0.03 (0.02)	0.04 (0.03)	0.89	0.10 (0.03)	0.03 (0.02)	0.38	0.35
Dorea	2.51 (0.37)	2.08 (0.36)	0.32	3.88 (0.67)	2.61 (0.47)	**0.003**	3.75 (0.57)	3.14 (0.53)	0.16	4.54 (0.79)	3.09 (0.54)	**≤ 0.001**	0.57
Anaerostipes	4.54 (1.17)	4.68 (1.54)	0.85	4.23 (0.88)	3.85 (0.96)	0.61	2.73 (0.62)	3.25 (0.83)	0.50	2.75 (0.52)	2.99 (0.70)	0.75	0.90
Lachnospiraceae incertae sedi	5.77 (0.59)	3.89 (0.70)	**0.001**	5.21 (0.75)	3.10 (0.58)	**≤ 0.001**	3.91 (0.48)	3.6 (0.49)	0.60	4.00 (0.52)	2.95 (0.40)	0.07	0.42
Collinsella	3.00 (0.70)	1.61 (0.51)	**0.05**	2.11 (0.37	1.23 (0.25)	0.22	2.52 (0.61)	2.25 (0.53)	0.70	1.60 (0.27)	2.11 (1.02)	0.47	0.80

Relative Microbiome Profiling 16S rRNA (genus level) from samples collected at Day 0 and Day 10 of the intervention phase. 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 Day 10 group comparisons using D0 values as a baseline covariate (orange column)

Appendix 3.4 α-diversity measures recorded across all four interventions at Day 0 and Day 10

	Pure inulin (*n* =24)			Shortbread (*n* =24)			Milk Chocolate (*n* =24)			Rice Drink (*n* =24)			*P* (b)
Measure	**Day 0**	**Day 10**	***P* (a)**	**Day 0**	**Day 10**	***P* (a)**	**Day 0**	**Day 10**	***P* (a)**	**Day 0**	**Day 10**	***P* (a)**	
Shannon Index	3.23 (0.08)	2.86 (0.14)	**0.003**	3.28 (0.11)	3.06 (0.13)	0.06	3.35 (0.09)	3.14 (0.07)	0.07	3.32 (0.12)	3.06 (0.13)	**0.03**	0.517
Richness (no. of species)	102.70 (6.58)	86.46 (7.33)	**0.01**	110.10 (8.12)	101.40 (7.37)	0.16	102.50 (6.80)	97.58 (4.07)	0.43	120.90 (7.63)	106.70 (6.92)	**0.03**	0.173
Simpson Index	0.08 (0.01)	0.14 (0.03)	**0.01**	0.08 (0.01)	0.11 (0.02)	0.12	0.07 (0.01)	0.10 (0.01)	0.28	0.09 (0.02)	0.12 (0.02)	0.20	0.631

α-diversity measures across the four interventions from samples collected at Day 0 and Day 10. 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 Day 10 group comparisons utilising Day 0 values as a baseline covariate (orange column)

Appendix 3.5 Quantitative microbiome profiling (QMP) data recorded across all four interventions at Day 0 and Day 10

Phylum	Intervention											*P* (b)
	Pure inulin (*n* =24)			Shortbread (*n* =24)			Milk Chocolate (*n* =24)			Rice Drink (*n* =24)			
	Day 0	D 10	*P* (a)	Day 0	D 10	*P* (a)	Day 0	D 10	*P* (a)	Day 0	Day 10	*P* (a)	
Actinomycetota (Actinobacteria)	1.83×10^9 (3.82×10^8)	2.88×10^9 (6.15×10^8)	**0.003**	1.23×10^9 (2.37×10^8)	2.36×10^9 (6.83×10^8)	**0.001**	1.05×10^9 (1.83×10^8)	1.92×10^9 (2.91×10^8)	**0.02**	1.05×10^9 (1.80×10^8)	2.10×10^9 (2.41×10^8)	**0.003**	0.61
Bacteroidota (Bacteroidetes)	3.00×10^8 (1.42×10^8)	4.51×10^8 (2.31×10^8)	0.19	1.63×10^8 (8.50×10^7)	1.57×10^8 (4.32×10^7)	0.96	1.27×10^8 (3.00×10^7)	1.96×10^8 (5.04×10^7)	0.54	1.24×10^8 (2.54×10^7)	2.08×10^8 (5.75×10^7)	0.46	0.34
Pseudomonadota (Proteobacteria)	1.63×10^8 (7780×10^7)	3.92×10^8 (2.15×10^7)	0.24	8.15×10^7 (3.18×10^7)	5.51×10^7 (1.87×10^7)	0.89	6.39×10^7 (2.77×10^7)	1.00×10^8 (7.00×10^7)	0.27	2.31×10^8 (1.17×10^8)	4.49×10^8 (2.69×10^8)	0.65	0.40
Verrucomicrobiota (Verrucomicrobia)	1.35×10^8 (5.38×10^7)	1.37×10^8 (4.60×10^7)	0.90	2.06×10^8 (7.55×10^7)	4.22×10^8 (1.66×10^8)	0.03	6.56×10^7 (2.69×10^7)	6.25×10^7 (2.38×10^7)	0.97	2.58×10^8 (7.18×10^7)	4.12×10^8 (1.79×10^8)	0.11	**0.01**
Euryarchaeota	3.97×10^7 (1.31×10^7)	3.94×10^7 (1.68×10^7)	0.99	7.90×10^7 (2.81×10^7)	5.70×10^7 (3.24×10^7)	0.46	3.97×10^7 (1.85×10^7)	1.49×10^7 (7.71×10^6)	0.40	6.90×10^7 (2.88×10^7)	1.35×10^8 (6.49×10^7)	**0.03**	0.13

Quantitative Microbiome Profiling 16S rRNA (phylum level) from samples collected at Day 0 and Day 10 of the intervention phase. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces. **(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 Day 10 group comparisons using Day 0 counts as a baseline covariate (orange column)

Phylum (continued)	Intervention											
	Pure inulin (*n* =24)			Shortbread (*n* =24)			Milk Chocolate (*n* =24)			Rice Drink (*n* =24)			*P* (b)
	Day 0	D 10	*P* (a)	Day 0	D 10	*P* (a)	Day 0	D 10	*P* (a)	Day 0	Day 10	*P* (a)	
Cyanobacteria	9.25×10^5 (4.84×10^5)	4.52×10^5 (4.52×10^5)	0.46	1.62×10^6 (8.06×10^5)	9.57×10^6 (7.11×10^5)	0.57	1.54×10^6 (1.49×10^6)	9.50×10^5 (8.72×10^5)	0.62	2.09×10^6 (1.38×10^6)	1.73×10^6 (1.30×10^6)	0.76	0.59
***Mycoplasmatota* (*Tenericutes*)**	1.36×10^9 (5.00×10^8)	9.45×10^8 (4.17×10^8)	0.85	3.60×10^9 (2.38×10^9)	4.26×10^9 (3.32×10^9)	0.77	5.82×10^9 (4.03×10^9)	5.28×10^8 (3.10×10^8)	**0.02**	2.63×10^9 (8.04×10^8)	3.87×10^9 (1.71×10^8)	0.58	0.38
***Bacillota* (*Firmicutes*)**	4.64×10^9 (5.87×10^8)	3.78×10^9 (5.69×10^8)	**0.02**	5.26×10^9 (7.83×10^8)	3.97×10^9 (4.79×10^9)	≤ **0.001**	4.26×10^9 (6.41×10^8)	4.44×10^9 (6.21×10^8)	0.61	4.78×10^9 (6.51×10^8)	4.55×10^9 (5.87×10^8)	0.51	0.74

Quantitative Microbiome Profiling 16S rRNA (phylum level) from samples collected at Day 0 and Day 10 of the intervention phase. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces. **(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 Day 10 group comparisons using Day 0 counts as a baseline covariate (orange column)

Genus	Intervention											
	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)	
Bifidobacterium	1.48×10^9 (3.85×10^8)	2.68×10^9 (6.16×10^8)	**0.004**	9.48×10^8 (2.27×10^8)	2.41×10^9 (7.9×10^8)	≤ **0.001**	7.86×10^8 (1.67×10^8)	1.64×10^9 (2.92×10^8)	**0.04**	7.76×10^8 (1.69×10^8)	1.81×10^9 (2.07×10^8)	**0.01**	0.47
Bacteroides	1.42×10^8 (7.57×10^7)	3.63×10^7 (1.92×10^8)	0.38	1.15×10^8 (7.54×10^7)	1.08×10^8 (3.10×10^7)	0.93	7.63×10^7 (2.13×10^7)	1.08×10^8 (3.06×10^7)	0.69	6.41×10^7 (1.46×10^7)	1.10×10^8 (3.24×10^7)	0.56	0.19
Prevotella	9.85×10^6 (4.04×10^6)	1.99×10^7 (1.18×10^7)	0.54	1.28×10^7 (4.62×10^7)	1.41×10^7 (4.44×10^6)	0.93	1.84×10^7 (3.80×10^6)	5.65×10^7 (2.99×10^7)	**0.02**	2.83×10^7 (1.33×10^7)	4.51×10^7 (2.08×10^7)	0.31	0.36
Alistipes	1.38×10^8 (1.12×10^8)	4.65×10^7 (2.66×10^7)	0.12	2.93×10^7 (1.27×10^7)	2.58×10^7 (9.39×10^6)	0.95	2.40×10^7 (5.88×10^6)	3.32×10^7 (7.80×10^6)	0.87	2.43×10^7 (5.82×10^6)	3.03×10^7 (8.84×10^6)	0.92	0.80
Roseburia	1.89×10^8 (5.00×10^7)	2.02×10^8 (6.15×10^7)	0.89	1.88×10^8 (6.40×10^7)	3.12×10^8 (9.37×10^7)	**0.03**	2.24×10^8 (6.51×10^7)	2.16×10^8 (5.11×10^7)	0.88	1.54×10^8 (3.09×10^7)	2.46×10^8 (6.23×10^7)	0.10	0.68
Clostridium cluster IVXA + IVXB	5.88×10^7 (1.15×10^7)	3.71×10^7 (8.45×10^6)	**0.01**	5.33×10^7 (8.37×10^6)	4.69×10^7 (7.66×10^6)	0.42	5.40×10^7 (1.13×10^7)	3.70×10^7 (9.08×10^6)	**0.03**	5.43×10^7 (8.70×10^6)	3.90×10^7 (6.16×10^6)	0.06	0.79
Faecalibacterium prausnitzii and relatives	2.73×10^8 (7.37×10^7)	3.83×10^8 (1.18×10^7)	0.27	1.36×10^8 (3.50×10^7)	2.54×10^8 (5.96×10^7)	0.23	4.61×10^8 (1.20×10^8)	4.94×10^8 (1.44×10^8)	0.74	2.03×10^8 (5.69×10^7)	3.09×10^8 (8.04×10^7)	0.23	0.42

Quantitative Microbiome Profiling 16S rRNA (genus level) from samples collected at Day 0 and Day 10 of the intervention phase. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces**. (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 Day 10 group comparisons using Day 0 as a baseline covariate (orange column)

Genus (continued)	Intervention											
	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)	
Ruminococcaceae (excluding Fprau)	7.78×10^8 (1.25×10^8)	6.64×10^8 (1.34×10^7)	0.43	1.02×10^9 (2.51×10^8)	7.16×10^8 (1.03×10^8)	**0.04**	7.74×10^8 (1.19×10^8)	7.73×10^8 (1.27×10^8)	0.99	1.28×10^9 (2.28×10^8)	1.00×10^8 (1.63×10^7)	0.06	0.30
Ruminococcus 2	1.60×10^8 (4.56×10^7)	1.61×10^8 (5.74×10^7)	1.00	2.53×10^8 (9.56×10^7)	1.97×10^8 (5.48×10^7)	0.25	2.03×10^8 (4.31×10^7)	1.57×10^8 (3.83×10^7)	0.34	1.67×10^8 (2.31×10^7)	1.41×10^8 (1.96×10^7)	0.59	0.85
Coprococcus	1.10×10^8 (3.17 x 107)	6.74×10^7 (1.25×10^7)	0.11	8.76×10^7 (1.27×10^7)	8.69×10^7 (1.71×10^7)	0.98	6.72×10^7 (1.25×10^7)	8.12×10^7 (1.76×10^7)	0.60	1.16×10^8 (2.18×10^7)	1.56×10^8 (3.65×10^7)	0.13	0.36
Blautia	1.15×10^9 (2.04 x 108)	7.08×10^8 (1.65×10^8)	**0.01**	1.39×10^9 (2.83×10^8)	8.93×10^7 (1.72×10^8)	**0.003**	1.11×10^9 (2.57×10^8)	1.11×10^9 (2.33×10^8)	0.98	1.00×10^9 (1.41×10^8)	9.35×10^8 (1.62×10^8)	0.83	0.50
Lactobacillus/Enterococcus	1.00×10^8 (5.37×10^7)	4.77×10^7 (2.60×10^7)	0.71	2.50×10^8 (1.02×10^8)	4.56×10^8 (2.16×10^8)	0.15	6.87×10^7 (3.21×10^7)	2.70×10^8 (1.12×10^7)	0.16	2.41×10^8 (1.46×10^8)	1.00×10^8 (5.41×10^7)	0.32	0.10
Lactococcus	1.29×10^7 (7.92×10^6)	1.29×10^7 (5.91×10^6)	1.00	1.18×10^7 (7.14×10^6)	8.13×10^5 (3.82×10^5)	0.09	1.03×10^6 (6.61×10^5)	2.74×10^6 (1.75×10^6)	0.80	7.27×10^6 (3.18×10^6)	1.72×10^6 (8.50×10^5)	0.39	0.21
Dorea	2.11×10^8 (6.31×10^7)	1.70×10^8 (3.58×10^7)	0.18	2.89×10^8 (6.85×10^7)	2.25×10^8 (5.28×10^7)	0.05	2.03×10^8 (4.52×10^7)	2.06×10^8 (4.46×10^7)	0.95	2.57×10^8 (4.32×10^7)	2.40×10^8 (4.15×10^7)	0.44	0.68

Quantitative Microbiome Profiling 16S rRNA (genus level) from samples collected at Day 0 and Day 10 of the intervention phase. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces**. (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 Day 10 group comparisons using Day 0 as a baseline covariate (orange column)

Genus (continued)	Intervention											
	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)	
Anaerostipes	3.20×10^8 (8.06×10^7)	2.86×10^8 (7.38×10^7)	0.59	2.90×10^8 (5.92×10^7)	3.16×10^8 (9.73×10^7)	0.67	1.47×10^8 (3.90×10^7)	2.05×10^8 (5.46×10^7)	0.34	1.84×10^8 (5.19×10^7)	2.46×10^8 (7.32×10^7)	0.31	0.80
Lachnospiraceae incertae sedi	4.19×10^8 (8.89×10^7)	2.76×10^8 (6.58×10^7)	≤ **0.001**	3.56×10^8 (6.87×10^7)	2.42×10^8 (5.01×10^7)	**0.01**	2.13×10^8 (3.90×10^7)	2.41×10^8 (4.57×10^7)	0.49	2.53×10^8 (4.27×10^7)	2.26×10^8 (3.69×10^7)	0.52	0.91
Collinsella	1.99×10^8 (5.77×10^7)	1.00×10^8 (3.50×10^7)	**0.04**	1.25×10^8 (2.14×10^7)	8.52×10^7 (1.65×10^7)	0.41	1.22×10^8 (2.75×10^7)	1.52×10^8 (4.56×10^7)	0.53	1.18×10^8 (3.06×10^7)	1.60×10^8 (6.33×10^7)	0.39	0.54

Quantitative Microbiome Profiling 16S rRNA (genus level) from samples collected at Day 0 and Day 10 of the intervention phase. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces**. (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 Day 10 group comparisons using Day 0 as a baseline covariate (orange column)

Appendix 3.6 Gastrointestinal sensation and bowel habit scores across the four interventions at Day 0 and Day 10

GI sensation/ Bowel habit	Intervention Group												*P* (b)
	Pure inulin (*n* =24)			Shortbread (*n* =24)			Milk Chocolate (*n* =24)			Rice Drink (*n* =24)			
	Day 0-5	Day 6-10	*P* (a)	Day 0-5	Day 6-10	*P* (a)	Day 0-5	Day 6-10	*P* (a)	Day 0-5	Day 6-10	*P* (a)	
Flatulence	0.80 (0.11)	0.81 (0.12)	0.0923	0.88 (0.10)	0.88 (0.13)	0.99	0.78 (0.11)	0.66 (0.10)	0.21	0.81 (0.12)	0.95 (0.14)	0.18	0.262
Intestinal Bloating	0.37 (0.09)	0.32 (0.11)	0.625	0.53 (0.10)	0.58 (0.14)	0.70	0.44 (0.11)	0.39 (0.10)	0.62	0.34 (0.08)	0.39 (0.10)	0.63	0.657
Abdominal Pressure	0.27 (0.10)	0.23 (0.07)	0.657	0.37 (0.08)	0.34 (0.11)	0.75	0.21 (0.06)	0.18 (0.05)	0.69	0.26 (0.07)	0.31 (0.08)	0.54	0.655
Abdominal Pain	0.19 (0.08)	0.17 (0.05)	0.744	0.26 (0.07)	0.26 (0.09)	0.93	0.08 (0.02)	0.10 (0.05)	0.79	0.13 (0.05)	0.23 (0.07)	0.15	0.576
Feeling of Fullness	0.65 (0.10)	0.38 (0.07)	**0.002**	0.72 (0.11)	0.65 (0.15)	0.39	0.63 (0.12)	0.58 (0.12)	0.56	0.77 (0.10)	0.77 (0.11)	0.96	0.058
Stool Consistency	3.74 (0.22)	4.29 (0.21)	0.001	3.69 (0.16)	3.76 (0.22)	0.60	3.60 (0.21)	3.69 (0.21)	0.57	3.18 (0.22)	3.35 (0.21)	0.31	**0.017**
Stool Frequency	1.62 (0.13)	1.77 (0.15)	0.08	1.58 (0.12)	1.52 (0.10)	0.45	1.55 (0.12)	1.57 (0.11)	0.84	1.50 (0.15)	1.54 (0.15)	0.58	0.759

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 Day 10 group comparisons using Day 0 scores as a baseline covariate (orange column)

University of Reading

An exploration of the prebiotic concept: from food matrix to mood state

Volume II: Effect of prebiotics and prebiotic candidates on microbial composition and mood state

A thesis submitted as a partial fulfillment for the degree of Doctor of Philosophy

Food and Nutritional Sciences

Peter Jackson

February 2023

Table of Contents

General abstract

There is much interest in whether blending inulin-type fructans with other novel oligosaccharides including β-glucan and 2'fuscosyllactose can result in a more controllable approach in manipulating microbial composition and resulting metabolites. Additionally, anxiety and depression are two of the biggest mental health issues worldwide. Due to the relationship existing between the gut and the brain one way to potentially influence mood state may be through targeted manipulation of the gut microbiota. Yet, much remains unknown about the ability of prebiotics to influence mood state.

To test our hypothesis that blending the prebiotic inulin-type fructans with β-glucan and 2'fuscosyllactose would result in a more controllable approach in targeted manipulation of the gut microbiota and resulting metabolites. We firstly conducted *in vitro* batch culture fermentations monitoring changes in microbial load, organic acid and neurotransmitter production. Results demonstrating that combinations of oligofructose/β-glucan and oligofructose/2'fuscosyllactose increased numbers of *Roseburia*, *Clostridum* cluster IX and *faecalibacterium*. Along with sustained propionate and butyrate production compared to sole supplementation ($P \leq 0.05$). While sole oligofructose and oligofructose/2'fucosyllactose combination induced physiologically relevant increases in γ-aminobutyric acid production ($P \leq 0.05$).

Next, we conducted a 5-week, 4-arm parallel, randomised placebo-controlled trial looking for difference in microbial composition and mood state parameters using sole oligofructose and 2'fucosyllactose and combination of oligofructose/2'fucosyllactose as interventions. Considerable differences in microbial composition were detected between substrates with

both oligofructose and oligofructose/2'fucosyllactose combinations inducing large microbial shifts in *Bifidobacterium*, *Roseburia* and *faecalibacterium* ($P \leq 0.05$). Along with remarkable improvements in mood state parameters ($P \leq 0.05$) compared to sole 2'FL supplementation.

In conclusion, the results suggests of this thesis suggest that combinations of oligofructose with β-glucan and 2'fucosyllactose can provide a more controlled approach to targeted manipulation of the gut microbiota and resulting metabolites. Along with inducing meaningful improvements in microbial composition and mood state parameters *in vivo.*

List of Tables

List of Figures

List of Appendices

Abbreviations

2’FL	2’fucosyllactose
3’FL	3’fucosyllactose
3’S3FL	3’-Sialyl-3-fucosyllactose
3’SL	3'-sialyllactose
3’SLN	3′sialyllactosamine
6’SL	6'-sialyllactose
ABC	ATP-binding cassette
ASV	Amplicon Sequencing Variants
ATP	Adenosine triphosphate
BDI	Becks’ Depression Inventory
BMI	Body Mass Index
$CaCl_2.6H_2O$	Calcium Chloride hexahydrate
CAR	Cortisol awakening response
DFL	Difucosyllactose
DF-LNnH	Difucosylated lacto-*N*-neohexaose
DSLNT	Disialyllacto-*N*-tetraose
DSS	Dextran sulphate sodium
FDR	False discovery rate
FID	Free induction decay
FISH-FLOW	Fluorescence *in situ* hybridization – flow cytometry
FOS	Fructo-oligosaccharides
FSC-A	forward scatter area
Fuc	Fucose
GABA	γ-aminobutyric acid
GAD-7	Generalised Anxiety Disorder Assessment-7

Gal	Galactose
GC-FID	Gas chromatography – flame ionisation detection
GH	Glycosidase hydrolase
GI	gastrointestinal
GlcNAc	*N*-acetylglucosamine
GLN	General linear model
Glu	Glucose
GOS	Galacto-oligosaccharides
HCl	Hydrochloric acid
HMO	Human milk oligosaccharides
HP	High performance
HPA	Hypothalamic-pituitary-adrenal axis
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HPLC-MS	High-performance liquid chromatography - mass spectrometry
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ITF	Inulin-type fructan
K_2HPO_4	Dipotassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
Lac	Lactose
LacNAc	*N*-acetyllactosamine
LDFT	Lactodifucotetrarose
LMM	Linear marginal model
LNB	Lacto-*N*-biose
LNDFH I	Lacto-*N*-difucohexaitol I
LNDFH I	Lacto-N-difucohexaose I

LNDFH II	Lacto-N-difucohexaose II
LNFP I	Lacto-*N*-fucopentaose
LNFP II	Lacto-*N*-fucopentaose II
LNFP III	Lacto-*N*-fucopentaose III
LNFP V	Lacto-*N*-fucopentaose V
LNnH	Lacto-*N*-neohexaose
LNnT	Lacto-*N*-neotetraose
LNT	Lacto-*N*-tetraose
LST a	Sialyllacto-*N*-tetraose a
LST b	Sialyllacto-*N*-tetraose b
LST c	Sialyllacto-*N*-tetraose c
MALDI FT-ICR MS	Matrix-assisted laser desorption/ionization fourier-transform ion cyclontron resonance – mass spectrometry
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization-time of flight – mass spectrometry
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
NA	Negative Affect
NaCl	Sodium Chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium Hydroxide
NEC	Necrotising enterocolitis
Neu5Ac	Sialic acid
nHPLC-PGC-chip-TOF MS	nano-high performance liquid chromatography - porous graphitised - carbon - chip - time of flight – mass spectrometry
NMR	Nuclear Magnetic Resonance
NR	Non-responder
OF	Oligofructose
OFI	Oligofructose inulin

PA	Positive Affect
PANAS-SF	Positive and Negative Affect Schedule – Short Form
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PHQ-9	Patient Health Questionnaire-9
PSQI	Pittsburgh Sleep Quality Index
QMP	Quantitative microbiome profiling
RCT	Randomised Controlled Trial
REDCap	Research Electronic Data Capture
RES	Responder
SCFA	Short-chain fatty acids
SHIME	Simulator of Human Intestinal Microbial Ecosystem
SPSS	Statistical Package for Social Science version
SSC-A	Side scatter area
STAI	State Trait Anxiety Inventory

1.1 Study rationale and hypothesis

There is a growing interest amongst the scientific community on finding means of more controlled and selective ways of stimulating propionate and butyrate production. This in part due to the high levels of selectivity that current prebiotics namely inulin-type fructans display towards bifidobacteria, where solely relying on cross-feeding to result in the generation of propionate and butyrate can lead to random generation of these beneficial compounds. While, several other oligosaccharides have been investigated for their prebiotic potential including β-glucan and human milk oligosaccharides the data regarding their prebiotic efficacy remains unclear. Thus, one way to potentially overcome the issues associated with an overreliance on cross-feeding may be by combining prebiotics with other potential prebiotic oligosaccharides hopefully leading to a more controlled approach to selective fermentation. In addition, anxiety and depression are two of the most common mental health disorders worldwide costing the health services billions of pounds per year. While, there are also increasing concerns with the side effects associated with long-term antidepressant medication. As a result in order to try and overcome the economic burden associated with increasing cases of mental illnesses more novel and cost-effective treatments including probiotics and prebiotics are currently being explored.

This study firstly investigated the influence that combining the prebiotic inulin-type fructans with prebiotic candidates β-glucan and 2'fucosyllactose had on microbial composition, organic acid and neurotransmitter production. This was investigated using 48 h *in vitro* batch culture fermentations at both pH 6.7 and 5.5. The hypothesis to be tested was that combining oligofructose with prebiotics candidates β-glucan and human milk oligosaccharides would result in a more targeted and controlled approach to microbial manipulation, SCFA and

neurotransmitter production. Thereafter we investigated the effects of the prebiotic oligofructose and prebiotic candidate 2'fucosyllactose alone and combination had on microbial composition and mood state *in vivo* using a 4-arm parallel, 5-week double blind randomised placebo-controlled trial.

1.2 Literature review

Determining the metabolic fate of human milk oligosaccharides: It may just be more complex than you think?

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Abstract

Human milk oligosaccharides (HMOs) are a class of structurally diverse and complex unconjugated glycans present in breast milk, which act as selective substrates for several genera of select microbes and inhibit the colonisation of pathogenic bacteria. Yet, not all infants are breastfed, instead being fed with formula milks which may or may not contain HMOs. Currently, formula milks only possess two HMOs: 2'fucosyllactose (2'FL) and lacto-*N*-neotetraose (LNnT), which have been suggested to be similarly effective as human breast milk in supporting age-related growth. However, the *in vivo* evidence regarding their ability to beneficially reduce respiratory infections along with altering the composition of an infant's microbiota is limited at best. Thus, this review will explore the concept of HMOs and their metabolic fate, and summarise previous *in vitro* and *in vivo* clinical data regarding HMOs, with specific regard to 2'FL and LNnT.

1.2.1 The History of HMOs

Human breast milk is considered the gold standard nutrient source for infants in the early stages of life due to the presence of several remarkable functional ingredients (Van den Abbeele *et al*., 2019). One group of such ingredients is the human milk oligosaccharides (HMOs). A classification which is given to a group of structurally diverse and complex unconjugated glycans present in human breast milk (Ninonuevo *et al*., 2006). HMOs were first "discovered" towards the end of the nineteenth century after French biochemist Georges Denigés noted that in addition to lactose, human and bovine milk possessed several other carbohydrate structures with Polonowski and Lespagnol originally terming these unknown fractions as gynolactoses (Polonowski and Lespagnol, 1929; Polonowski and Lespagnol, 1931). The levels of HMOs present in breast milk range from 5 to 25 g/L throughout the course of lactation, making the levels of oligosaccharides present in human milk the highest amongst mammalian species. This is over 100 times greater than the oligosaccharide content found in bovine milk, which has been estimated at around 100 mg/L (Robinson, 2019; Zivkovic and Barile, 2011).

1.2.2 Structural complexity of HMOs

Virtually, all HMOs possess a lactose (Lac) core to which a multitude of different monosaccharide "building blocks," including galactose (Gal), glucose (Glc), fucose (Fuc), sialic acid (Neu5Ac) and N-acetylglucosamine (GlcNAc), can be attached via the action of specific glycosyltransferases in the presence of α-lactalbumin (Smilowitz *et al*., 2014). The synthesis of HMOs begins with the enzymatic elongation of lactose (Lac) by either β1-3 or β1-6 linkages of Gal to lacto-N-biose (LNB) or N-acetyllactosamine (LacNAc), respectively. Based on this, HMOs can be classified as either Type-I or Type-II chains. Type-I chain HMOs possess lacto-N-tetraose

(LNT), which is lactose coupled to LNB. While Type-II chains, are composed LNT isomer lacto-N-neotetraose (LNnT), and is Lac linked to LacNAc (James *et al*., 2016). These core HMO structures can then be further elongated and additionally categorised as neutral, fucosylated or sialylated (Plaza-Diaz, Fontana and Gil, 2018; Zivkovic and Barile, 2011). Neutral HMOs possess structures similar to galacto-oligosaccharides (GOS) containing both Glc and Gal (Barile and Rastall, 2013), and may also accommodate several GlcNAc or LNB units attached via β1-3 and β1-6 linkages (Ayechu-Muruzabal *et al*., 2018). At this point, Fuc units can be enzymatically attached via α1-2, α1-3 or α1-4 linkages generating fucosylated HMOs. Hereafter, one or more molecules of Neu5Ac may be attached via α2-3 or α2-6 linkages in the presence of sialyl-transferases generating sialylated HMOs (Smilowitz *et a*l., 2014). Figure 1.1 gives a generalised overview of the complexity and structural diversity of HMOs present in breast milk.

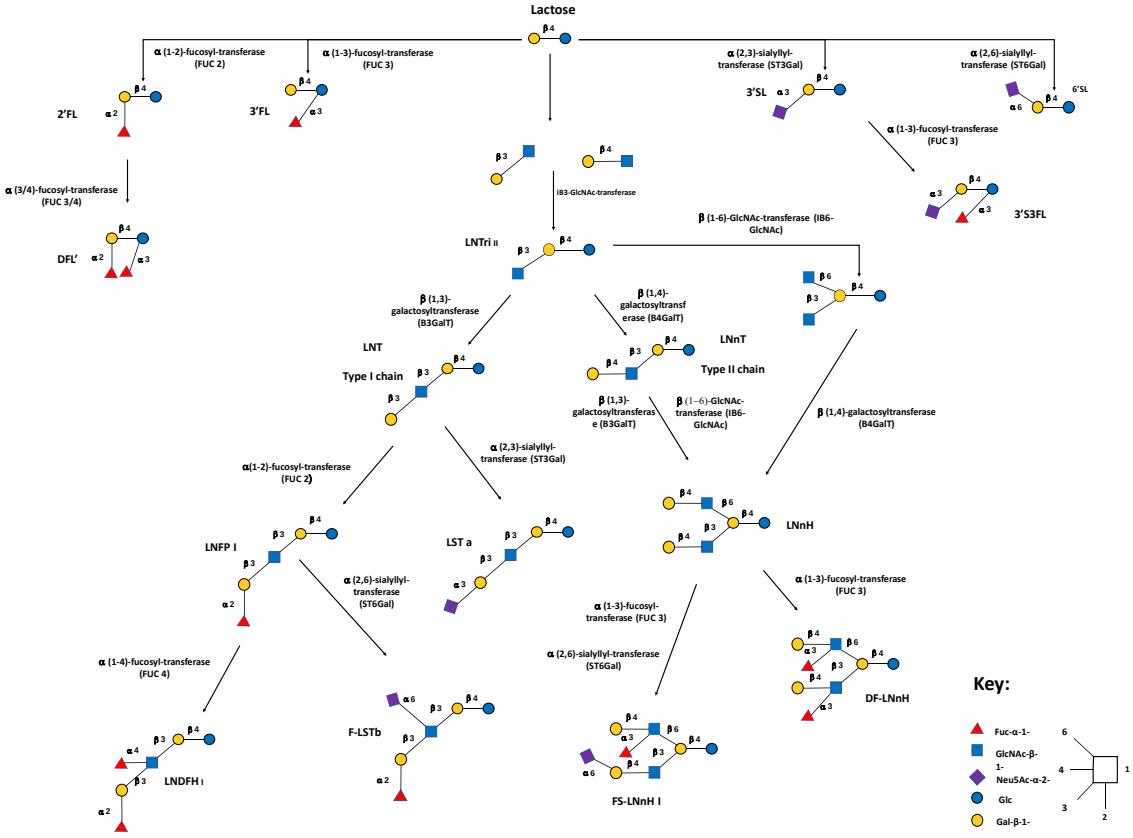

Figure 1.1. Generalised overview of the complexity and structural diversity of HMO present in breast milk.

Abbreviations: Neutral HMO: (2'FL) 2'fucosyllactose; **(3'FL)** 3'fucosyllactose; **(DFL)** difucosyllactose; **(LNT)** lacto-*N*-tetraose; **(LNnT)** lacto-*N*-neotetraose; **(LNFP I)** lacto-*N*-fucopentaose I; **(LNDFH I)** lacto-*N*-difucohexaitol I; **(LNnH)** lacto-*N*-neohexaose; **(DF-LNnH)** difucosylated lacto-*N*-neohexaose

Acidic nonfucosylated HMO (3'SL) 3'sialyllactose; **(6'SL)** 6'sialyllactose; **(LST a)** sialyllacto-*N*-tetraose a

Acidic fucosylated HMO: (3'S3FL) 3'sialyl-3-fucosyllactose; **(F-LST b)** sialylfucosyllacto-*N*-tetarose b; **(FS-LNH I)** fucosylsiallacto-*N*-hexose I

1.2.3 Composition of human milk oligosaccharides in breastmilk and factors affecting composition

To date, somewhere in the region of 200 HMOs have been identified in the breast milk of mothers with the most widely recognised HMOs being 2'-fucosyllactose (2'FL), LNT and LNnT (Barile and Rastall, 2013; Egge, Dell and Von Nicolai, 1983; Urashima *et al*., 2018). The composition and concentration of HMOs present in breast milk are highly dependent on several critical factors, including geographical location, ethnicity, length of gestation and secretor status. In general, the levels of HMOs present in breast milk are highest immediately following birth and decrease throughout lactation with the concentration of 2'FL appearing to be highest during the first month postpartum (Thurl *et al*., 2010; Thurl *et al*., 2017; Xu *et al*., 2017). Yet, the rates at which HMOs decline are not constant across all HMOs. For example, while 2'FL, difucosyllactose (DFL), lacto-N-fucopentaose-2 (LNFP II), 3'-Sialyllactose (3'SL) and 6'-Sialyllactose (6'SL) all decline in concentration throughout lactation, the rate of decline does not appear to however significantly alter during days 30–120 (Gabrielli *et al*., 2011; Spevacek *et al*., 2015; Thurl *et al*., 2010), whereas lacto-N- fucopentaose-1 (LNFP I) appears to decline just 3 days after birth recording a twofold decline by the end of lactation, respectively (Bao, Chen and Newburg, 2013; Smilowitz *et al*., 2013). Other HMOs, including 3'-fucosyllactose (3'FL), appear to increase in concentration throughout lactation by 1.67–1.8-fold (Austin *et al*., 2019; Gabrielli *et al*., 2011; Samuel *et al*., 2019; Smilowitz *et al*., 2013).

However, a significant percentage of mothers can only synthesise certain HMOs, including 2'FL, dependant on their secretor status. Secretor status is based on the Lewis blood group and depends on the expression of the specific glycosyltransferases, α1-2-fucosyltransferase (FUT2 encoded by the Se gene) and α1-3/4-fucosyltransferase (FUT3 encoded by the Le gene),

and can result in marked differences in which HMOs are synthesised (Blank *et al*., 2012; Hegar *et al*., 2019). Table 1.1 summarises these findings.

Gene	Lewis gene +	Lewis gene -
	Se+ Le+	**Se+ Le-**
Secretor gene +	Secretes all HMO	Able to secrete 2'FL, 3'FL, LNFP I, LNFP III
	Se- Le+	**Se- Le-**
Secretor gene -	Able to secrete 3'FL, LNFP-II, LNFP III	Able to secrete 3'FL, LNFP III, LNFP V

Table 1.1. Human milk oligosaccharide composition of breast milk based on the genetic background of the mother Source: (Vandenplas *et al.*, 2018).

However, the Lewis antigen system and secretor status can be described as an over generalisation of an extremely complex situation (Plaza-Diaz, Fontana and Gil, 2018) as even when these factors are accounted for, substantial differences in HMO profile can still occur. For example, FUT2 and FUT3 have been shown to compete for several of the same substrates. As a result, differences in levels of expression can alter the synthesis of 2'FL by a "secretor" mother (McGuire *et al*., 2017; Plaza-Diaz, Fontana and Gil, 2018).

Additionally, levels and composition of HMOs present in breast milk may also vary between the mothers of preterm and full-term infants regardless of secretor status. However, reports on this are contradictory with several papers seemingly reporting that HMOs may or may not vary between preterm and term infant mothers (Austin *et al*., 2019; De Leoz *et al*., 2012; Kunz and Rudloff, 2017; Thurl *et al*., 2017).

Furthermore, the sizeable heterogeneity in the levels and composition of HMOs detected in breast milk may be in part due to differences in analytical techniques used in studies, including high-performance liquid chromatography mass spectrometry (HPLC MS) and HPLC time-of-flight mass spectrometry, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), as well as differences in the number of preparation steps taken (van Leeuwen, 2019; Zivkovic *et al*., 2011). A further complication is the lack of analytical standards needed in order to quantitatively assess the concentration of HMOs present (Wicinski *et al*., 2020). As a result, it could be argued that determining the average composition and quantity of HMOs in breast milk, based on current published information, is not achievable due to too many confounding variables. Furthermore, to reiterate (Thurl *et al*., 2017), to be able to determine the average composition of HMOs in breast milk, a worldwide multicentre study following the same protocols would be required. However, even if the average HMOs content of breast milk was determined, what relevance this would have regarding clinical significance remains uncertain.

1.2.4 Human milk oligosaccharides, health benefits and clinical data

It is well documented that breastfeeding is highly associated with several health benefits, including improved growth rate, lower prevalence of respiratory, intestinal and urinary infections (Li *et al*., 2014) and lower incidence of allergies and autoimmune conditions, with some of this being put down to the presence of HMOs in breast milk (Doherty *et al*., 2018; Triantis, Bode and van Neerven, 2018).

The structural nature of HMOs renders them resistant to digestive enzymes found within the GI tract (Garrido, Barile and Mills, 2012) with >90 percent of HMOs reaching the colon intact.

HMOs likely function as prebiotics, stimulating the growth of beneficial bacteria, including bifidobacteria (Barile and Rastall, 2013; Bode, 2009). HMOs can also act as soluble decoys, preventing the adhesion of pathogenic bacteria to cell surface receptors due to their resemblance to the glycans found on the surface of epithelial cells in the intestinal tract (Wicinski *et al*., 2020). In addition, the formation of the short-chain fatty acids (SCFAs) butyrate, acetate and propionate from saccharolytic fermentation plays vital roles in the activation and differentiation of immune cells and may also reduce the risk of infections and allergies (Ayechu-Muruzabal *et al*., 2018; Kumari and Kozyrskyj, 2017). Additionally, it has been shown in infants that a lack of bifidobacteria, particularly those associated with HMO degradation and utilisation, is associated with increases in systemic inflammation and immune dysfunction (Henrick *et al*., 2021). Furthermore, supplementation of 2'FL in combination with *B. pseudocatenulatum* MP80 was associated with changes in gene expression of both anti-inflammatory and pro-inflammatory markers in the cecum of healthy mice, whereas in mice with dextran sulphate sodium (DSS)-induced colitis, supplementation of 2'FL in combination with *B. pseudocatenulatum* MP80 resulted in attenuations of bodyweight loss, along with a reduction in DSS-induced immune cell infiltration, increase in colon length and disrupted mucosal architecture along with preventing a reduction in occluding expression (Heiss *et al*., 2021).

However, not all infants are breastfed with many being fed formula milk which may or may not be supplemented with HMOs. The first two HMOs to be commercially produced were 2'FL and LNnT, and while these compounds are referred ta as HMOs, they are not sourced from human milk, but are produced by microbial fermentation using strains of *E. coli* and yeasts which have been genetically modified (Sprenger *et al*., 2017).

Several studies have suggested that infants fed HMO-supplemented formula milk present lower risks of parent-reported bronchitis and respiratory infections, along with reduced use of antibiotics, less waking at nights and improved age-appropriate growth (Marriage *et al*., 2015; Puccio *et al*., 2017). In a study on 2'FL, sole or in combination with *Bifidobacterium longum* subsp. *infantis* (Bi-26), on cognitive and structural development in young pigs (Sutkus *et al*., 2022), the authors noted that synbiotic administration of 2'FL and Bi-26 had several interactive effects on microstructural brain components; however, it appeared to have no effect on memory. Preclinical *in vitro* and *in vivo* studies propose that both 2'FL and LNnT promote the growth of several *Bifidobacterium* and *Bacteroides* species, strains, and subspecies including *Bifidobacterium longum* subsp. *infantis*, *Bacteroides fragilis*, and *Bacteroides vulgatus* (Marcobal *et al*., 2010; Yu, Chen and Newburg, 2013).

However, while the importance of breast milk on infant health outcomes is well supported, there remains a great deal unknown regarding the efficacy of HMOs added to infant formulas on infant health outcomes. Furthermore, it has been demonstrated that several species, strains and subspecies of microorganisms found within the gut microbiota, including *B. adolescentis* and *B. animalis*, do not grow well on HMOs, including 2'FL and LNnT on their own (Lawson *et al*., 2020; LoCascio *et al*., 2010; Marcobal *et al*., 2010; Sela *et al*., 2012; Xiao *et al*., 2010). This suggests that the composition of gut microbiota may be key if formula milks containing 2'FL and LNnT are to be effectively utilised. Therefore, the rest of this review will focus on the effects that HMOs have on the composition of the infant gut microbiota both *in vitro* and *in vivo* and will attempt to determine the metabolic fate of HMOs.

1.2.5 The metabolic fate of human milk oligosaccharides infant and rodent studies

It was established as early as the 1970s that HMOs are present in the urine of expecting mothers as soon as 8 weeks (Hallgren, Lindberg and Lundblad, 1977) and can be detected in the mammary glands using 13C isotopes (Dotz *et al*., 2014), suggesting that HMOs circulate throughout maternal serum. Hirschmugl *et al*., (2019) investigated individual and temporal variations in the composition and levels of HMOs in maternal serum throughout the course of pregnancy. In this study, serum samples were collected from healthy pregnant woman throughout the course of gestation at weeks 10–14 (Visit 1), 20–24 (Visit 2) and 30–35 (Visit 3) and at the time of admission to delivery at the Department of Obstetrics, Medical University of Graz, Graz, Austria. In total, 16 HMOs – 2'FL, 3'FL, DF', 3'SL, 6'SL, LNT, LNnT, LNFP I, II, and III, LST a, b, and c, LNDFH, LNH and disialyllacto-N-tetraose (DSLNT) were detected in serum with the authors reporting a steady increase in the presence of HMOs, in particular, fucosylated HMOs in circulation throughout the course of pregnancy.

Based on this, one could speculate that HMOs may undergo maternal-to-foetal transport. Indeed, there are data suggesting that several HMOs, including 2'FL, 3'FL, DFL and 6'SL, were present in amniotic fluid (Wise *et al*., 2018). Likewise, it has also been demonstrated, using an *ex vivo* experimental model involving isolated placental cotyledons perfused with 2'FL, that 2'FL was able to cross the placenta (Hirschmugl *et al*., 2019). Furthermore, given that HMOs function as signalling molecules and that similar glycan structures can act as receptors in cells and tissues (Bhargava *et al*., 2012), it is likely that HMOs may also contribute towards the development and functioning of immune (Plaza-Diaz, Fontana and Gil, 2018) and endothelial cells (Donovan and Comstock, 2016) of infants *in utero*. However, since fetal circulation and

cord blood are not accessible during pregnancy, these findings should be interpreted with a great deal of care (Hornef and Penders, 2017; Walker *et al*., 2017).

To date, the ability of HMOs to end up in the urine of infants has been extensively studied using 13C-labelled glycans along with several analytical techniques, including MALDI-TOF-MS, HPAEC- PAD, nano-LC–MS and MALDI FT-ICR MS. The results of these studies suggest that fully and partially intact HMOs, including 2'FL, 3'SL, 6'SL, LNT and LNnT, can be present in the urine of infants, albeit in far lower concentrations than that found in milk at just 4 per cent, with secretor/non-secretor status massively impacting on which HMOs are detected (Borewicz *et al*., 2020; De Leoz *et al*., 2013; Dotz *et al*., 2014; Dotz *et al*., 2015; Goehring *et al*., 2014).

Given that HMOs can end up in the urine of infants, HMOs can clearly be absorbed through the epithelial cells of the gastrointestinal tract and enter the bloodstream (Dotz *et al*., 2014; Rudloff and Kunz, 2012). To date, the potential for HMOs to enter circulation has been extensively studied in animal models. In one study, conducted in rats fed both mixed and isolated HMOs, including 2'FL, it was noted that when mixed HMOs were ingested, 2'FL was detected in circulation 30 min later in a dose-dependent manner, reaching a maximum at 60 min (Vazquez *et al*., 2017), whereas in rats fed isolated HMOs, levels of 2'FL detected in circulation similarly increased in concentration over time, again in a dose-dependent manner. However, they did not reach a maximum peak within the 4-h sampling period. In contrast, Jantscher-Krenn, Marx and Bode, (2013) reported that only 3'SL, along with very few other HMOs, was detected in the serum of rats. The discrepancies in findings between these studies probably result from biological differences in the metabolism of HMOs due to the ages of rats used in each respective study.

The potential for HMOs to enter into the circulation and plasma of infants was investigated by Ruhaak *et al*., (2014) in 13 full-term infants using solid-phase extraction followed by an analysis by nano-high performance liquid chromatography - porous graphitised carbon - chip - time of flight – mass spectrometry (nHPLC-PGC-chip-TOF-MS). In total, 15 oligosaccharides, including LNT, LDFP, LNFT, 3'SL, 6'SL, 3'sialyllactosamine (3'SLN), 6'sialyllactosamine (6'SLN), LNFP III and 2'FL, were detected in the plasma of infants, albeit in lower concentrations than levels found in breast and formula milk, with over a 10-fold variation in LNT being recorded between partially breastfed and formula-fed infants. Interestingly, an unknown isomer of SLN was found. Given SLN is usually derived from bovine milk (McGrath *et al*., 2016; Ruhaak *et al*., 2014) and the relative abundances detected in the infants of this study far exceed those found in bovine milk (Fong, Ma and McJarrow, 2011). From this, one could theorise that new SLNs detected in the circulation of infants may have been produced via interactions between milk, milk by-products and bacterial glycosidases (Lis-Kuberka and Orczyk-Pawilowicz, 2019).

Moreover, using isotopically labelled standards, coupled with ultra-performance liquid chromatography and HPLC, Goehring *et al*., (2014) investigated the presence of HMOs in both breastfed and formula-fed infants. The authors reported the detection of several HMOs, including 2'FL, 3'FL and LNnT, in the plasma of breastfed but not formula-fed infants, again albeit in far lower levels. A similar finding was reported by (Radzanowski *et al*., 2013), who documented that the concentration of HMOs present in infant plasma was substantially less (3'SL: 0.10–0.78 mg/L; 6'SL: 0.05–0.68 mg/L; 2'FL: 0–2.25 g/L) compared with breast milk (3'SL: 54.3–225 mg/L; 6'SL: 29.3–726 mg/L; 2'FL: 0–3.8 g/L) at just 0.1 per cent, respectively.

1.2.6 The degradation and transportation of HMOs

As previously discussed, virtually, all HMOs possess a lactose core to which a multitude of different monosaccharide "building blocks," including Gal, Glc, Fuc, sialic acid (Neu5Ac) and N-acetylglucosamine (GlcNAc), can be attached via the action of specific glycosyltransferases in the presence of α-lactalbumin (Smilowitz *et al*., 2014). In order to stimulate the fructose 6-phosphate phosphoketolase-dependent glycolytic pathway more commonly termed the bifid shunt active in bifidobacteria, these complex milk glycans must be degraded (Pokusaeva, Fitzgerald and van Sinderen, 2011). The mechanisms by which HMOs undergo degradation can be characterised as intracellular (transport-dependant) and extracellular (glycosidase-dependent). Both mechanisms require the use of specific ATP (adenosine triphosphate)-binding cassette (ABC) transporters to either import intact or processed glycans inside the bacterial cell (Garrido *et al*., 2011) with the most common species and strains of bifidobacteria (*B. breve*, *B*. *longum*, *B*. *bifidum* and *B*. *infantis*) preferring to utilise specific mechanisms of action (Sakanaka *et al*., 2020) (Figure 1.2).

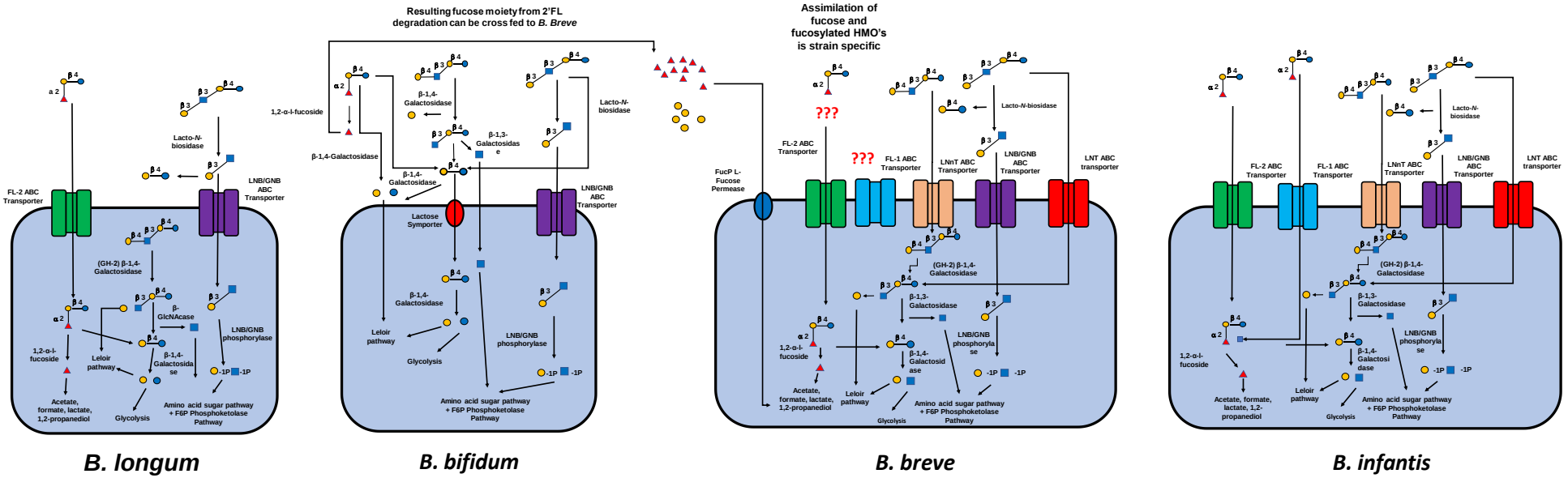

Figure 1.2. Intracellular and extracellular degradation of 3 main HMOs: 2'FL, LNT and LNnT and resulting metabolites by 4 common *Bifidobacterium: B. longum, B. bifidum, B. breve, and B. infantis* and selective fermentation pathways **Abbreviations:** ABC: ATP-binding cassette; LNB - lacto-*N*-biose; GNB - galacto-*N*-biose; FL - fucosyllactose; LNnT - lacto-*N*-neotetraose : **Key:** ▲ Fucose; ● Glucose; ● Galactose; ■ N-acetylglucosamine

1.2.6.1 Fucosidases and fucosylated human milk oligosaccharide transporters

To hydrolyse fucosidic-linked HMOs, two distinct glycoside hydrolase (GH) families, GH95 and GH29, are required for the degradation of specific fucosidic linkages (Sakanaka *et al*., 2019). While both GH95 and GH29 can target the 1,2-, 1,3- and 1-4-α-l-fucosides, GH29 displays a higher affinity towards both 1,3- and 1,4-α-l-fucosides, whereas, GH95 displays a higher preference towards the hydrolysis of 1,2-α-l-fucosides (Matsuki *et al*., 2016; Shani *et al*., 2022; Zeuner *et al*., 2018). The transporters responsible for the uptake of fucosyllactose (FL') were first discovered in *B*. *longum* subsp. *infantis* ATCC 15697T (Sela *et al*., 2008). *B*. *longum* subsp. *infantis* ATCC 15697T possesses two paralogous FL' transporters which share up to 60 per cent of the same solute-binding proteins (SBPs), suggesting that there is some degree of overlap in their ability to transport various HMOs (Sakanaka *et al*., 2020). This overlap was demonstrated in a recent study conducted by Sakanaka *et al*., (2019) with the authors concluding that FL transporter-1 was only able to import low molecular weight HMOs, including 2'FL and 3'FL. In contrast, FL transporter-2 was able to import not only 2'FL and 3'FL, but also LDFT and LNFP I. Yet, the ability of different species and strains of bifidobacteria to express FL' transporters is not identical. As research by Garrido *et al*., (2016) and Matsuki *et al*., (2016) revealed, there was a remarkable difference in the ability of *B. longum* subsp. *infantis* and *B*. *bifidum* to utilise FL' due to the presence of different intracellular and extracellular ABC-type transporters (K02025 and K02026). Outside of *Bifidobacterium* spp. GH29 and GH95 have also been detected in *Roseburia inulinivorans* and GH29 detected in *Akkermansia muciniphila*, enabling it to cleave the α1-2-fucosyl linkage to Gal (Kostopoulos *et al*., 2020).

1.2.6.2 Lacto-N-biosidase and LNB transporter

LNT is one of the most abundant neutral HMOs present in breast milk and is hydrolysed by lacto-N- biosidase (lnb), resulting in the formation of LNB and Lac (Yamada *et al*., 2017). The specificity of lnb present in *Bifidobacterium* also varies remarkably between species (Sakanaka *et al*., 2020). On this basis, lnb present in *B*. *bifidum* is classified as GH20, whereas in *B. longum*, lnb is categorised as GH136. The differences being that GH136 requires an additional chaperonin for proper protein folding and is also capable of accepting sialyllacto-N-tetraose a (LST a) in addition to LNT (Sakurama *et al*., 2013; Yamada *et al*., 2017).

In *B*. *bifidum*, degradation of HMOs to LNB and monosaccharides begins extracellularly with the hydrolysis of LNFP I, II and lacto-N-difucohexaose I, II (LNDFH I, II) to LNT and Fuc with the aid of additional fucosidases (Marcobal and Sonnenburg, 2012). LNB is then hydrolysed from LNT by Lnb and then transported inside of the cell leaving any remaining fucosyl residues behind. Lnb phosphorylase then converts LNB into its respective monosaccharides Gal and GlcNAc, which then undergo further assimilation (Zivkovic *et al*., 2011). In *B. longum*, the degradation of LNT seemingly follows similar principles to that of *B. bifidum* with LNB being extracellularly hydrolysed from LNT. However, unlike *B. bifidum*, the hydrolysis of LNB from LNFP I by *B*. *longum* occurs intracellularly, meaning that no Fuc residues are left behind (Xiao *et al*., 2010; Yamada *et al*., 2017). In contrast to both *B*. *bifidum* and *B*. *longum*, the degradation of LNT and LNnT to LNB by *B*. *longum* subsp. *infantis* occurs entirely intracellularly with LNB constituent monosaccharides Gal and GlcNAc entering selective fermentation pathways (Ozcan and Sela, 2018). More recently, the presence of GH136 has been discovered in *Roseburia* with the degradation of fuscoylated pentose and hexose HMOs said to occur extracellularly (Pichler et al., 2020).

1.2.6.3 Sialidase

For microorganisms to be able to utilise sialic acids, they must possess the necessary sialidases required to hydrolyse the α-2,3 and α-2,6 linkages of sialylated HMOs (Kiyohara *et al*., 2011; Zivkovic *et al*., 2011). In bifidobacteria, the most common sialidases are classified as GH33 with each member of the GH33 family exhibiting preference for specific sialic acid linkages. For example, in *B*. *bifidum*, the sialidase present is categorised as SiaBb2 and exhibits a preference for α-2,3 linkages, whereas the sialidase present in *B*. *longum* subsp. *infantis* is classified as NanH2, but unlike SiaBb2 appears to exhibit an equal preference for both α-2,3 and α-2,6 linkages (Juge, Tailford and Owen, 2016).

1.2.6.4 LNT β-1,3-galactosidase

In bifidobacteria, β-1,3-galactosidases are categorised as GH42 and GH35 and are responsible for cleaving HMOs possessing β-1,3 Gal linkages, displaying hydrolytic ability for both Type-I and Type-II chains with highest activity being displayed on LNT, followed by Lac, LNB and LNnT (James *et al*., 2016; Yoshida *et al*., 2012).

1.2.6.5 β-1,4-galactosidase

Several other glycosidases foe the assimilation of HMOs exist, including β-1,4-galactosidases (Zeuner *et al*., 2019). In *B*. *bifidum* and *B*. *breve*, the β-1,4-galactosidases belong to the GH2 family and are categorised as BbgIII and LacZ2 and LacZ6, respectively, and are responsible for the hydrolysis of HMOs possessing Lac and Type-II chains (James *et al*., 2016; Yoshida *et al*., 2012). This mechanism of HMOs degradation is particularly prominent in *B*. *bifidum* and functions extracellularly cleaving LNnT at its Galβ-1,4 residue liberating Gal and LNT. Thereafter, LNT is further hydrolysed producing GlcNAc and lactose with lactose undergoing

additional hydrolysis resulting in the formation of Glc and Gal, respectively (James *et al*., 2016; Miwa *et al*., 2010).

1.2.6.6 β-D-hexosaminidases

N-acetyl-β-D-hexosaminidases are another set of hydrolytic enzymes, which belong to the GH20 family with three enzymes seemingly responsible for the hydrolysis of HMOs. N-acetyl-β-D-hexosaminidases have been detected in *B*. *longum* subsp. *infantis* (Garrido, Ruiz-Moyano and Mills, 2012) with these GHs being categorised as Blon_2355 and Blon_0732 and Blon_0459. Blon_2355 displays a preference for GlcNAc β1-3 Gal linkages, whereas Blon_0732 and Blon_0459 can additionally release GlcNAc from branched HMOs characterised by GlcNAc β1-6 Gal linkages (Garrido, Ruiz-Moyano and Mills, 2012). Additionally, N-acetyl-β-D-hexosaminidases have been discovered in *B. bifidum* JCM 1254, categorised as BbhI and BbhII, with BbhI being shown to hydrolyse lacto-N-triose II into GlcNAc and lactose, respectively (Miwa et al., 2010).

It is clear that different species, strains and subspecies of *Bifidobacterium* possess several different mechanisms for the assimilation of HMOs and it is likely that substantial differences in rates of consumption and fermentation occur.

1.2.7 The *in vitro* assimilation and consumption of HMOs

Differences in the consumption behaviour between different microorganisms found within in the gut microbiota have been assessed *in vitro* using either pooled or singular HMOs as sole carbon sources. To date, bifidobacteria are the most widely studied microorganisms, both singular and in combination, in relation to their ability to utilise HMOs due to their predominance in the breastfed infant gut microbiota. Yet, it is not only bifidobacteria that

have the ability to degrade HMOs, several other genera within the gut, including *Bacteroides*, *Roseburia* and *Akkermansia* amongst others, appear to play a role in HMO utilisation.

1.2.7.1 Single cultures

As mentioned, bifidobacteria are one of the most well-documented microorganisms in the gut regarding HMO consumption. In one study, conducted by Gotoh *et al*., (2018), the authors aimed to determine the ability of various strains of bifidobacteria to utilise HMOs, including 2'FL. The four strains of *B*. *bifidum* tested (JCM1254, JCM7004, TMC3108 and TMC3115) were isolated from either infant faecal samples or obtained from other researchers at the Riken Bioresource centre and subjected to *in vitro* assays using GAM broth and HMOs with concentrations of HMOs being analysed in the spent media. There was a large amount of Fuc and Lac in the supernatants of all four *B*. *bifidum* strains by the 15 h mark, with the degradation of 2'FL beginning even before cells entered the exponential phase. This suggests that each strain of *B*. *bifidum* possessed the necessary 1,2-α-fucosidase and 1-3- and 1-4-α-fucosidases required for the degradation of fucosylated HMOs. Furthermore, the fermentation of 2'FL appeared to occur rapidly and extracellularly which may have important implications regarding the utilisation of HMOs within bifidobacterial communities and subsequent microbial diversity (Sakanaka *et al*., 2020).

Garrido *et al*., (2015) also examined the ability of several strains of *B*. *longum* subsp. *infantis* and *B*. *bifidum* utilised from breastfed infants to utilise HMOs, including 2'FL, 3'FL, 6'SL and LNT, as sole carbon sources. All *B*. *longum* subsp. *infantis* strains displayed excellent growth on all HMOs, while the ability of various *B*. *bifidum* strains was highly variable with several strains showing little growth on pooled HMOs. These findings are similar to those of Gotoh *et al*., (2018), who recorded that the growth of *B*. *bifidum* strains (JCM1254, TMC3108 and TMC3115) resulted in higher cell biomass compared with *B*. *bifidum* strain JCM7004. There is

clearly a remarkable variability in the ability of *Bifidobacterium* strains, even of the same species, to utilise HMOs with several species, strains and subspecies appearing to be better adapted than others (Sakanaka *et al*., 2020).

The differences in the ability of *Bifidobacterium* to utilise HMOs were also demonstrated by Bunesova, Lacroix and Schwab, (2016), who tested several strains of *Bifidobacterium*, including *B*. *longum* subsp. *infantis*, *B*. *longum* subsp. *suis* BSM11–5 and *B*. *bifidum* and *B*. *kashiwanohense* isolated from the stool samples of 6-month infants. There was substantial variability in the ability of bifidobacterial strains and subspecies to utilise HMOs with *B*. *longum* subsp. *infantis* being able to utilise 2'FL, 3'FL, 3'SL and LNnT. *B*. *bifidum* BSM28-1 was able to utilise 6'SL in addition to 2'FL, 3'FL, 3'SL and LNnT, while *B*. *longum* subsp. *suis* BSM11-5 and *B. kashiwanohense* strains all grew in the presence of 2'FL and 3'FL. Several strains and subspecies of bifidobacteria, however, including *B*. *bifidum* DSM 20215 and *B*. *breve* DSM 20213, were unable to utilise HMOs to any real degree, with *B*. *pseudolongum* not being able to utilise HMOs at all. Furthermore, all *B*. *longum* strains and subspecies tested, including *B*. *longum* subsp. *suis* BSM11-5 and *B*. *longum* subsp. *infantis* DSM 20088, were able to utilise the resulting Fuc moiety, albeit with a varying degree of success with *B*. *kashiwanohense* not being able to metabolise Fuc at all. This adds to the evidence that the composition of the gut microbiota appears to be critical if HMOs are to be effectively utilised.

Ward *et al*., (2007) also investigated the capability of *Bifidobacterium* to effectively degrade 2'FL and utilise the resulting Fuc and sialic acid moiety with *B*. *longum* bv. *infantis* ATCC 15697 achieving both the highest growth rate and the highest Fuc consumption amongst the majority of species and strains of *Bifidobacterium* tested, whereas *B*. *breve* ATCC 1570 was only able to achieve intermediate levels of growth with only moderate Fuc usage and *B*. *adolescentis* and *B*. *bifidum* ATCC 15696 exhibiting no growth on either Fuc or sialic acids. These findings are

similar to those recorded by Garrido *et al*., (2015), who noted that while *B*. *longum* subsp. *infantis* ATCC 15697 exhibited excellent growth on both 2'FL and 3'FL, *B*. *bifidum* JCM 7004 only exhibited moderate growth on 2'FL, 3'FL and 6'SL with *B*. *animalis* JCM 10602 exhibiting no growth, on HMOs at all.

Accordingly, (Locascio *et al*., 2007) reported that *B*. *adolescentis* ATCC 15703 presented little-to-no sign of growth in the presence of several HMOs, whereas Xiao *et al*., (2010) found that *B*. *adolescentis* ATCC 15704 and 15705 appeared to be unable to utilise HMOs including LNB. This infers that *B*. *adolescentis* ATCC 15703, 15704 and 15705 lack the glycosidases and transporters required to assimilate HMOs (LoCascio *et al*., 2010).

The ability of the *B*. *longum* subsp. *infantis* ATCC 15697 to effectively utilise HMOs can be put down to the presence of five distinct gene clusters (Sela *et al*., 2008). In the genomic sequencing of *B*. *longum* subsp. *infantis* ATCC 15697 a 43-kb gene cluster dedicated to HMO import encoding 21 genes was identified with one of its four loci encompassing all of the necessary sialidases, fucosidases, galactosidases and hexosaminidases required for transporting and metabolising HMOs (Locascio *et al*., 2007; Sela *et al*., 2008). Several other strains and subspecies of bifidobacteria, including *B*. *longum* subsp. *longum* DJO10AB and *B*. *adolescentis* ATCC 15703, possess fewer than 11 genes where the lack of SBPs results in an inability to effectively utilise HMOs (Lee *et al*., 2008; LoCascio *et al*., 2009).

It is not just *Bifidobacterium* which possesses the ability to utilise and exhibit growth on HMOs. In addition to *Bifidobacterium*, several strains of *Bacteroides*, including *B*. *fragilis* and *B*. *vulgatus*, are also known prominent consumers of HMOs (Marcobal *et al*., 2010). Of these, two strains of *B*. *fragilis* ATCC 2585 can effectively utilise a full range of HMOs, albeit displaying a preference for non-fucosylated HMOs, recording an overall consumption between 25 and 90

per cent. These findings are substantially higher than *B*. *vulgatus* ATCC 8482, which can also utilise a full range of HMOs, again displaying a preference for fucosylated HMOs. This strain displayed much lower consumption rates of total HMOs than *B*. *fragilis* ATCC 2585 at 16–40 per cent, respectively (Marcobal *et al*., 2010), the difference being the presence of Fuc-specific GH95 and GH29 glycoside hydrolases.

Furthermore, in contrast to *Bifidobacterium* and *Bacteroides*, several strains of *Enterobacteriaceae*, including EC1000, EC11775, EC29425 and SD13313, appear to be unable to utilise several HMOs, including 2'FL and 6'SL while displaying limited growth on LNnT. However, these strains could also readily utilise Glc, maltodextrin and GOS as a sole carbon source in pure cultures (Hoeflinger *et al*., 2015).

1.2.7.2 Mixed culture/faecal inoculum

The ability of 2'FL to alter the composition of the gut microbiota has been investigated using an *in vitro* Simulator of Human Intestinal Microbial Ecosystem (SHIME) model using faecal samples from 6-month-old infants had been exclusively formula-fed (Van den Abbeele *et al*., 2019). The authors noted that 2'FL increased the relative abundance of bifidobacteria and butyrate-producing bacteria, shifting the distribution of *Bifidobacterium* spp. from *B*. *bifidum* towards *B*. *adolescentis*: and interesting finding given that, as previously discussed, *B*. *adolescentis* appears to be unable to utilise whole HMOs. This likely indicates that *B*. *adolescentis* can exploit products of the degradation of HMOs by other microbial community members (Thongaram *et al*., 2017).

Increases in the concentration of acetate and butyrate were seen in both parts of the distal and proximal colon of the SHIME model, with levels of propionate displaying a more rapid increase in the distal part of the colon upon 2'FL dosing. This is consistent with (Boler *et al*.,

2013) who reported that 2'FL was rapidly fermented upon inoculation with mixed faecal cultures. However, the bifidogenic effect of 2'FL appeared to be donor-specific with increases in numbers of bifidobacteria only being observed in the proximal colon of one donor. In another, donor increases in bifidobacteria were observed in both the proximal and distal colon.

Additionally, the authors reported that increases in the concentration of acetate and butyrate detected were seen in both parts of the distal and proximal colon the SHIME model with levels of propionate recording a more rapid increase in the distal part of the colon upon 2'FL dosing. Thus, from these results, it suggests that the microbiota dependence of individual rates of fermentation of 2'FL is likely to be highly variable between subjects (Marcobal and Sonnenburg, 2012). Yet, the supplementation of 2'FL in this study was undertaken at 2 g/L, approximately twice the concentration of 2'FL found in formula milks currently for sale on the market (SMA Nutrition, 2020), indicating that the results generated by this study may not give a fair reflection of what might transpire in real life.

In another study conducted by (Salli *et al*., 2019) using faecal samples from healthy infants aged below 1 year, the effects of 2'FL on the composition and metabolites of the infant microbiota were investigated using a semi-continuous colon simulator and was compared against GOSs with Lac as a control. Changes in microbial composition and metabolites were measured via 16S RNA amplicon sequencing and gas chromatography. From the results, it was noted that 2'FL recorded similar increases in numbers of total bacteria, *Firmicutes* and *Actinobacteria* (including bifidobacteria) compared to GOS, but 2'FL was unable to match GOS in reductions of numbers of *Proteobacteria*. Furthermore, levels of SCFAs and lactic acid produced by 2'FL were only half compared with those of GOS, suggesting that at least in this

regard GOS results in a greater generation of metabolites associated with beneficial health outcomes than supplementation of 2'FL on its own.

Interestingly, (Li *et al*., 2012) noted that in the *in vitro* fermentation of piglet faeces, LNnT recorded the largest increases in levels of acetate and butyrate compared with FOS, GOS/polydextrose mixture and pooled HMOs, whereas pooled HMOs and FOS recorded higher levels of propionate and lactate. Furthermore, both pooled HMOs and LNnT were able to stimulate changes in the microbial composition, including increasing numbers of total bacteria, *Bifidobacterium*, *Lactobacillus*, *B. vulgatus* and *Clostridium* cluster XIVa along with resulting in reductions in *Clostridium* cluster IV, suggesting that both pooled and single HMOs can drive beneficial changes in microbial composition. However, despite differences being detected in microbial composition, both pooled HMOs and LNnT appeared to be no more effective in stimulating changes in microbial composition compared with both FOS and the GOS/polydextrose mixture, respectively.

The metabolic by-products and fermentation characteristics of prebiotics, including GOS, 2'FL, LNnT, 6'SL, high-performance inulin (HP) and gum arabic, were investigated by (Boler *et al*., 2013) using faecal samples isolated from both breast and formula-fed infants using an *in vitro* fermentation model. From the results, it was noted that the rates of fermentation of prebiotics differed significantly between breastfed and formula-fed infants inocula. For example, formula-fed inocula generated higher levels of acetate compared with breastfed inocula ($P \leq 0.001$) with 6'SL producing the largest concentration of acetate after 12-h fermentation. However, acetate production also varied over time between substrates with GOS generating large quantities of acetate regardless of diet. Butyrate appeared to be less affected by substrate or diet, but was higher in formula-fed inocula compared with breastfed inocula overall ($P \leq 0.01$); however, no differences were detected at any individual time point.

Conversely, propionate was affected by diet, but more so by substrate and time with 6'SL producing large amounts of propionate after 12 h of fermentation. Moreover, the fermentation of 2'FL seemingly levelled off after 6 h, further indicating that 2'FL likely undergoes rapid fermentation upon inoculation (Salli *et al.,* 2019). Finally, regarding microbial composition, numbers of bifidobacteria increased, whereas numbers of *E. coli* and *Clostridium perfringens* decreased regardless of the substrate used.

While using a pH-controlled *in vitro* fermentation model involving faecal donors from healthy, Irritable bowel syndrome (IBS) and ulcerative colitis patients, the most noticeable changes in gut microbiota composition were in *Bifidobacterium* ($P \leq 0.01$), while supplementation of 2'FL also resulted in increased numbers of *Eubacterium rectale* and *Clostridium coccoides* after 8- and 24-h fermentation $P \leq 0.01$ (8 h) and $P \leq 0.05$ (24 h) in healthy and $P \leq 0.01$ (8 and 24 h) IBD-ulcerative colitis donors. Significant increases in *Roseburia* at 8 h fermentation were seen in both healthy and IBS but not inflammatory bowel disease (IBD)-ulcerative colitis donors. Interestingly, in both IBD-ulcerative colitis and IBS patients but not healthy donors, there were significant increases in *Atopobium* cluster at 8 and 24 h ($P \leq 0.01$) (Ryan *et al.,* 2021).

These results further add to the evidence that the ability of HMOs and 2'FL to stimulate changes in the microbiota and its resulting metabolites appears to be highly specific and restricted to certain species, strains and subspecies of microbes as well as the initial composition of the gut microbiota (Gotoh *et al*., 2018; Sakanaka *et al*., 2020; Yu, Chen and Newburg, 2013).

1.2.8 Cross-feeding: a strategy to ensure dominance?

As previously discussed, the gut microbiota, in particular bifidobacteria, have developed several strategies to colonise and dominate the microbiota of an infant's gut with some strains, species and subspecies, displaying better potential than others. Interestingly, to help drive the colonisation of the gut, *Bifidobacterium*, *Bacteroides* as well as several other genera, including *Akkermansia*, *Anaerostipes* and *Roseburia*, have developed another strategy based on cross-feeding. Strains and species of *Bifidobacterium* and *Bacteroides* that are not able to utilise whole HMOs can feed on metabolites resulting from exploitation of HMOs by other species and strains (White *et al*., 2014).

In breastfed infants, *B*. *bifidum* is said to make up of over 10 per cent of the total number of bifidobacteria present within their gut (Sakanaka *et al*., 2019). When *B. bifidum* is in abundance, the corresponding microbiota appears to follow suit with higher numbers of several other bifidobacterial species and strains also being recorded (Tannock *et al*., 2013). The potential for *B*. *bifidum* to act as cross-feeders for other members of the *Bifidobacterium* genus was noted by Asakuma *et al*., (2011), who documented that *B*. *bifidum* left several HMO components, including Fuc and Gal in spent media, indicating that extracellular degradation had occurred and suggesting that non-HMOs utilising species/subspecies may be able to exploit these monosaccharide moieties (Kitaoka, 2012).

Additionally, using faecal suspensions isolated from infants, children and adults in a mucin-based medium supplemented with HMOs, Egan *et al*., (2014) and Gotoh *et al*., (2018) recorded the ability of several species and strains of bifidobacteria to grow in the presence and absence of *B*. *bifidum*. The overall findings of these studies suggest that in faecal suspensions possessing *B*. *bifidum*, the numbers of several bifidobacteria species and strains, including *B*.

longum 105-A and *B*. *breve* UCC2003, increased; strains not known to effectively utilise whole HMOs to any real extent. It seems that *B*. *bifidum* is likely to be a prominent player in the establishment of the microbiota in early life (Kitaoka, 2012).

It was reported in a single ecosystem that 2'FL derived metabolites from *B. pseudocatenulatum* strains LH9, LH13 and LH14 supported the growth of several non-HMOs utilising strains including *B*. *longum* LH12. However, *B*. *longum* subsp. *infantis* LH23 2'FL degradation products did not support the growth of *B*. *breve* (LH21 and LH24), respectively. Additionally, with *B*. *longum* LH206 2'FL conditioned media, increases in numbers of all strains of *B*. *longum* subsp. *infantis* and *B*. *pseudocatenulatum* tested were seen. This indicates that the metabolism of 2'FL by *B*. *infantis* LH206 may generate a wider variety of growth-promoting compounds (Lawson *et al*., 2020).

It has been documented in a co-culture experiment that *Anaerostipes cacae* was able to utilise monosaccharides, as well as lactate and acetate, resulting from HMOs degradation by *B*. *infantis* (Chia et al., 2021). *Roseburia* spp. were able to grow in the presence of *A*. *muciniphila*, whereas in pure culture *Roseburia* spp. showed little-to-no sign of growth (Pichler *et al*., 2020). The ability of *Bacteroides* to act as primary degraders of HMOs was demonstrated in mice-fed sialylated HMOs, including 3'SL and 6'SL, when a marked increase in *Enterobacteriaceae* was seen. This led to an exacerbation of the pro-inflammatory response (Huang *et al*., 2015).

Additionally, in antibiotic-treated germ-free mice infected with either *Salmonella typhimurium* or *C*. *difficile*, it has been demonstrated that *S*. *typhimurium* was able to access both Fuc and sialic acid and *C*. *difficile* was able to readily utilise sialic acid as a result of breakdown of host carbohydrates by *Bacteroides thetaiotaomicron* (Ng *et al*., 2013). However, while the expansion of enteric bacterial pathogens via the utilisation of HMOs is sometimes seen *in vitro*

in co-cultures or using *in vivo* mechanistic disease state rodent models, in the highly complex ecosystem in the human gut, this has never been reported.

Not all microorganisms found within the gut can participate in cross-feeding due to intracellular metabolism of polysaccharides/glycans. The inability of specific bifidobacteria to act as cross-feeders was demonstrated by (Garrido *et al*., 2016) *B. longum* SC596 exhibited excellent growth on both Type-I and Type-II chain HMOs, albeit displaying a preference for fucosylated HMOs; however, no monosaccharides from HMOs degradation were detected in the medium. As the degradation of HMOs by *B*. *longum* SC596 appears to be similar to that of *B*. *longum* subsp. *infantis* ATCC 15697, which uses intracellular metabolism (Garrido, Dallas and Mills, 2013), this suggests that *B*. *longum* SC596 cannot partake in the cross-feeding of other microorganisms.

These results suggest that the mutualistic behaviour which exists between microorganisms found in the gut likely influences the rates at which metabolites such as SCFAs are generated (Comstock, 2009). From this, one could conclude that the degree to which this mutualistic behaviour exists between microorganisms found in the gut not only increases the diversity of the gut microbiota, but is maybe one of the most critical characteristics in helping to shape a flexible, healthy ecosystem (Gotoh *et al*., 2018).

1.2.9 The influence of human milk oligosaccharides on infant microbiota composition *in vivo*

As previously discussed, the ability of HMOs to alter microbial composition in infants has been studied extensively using *in vitro* test conditions, but less so *in vivo* with only a limited number of studies being undertaken to date.

In a proof-of-concept study (De Leoz *et al*., 2015), serial faecal samples were collected from two vaginally born infants. Infant A was breastfed directly from birth, whereas infant B received formula supplementation for 4 days from days 2–6 and then was solely breastfed thereafter. Faecal samples were collected twice per week for the first month, twice per month in the second month and once or twice per month thereafter. Microbial compositions were analysed via 16S rRNA sequencing. The results demonstrated that after an initial rise in non-HMO-consuming bacteria, including *Enterobacteriaceae* and *Staphylococcaeae*, large shifts in microbial composition from non-HMO-consuming bacteria to HMO-consuming bacteria *Bacteroidaceae* and *Bifidobacteriaceae* were seen. Yet, large differences were seen between both donors whereby week 13 *Bifidobacterium* spp. dominated in infant A, and levels of most faecal HMOs dropped dramatically, whereas by week 14, *Bacteroides* spp. were most dominant in infant B.

Borewicz *et al*., (2019) and Borewicz *et al*., (2020) aimed to correlate the HMOs in breast milk with changes in faecal microbiota composition, analysed via Illumina HiSeq 16S rRNA gene sequencing, in healthy 2-, 4-, 6- and 12-week-old breastfed infants. Unsurprisingly, the ability of infants to utilise HMOs, including 2'FL, was associated with differences in the faecal microbiota composition, with those infants possessing relatively high abundances of *Bifidobacterium* 418, 614 and 681 and *Lactobacillus* 744 (FDR ≤ 0.05) reporting higher rates of 2'FL consumption. Additionally, infants who recorded higher consumptions rates of LNT and LNnT, LNFP III, LNFP II and lacto-N-hexaose (LNH) possessed significantly higher relative abundances of *Bifidobacterium* OTUs 418, 406, 643, 658, 423, 1335 and 597 and *Bacteroides* 144 (FDR ≤ 0.05). Moreover, the degradation of sialylated HMOs 3'SL, 6'SL, LST a, LST b and LST c appeared to be more highly associated with *Bacteroides*; a finding confirming those reported by Yu *et al*., (2013) who demonstrated using *in vitro* models that *B*. *fragilis*, *B*. *vulgatus* and *B*. *thetaiotaomicron* could utilise 3'SL and 6'SL as sole carbon sources.

Interestingly, Borewicz *et al*., (2019) and Borewicz *et al*., (2020) also recorded that lactobacilli appeared to thrive in the presence of 2'FL, DFL, LNDFH I, LNT, LNnT and LNFP II. This is fascinating given that it has been shown repeatedly in several *in vitro* studies that lactobacilli appear to be unable to utilise HMOs (Schwab and Ganzle, 2011; Ward *et al*., 2006). This suggests that lactobacilli might be able to thrive in the infant's gut via cross-feeding, scavenging any resulting metabolites, including Fuc and lactose from the extracellular degradation of HMOs (Zuniga *et al*., 2018). This likely infers that the degradation of HMOs strongly correlates with the microbiota and specifically with the relative abundance of the phylotypes *Bifidobacterium*, *Bacteroides* and *Lactobacillus* present within an infant's gut.

Moreover, in a randomised, double-blind, multicentre clinical trial, healthy, full-term infants (aged 0–14 days) were fed infant formula with no added HMOs (control), or the same formula with the addition of 2'FL and LNnT for a timeframe of 6 months. Thereafter, all infants were fed the same non-HMO-containing infant formula (Berger *et al*., 2020). Results were analysed against a breastfed reference group with changes in microbial community types being analysed at 3 and 12 months via 16S rRNA gene sequencing. The results indicated that, compared with the breastfed reference group, the HMO-containing formula stimulated increases in *Bifidobacterium*, albeit to a lower degree than the reference breastfed group. Levels of *Escherichia* were, however, significantly lower in the HMO-containing formula group compared with the control group and were similar to those in the breastfed group. Numbers of *Peptostreptococcaceae* were far higher in the control and HMO-containing formula group compared with the breastfed group. Yet, at 12 months, no differences were detected between the two formula groups. This suggests that the supplementation of infant with HMO-containing formulae may offset some of the ill effects associated with not breastfeeding from birth. However, this study is not without limitation. First, only two faecal samples were collected: one at 3 months and one at 12 months. Second, no data were collected on day-care

attendance and when solid foods were introduced (weaning) which, due to the effects these factors have on the microbial composition (McBurney *et al*., 2019), may have introduced biases into the results. Consequently, further investigation into this area would be highly beneficial to determine the true effects that both 2'FL and LNnT have on altering the composition of healthy infants *in vivo*.

In another study, differences in gut microbiota composition between caesarean and vaginally born babies of α1-2 fucosylated secreting mothers were conducted by (Tonon *et al*., 2021). In this study, faecal microbiota composition from caesarean and vaginally born infants was analysed by 16S rRNA gene sequencing and qPCR with results being stratified by secretor status. The authors concluded that levels of *Bifidobacterium* were similar between caesarean and vaginally born infants of secretor mothers. Yet, there were differences between caesarean and vaginally born infant microbiotas with the caesarean born infants from secretors possessing higher amounts of *Kluyvera* and *Veillonella*. Vaginally born infants from secretor mothers possessed higher amounts of *Bacteroides*. This further adds to the evidence that mode of delivery may likely impact on proliferation of the gut microbiota and HMO utilisation.

In addition, to healthy infant's HMOs and their effects on the gut microbiota and also been studied in preterm infants. In one study 12 premature infants were randomised into 2 groups – one group containing formula and increasing doses of short-chain galacto-oligosaccharides (DP < 8) and the other group receiving formula + HMOs (Underwood *et al*., 2014). The authors noted that relative abundances of clostridia increased with increasing doses of HMOs. The authors also noted that there were trends towards increase of γ-proteobacteria over time/dose in preterm infants feed formula + HMOs.

Additionally, in a study involving preterm infants with necrotising enterocolitis (NEC), it was noted that infants with NEC possessed higher levels of *Proteobacteria* and lower levels of *Actinobacteria* at phylum levels, along with lower relative abundances of *B. longum* and higher relative abundances of *Enterobacter cloacae*. The authors also noted that the composition of breast milk, specifically lower concentration of DSLNT, was associated with the likelihood of developing NEC (Masi *et al*., 2021). While, similarly, Underwood *et al*., (2015) concluded that preterm infants of non-secretor mother possessed higher levels of *Proteobacteria* and lower levels of *Firmicutes*, secretor mothers possess specific fucosylated HMOs including LDFT and LNFP V, which may be associated with lower levels of *Enterobacteriaceae* and potentially protective effect against pathogens associated with NEC. These results potentially infer that the composition of HMOs present in breast milk may be a contributing factor towards the development of NEC in preterm infants.

Moreover, in another study conducted in healthy rats, (Chleilat *et al*., 2020) investigated the effects that the supplementation of 2'FL and 3'SL either together or on their own had on microbial composition compared to a non-HMO control. In general, all HMO-fortified diets altered gut microbiota composition. However, larger increases in *Bifidobacterium* spp. were recorded in the 2'FL group compared to the 3'SL-fortified group ($P = 0.03$). Additionally, *Akkermansia muciniphila* numbers were significantly lower in 3'SL + 2'FL group compared to the control ($P \leq 0.01$) respectively. Lastly, in a study involving mice supplemented with 2'FL and 2'FL consuming *B. pseudocatenulatum* MP80, it was noted that 2'FL created an environment that allowed *B. pseudocatenulatum* MP80 to thrive, along with finding that 2'FL increased *Bifidobacteriaceae* relative abundance, as well as resulting in higher log ratios of *Bacteroidaceae* and *Bifidobacteriaceae* relative to *Lachnospiraceae* and *Ruminococcaceae* amplicon sequencing variant ($P = 0.003$) (Heiss *et al*., 2021).

These results help to explain the large variability in the presence and levels of HMOs detected in the faecal samples of infants, even when secretor status is considered, with virtually no HMOs being detected in faecal samples of several infants, whereas, in the faecal samples of other infants, there was a strong presence of non-fucosylated HMOs, suggesting the presence of the fucoside-utilising microorganisms needed to degrade fucosylated HMOs (Asakuma *et al*., 2011). In faecal samples of several other infants, large quantities of LNnT were detected with LNnT not being detected in others. However, despite these differences, a common characteristic amongst nearly all infants used in these studies was what appeared to be the presence of several new, non or partially intact HMOs and HMOs by-products in faecal samples with the majority of new HMOs detected displaying a high proportion of HexNAc (Davis *et al*., 2016; De Leoz *et al*., 2013; Dotz e*t al*., 2015).

Thus, while results seemingly imply that the supplementation of 2'FL and LNnT may contribute towards a positive shift in the composition of the gut microbiota, in reality, the make-up of the infant's microbiota is shaped through several often complex and interacting factors from birth, including mode of delivery (vaginal vs. c-section), feeding practices (breast vs. bottle feeding) and age at which the introduction of complex dietary substrates occurs (weaning). The use of gut microbiome altering medications and supplements, namely antibiotics and probiotics (Bertelsen, Jensen and Ringel-Kulka, 2016; McBurney *et al*., 2019), will also have an impact. Consequently, the degradation of HMOs will differ greatly depending on the relative abundance of specific species, strains and subspecies of microorganisms present within an individual infant's gut microbiome. More detailed analysis is needed of infant microbiotas prior to supplementation in such studies. The supplementation of 2'FL and LNnT in infant formula milk may be of little-to-no benefit to many infants as several infants especially those

who were never breastfed may not possess the necessary microorganisms and thus glycosidases and transporters needed to effectively utilise these specific HMOs.

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Author contributions

Conceptualisation: P.P.J.J, R.A.R and A.W; Formal analysis: P.P.J.J.; Funding acquisition: R.A.R.; Investigation: P.P.J.J.; Methodology: all authors; Project administration: R.A.R.; Supervision: R.A.R and A.W.; Visualisation: all authors; Writing – original draft: P.P.J.J.; Writing – review and editing: all authors. P.P.J.J. drafted and revised all manuscript drafts following feedback from co-authors R.A.R and A.W.

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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 human milk oligosaccharides 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 pre-screening tool to determine combinations that could be taken forward for use in a human intervention trial.

Method 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 was assessed by fluorescence *in situ* hybridisation flow cytometry (FISH-FLOW) and 16S rRNA sequencing, and changes in SCFA via gas chromatography. All substrates tested generated changes in microbial load and profile, achieving peak microbial load at 8 h fermentation with largest changes in profile across all substrates in *Bifidobacterium* ($Q \leq 0.05$). This coincided with significant increases in acetate observed throughout fermentation ($Q \leq 0.05$). Finally, combinations of short-chain inulin-type fructans, β-glucan and 2'fucosyllactose induced substantial increases in both propionate and butyrate producing bacteria (*Roseburia*, *Clostridium* cluster IX and *Faecalibacterium* praunitzii), and higher levels of propionate and butyrate, the latter being maintained until the end of fermentation ($Q \leq 0.05$, $Q \leq 0.01$, and $Q \leq 0.001$).

Conclusions

Combinations of inulin-type fructans, specifically oligofructose, with β-glucan and 2'fucosyllactose induced selective changes in microbial combination and short-chain fatty acid production specifically *Roseburia*, *Clostridium* cluster IX, *Faecalibacterium praunitzii*, propionate and butyrate compared to sole supplementation.

Significance and importance of study

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

Abbreviations

Inulin-type fructans; β-glucan; 2'fucosyllactose; gut microbiota; short-chain fatty acids

2.0 Introduction

The term prebiotic was first defined by (Gibson and Roberfroid, 1995) as a "non-digestible 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 microbiotas and consequently improve health. Since then, the definition of a prebiotic has evolved where today prebiotics are categorised 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 (Louis and Flint, 2009; Louis and Flint, 2017; Reichardt *et al.*, 2014). We

hypothesise that blending combinations of prebiotics and prebiotics candidates will result in the stimulation of a wider range of organisms, resulting in increased and sustained generation of SCFA, particularly propionate and butyrate, compared to sole supplementation.

The most substantiated prebiotics are fructo-oligosaccharides (OF) and inulin, which belong to a class of non-digestible carbohydrates referred to as inulin-type fructans (ITF) (Scott *et al.*, 2019). One of the key concepts in prebiotics is 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, Meyer and Gibson, 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 utilised 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 human milk oligosaccharides (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'fucosyllactose (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, strains, and subspecies 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 non-starch 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 as 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 utilise β-glucan as a carbon sources appears to be somewhat mixed

(Shoukat and Sorrentino, 2021). This likely due to not all bifidobacteria possessing the necessary loci able to utilise high molecular weight and complex carbohydrates.

During the fermentation of these complex carbohydrates the short-chain fatty acids (SCFA) acetate and lactate are formed as products of fermentation by members of *Bifidobacterium*. However, *Bifidobacterium* do not produce either propionate or butyrate. Several bacteria within the gut including *Bacteroides, Roseburia* spp., *Faecalibacterium prausnitzii* and *Clostridium* cluster IX can utilise 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 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 short-chain fatty acid production) of OF and inulin, 2'FL and β-glucan from barley in singular and in combination using pH-controlled *in vitro* batch culture fermentation over 48 h.

2.1 Methods

2.1.1 Materials

2.1.1.1 Prebiotics

The ITF used was oligofructose (OF) (Orafti® P95, DP 3-9, average DP 4; BENEO-Orafti, Tienen, Belgium) and an 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 polymerisation (DP) ≥ 11 approx. 25–30% (on g/100 g DM), average DP of 7–8, 15-30 wt.-% (dry matter, based on total mass of carbohydrates) of Fm compounds with m=2 to 9, BENEO-Orafti, Tienen, Belgium).

2.1.1.2 Prebiotic candidates

2'fucosyllactose 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'fucosyllactose was supplied by BENEO-Orafti, (Tienen, Belgium). BENEO β-glucan rich barley flour was supplied by BENEO-Orafti, Tienen, Belgium). Per 100g, 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, lactose): 4 g.

2.1.1.3 Reagents

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

2.1.2 Starch removal

β-Glucan-rich barley flour (100 g) was mixed with 500 g of deionised distilled water. Amylase MT-3K (3000 μ/mL, Enzyme Supplies Limited, Oxford) was diluted to 10 units per mL and 2 mL were added. The β-glucan sample was then placed in a stirring water bath at 50°C for one hour. 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.

2.1.3 *In vitro* batch culture fermentation

2.1.3.1 Faecal sample preparation

Ethical approval of collecting faecal samples from healthy volunteers was obtained from University of Reading Research Ethics Committee. Freshly voided faecal samples were obtained from 3 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 homogenised in a stomacher (Seward, stomacher 80, Worthing, UK) for 120 seconds at 260 paddle-beats per min. 15 mL of faecal slurry was immediately used to inoculate each batch culture vessel.

2.1.3.2 Basal batch culture nutrient medium

To make 1 litre 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 litre of deionised water. 135 mL of medium was placed into glass jars and autoclaved at 121 °C for 15 minutes.

2.1.3.3 pH controlled, stirred batch culture fermentation

For each donor one independent batch cultures were run. For each batch culture vessels 14 x 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, ITF-mix + β-glucan (all 50/50,w/w). One vessel was set up as the negative control with no added carbohydrate. 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 fluorescence *in situ* hybridisation-flow cytometry (FISH-FLOW), 16S rRNA sequencing and gas chromatography-flame ionisation detection (GC-FID), respectively.

2.1.4 Enumeration of faecal microbial populations by flow cytometry fluorescence *in situ* hybridisation (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) were added and samples were stored at 4 °C for 4 hours. 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 2.1

Probe	Sequence (5' to 3')	Targeted groups	Reference
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	(Wallner, Amann and Beisker, 1993)
Eub338I	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)

Table 2.1: Name, sequence, and target group of oligonucleotide probes used in this study for FISH of bacterial enumeration

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 11337 × 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 11337 × g for 3 min. Supernatants were discarded, and pellets washed with 500 µL filtered cold PBS by aspiration to disperse the pellet. Samples were then centrifuged at 11337 × g for 3 min and supernatants discarded. Pellets were resuspended in 150 µL of hybridization buffer, aspirated using a pipette and gently vortexed. Samples were centrifuged at 11337 × g for 3 min and supernatants discarded. Pellets were resuspended in 1 mL of hybridisation buffer. Aliquots (50 µL) of samples were placed in labelled 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 hours in the dark.

Following incubation, 125 µL of hybridization buffer were added to each tube and gently vortexed. Samples were then centrifuged at 11337 × 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 11337 × g for 3 minutes. 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. Fluorescence measures were performed by a BD Accuri™ C6 Plus (BD, Erembodegem, Brussels) measuring at 488 nm and 640 nm. A threshold of 9000 in forward scatter area (FSC-A) and 3000 in side scatter area (SSC-A) was 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, limit of collection was 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.

2.1.5 Short-chain fatty acid analysis (SCFA) by gas chromatography-flame ionisation detection (GC-FID)

Samples (1.5 mL) of batch culture fluid were collected and centrifuged at 11337 × 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 performed with according to Richardson *et al.* (1989) with modifications. Briefly, 1 mL of sample was transferred into a labelled 100 mm ×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 minute. Samples were centrifuged at 2000 x g for 10 min. The resulting diethyl ether (upper) layer of each sample was transferred to clean 100 mL 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 hours to allow samples to completely derivatise. An Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS 30m × 0.25mm column with a 0.25µm coating (crosslinked (5%-phenyl)-methylpolysiloxane, Hewlett Packard, UK) was used for analysis of SCFA. Temperatures of injector and detector were 275 °C, with the column temperature programmed from 63 °C to 190 °C at 15 °C min^{-1} followed by 190 °C for 3 minutes. 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}.

2.1.6 16S RNA Sequencing

2.1.6.1 Bacterial DNA extraction

Bacterial DNA was extracted from batch culture samples pellets using the QIAamp Fast DNA Stool mini kit (QIAGEN) according to the manufacturer's instructions. Batch culture sample pellets diluted in 400 uL 0.1 mol l^{-1} 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 45s at speed 4). Raw extract (200 uL) was then used for DNA isolation.

2.1.6.2 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). Bead ratio was 1:1:2, Beads were washed with 75% Ethanol, airdried 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, 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 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, to not distort the statistical analysis. Remaining reads were denoised 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 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 corrected for by taking into considering the no. 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).

2.1.7 Statistical Analysis

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

2.2 Results

2.2.1 Organic Acids

Figure 2.1, 2.2, 2.3 and 2.4 reports the mean values for acetate, propionate, butyrate and total SCFA recorded throughout the course of fermentation across the 3 independent donors. Overall mean and Individual SCFA values are presented in Appendix 2.1 and 2.2.

Acetate was the most abundant SCFA detected representing between 52-73% of total SCFA produced by the end of fermentation. Acetate concentrations were highest at the end of fermentation (48 h) in all treatments vessels, and they were all significantly higher compared to their respective 0 h (Figure 2.1). Differences were detected in the magnitude of increase in acetate production between substrates and was greatest in 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. 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.

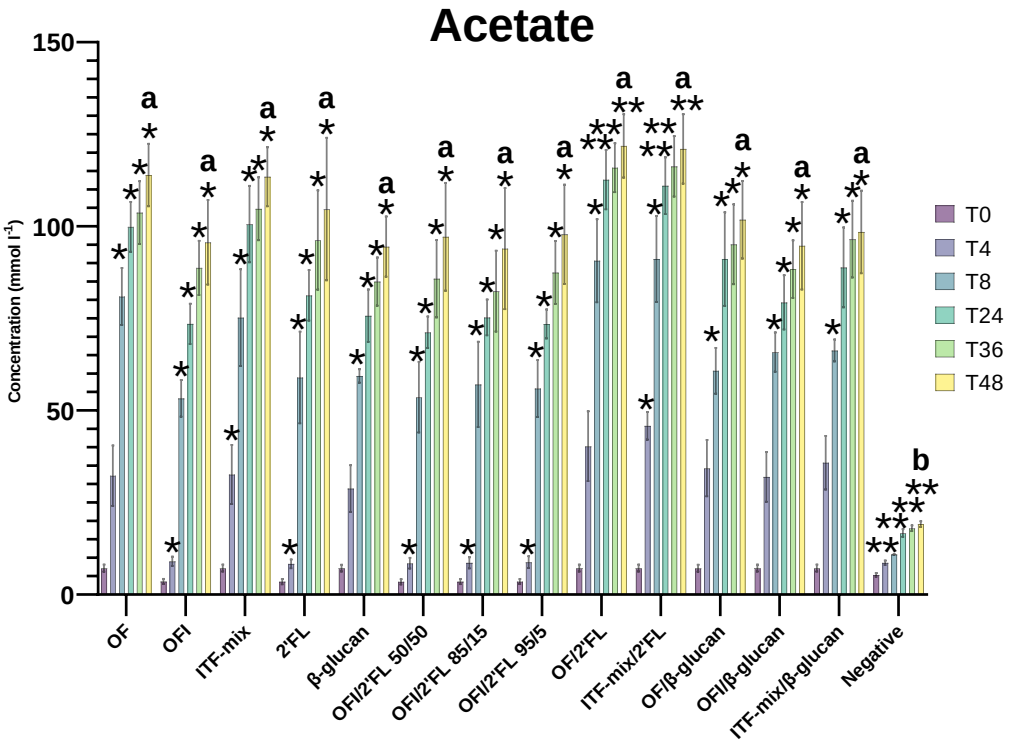

Figure 2.1. GC-FID analysis of acetate concentrations in the supernatant of effluents collected from vessel 1-14 at 0, 4, 8, 24, 36 and 48 h representing the mean (*n* = 3) and standard error (SE) of the data. Concentration reported in (mmol l^{-1}). * (*Q* = ≤ 0.05), ** (*Q* ≤ 0.01), and *** (*Q* ≤ 0.001) indicates significance compared with 0 h sampling. Significant differences between substrates at 48h are indicated by differing letters. **Abbreviations:** OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Propionate accounted for between 19-33% of 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 hours significant differences were detected in final propionate production between β-glucan singular/β-glucan-ITF combinations and several other substrates including OFI, 2'FL, OFI/2'FL combinations (50/50, 85/15 and 95/5) (Figure 2.2 and Appendix 2.1).

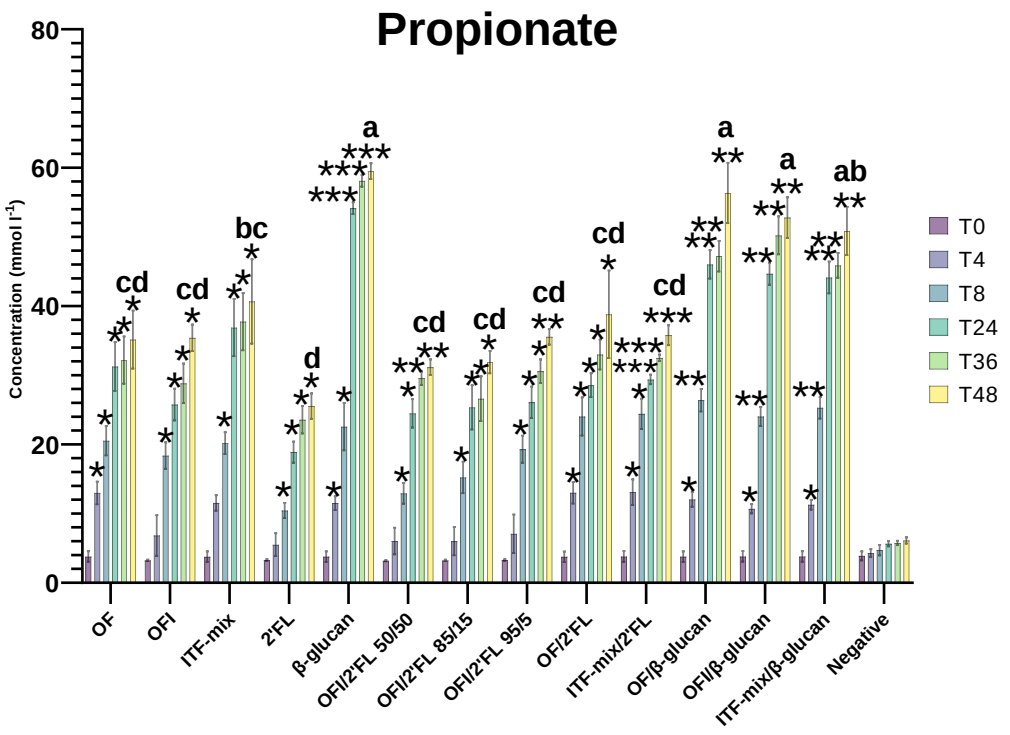

Figure 2.2. GC-FID analysis of propionate concentrations in the supernatant of effluents collected from vessel 1-14 at 0, 4, 8, 24, 36 and 48 h representing the mean (*n* = 3) and standard error (SE) of the data. Concentration reported in (mmol l^{-1}). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling. Significant differences between substrates at 48h are indicated by differing letters. **Abbreviations**: OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

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 mmol l^{-1} 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 utilising treatments (Figure 2.3 and Appendix 2.1). These data correlate strongly with changes seen in butyrate-producing bacteria (*Bacteroides, Roseburia* and *Faecalibacterium prausnitzii*) throughout fermentation.

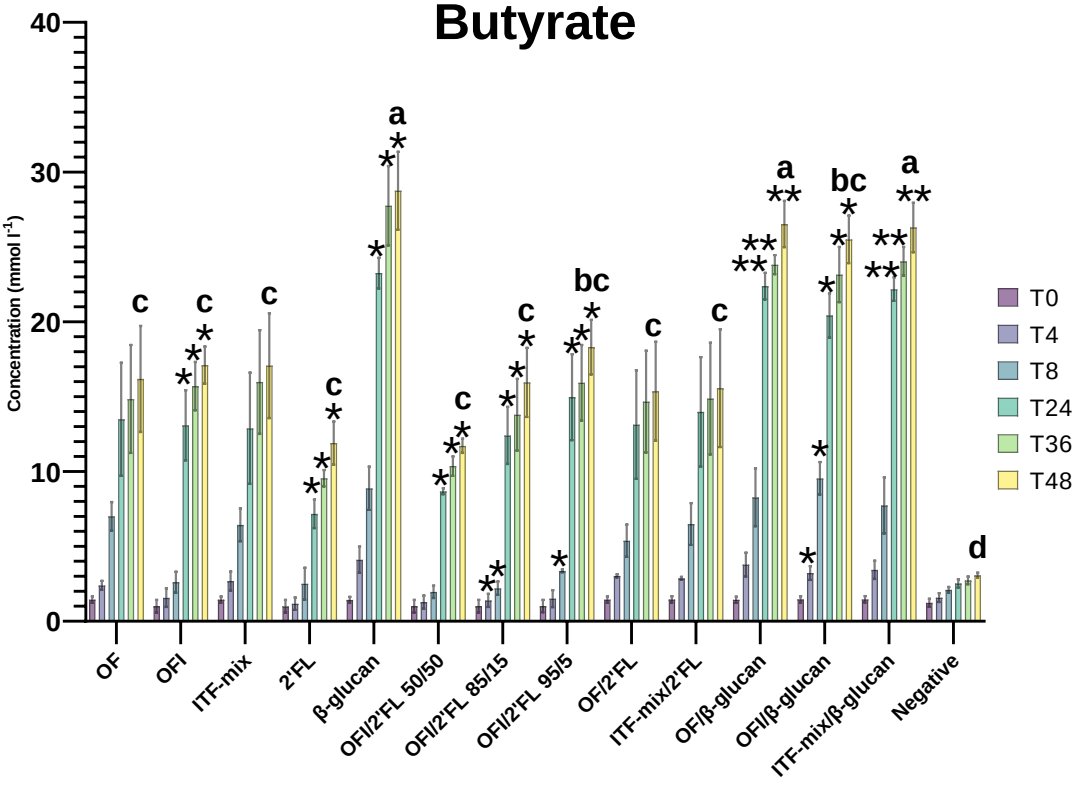

Figure 2.3. GC-FID analysis of butyrate concentrations in the supernatant of effluents collected from vessel 1-14 at 0, 4, 8, 24, 36 and 48 h representing the mean (n = 3) and standard errors (SE) of the data. Concentration reported in (mmol l^{-1}). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling. Significant differences between substrates at 48h are indicated by differing letters. **Abbreviations:** OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

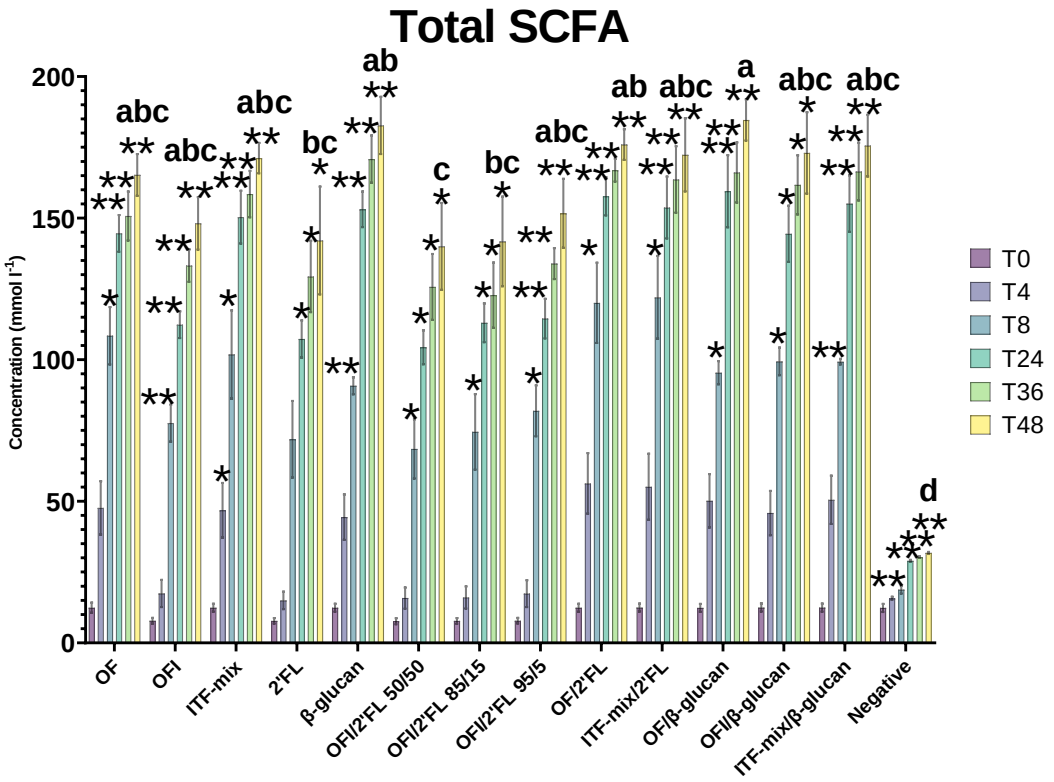

Figure 2.4. GC-FID analysis of total SCFA concentrations in the supernatant of effluents collected from vessel 1-14 at 0, 4, 8, 24, 36 and 48 h representing the mean (*n* = 3) and standard error (SE) of the data. Concentration reported in (mmol l^{-1}). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling. Significant differences between substrates at 48h are indicated by differing letters. **Abbreviations:** OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

2.2.2 Bacterial Enumeration

In order to determine changes in bacterial populations, twelve 16S-rRNA fluorescence *in situ* hybridisation probes were used to identify changes in 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 Figures 2.5.1, 2.5.2, 2.5.3, 2.5.4, 2.5.5 and 2.5.6. Overall mean and individual FISH-FLOW data is presented is presented in Appendix 2.3 and 2.4

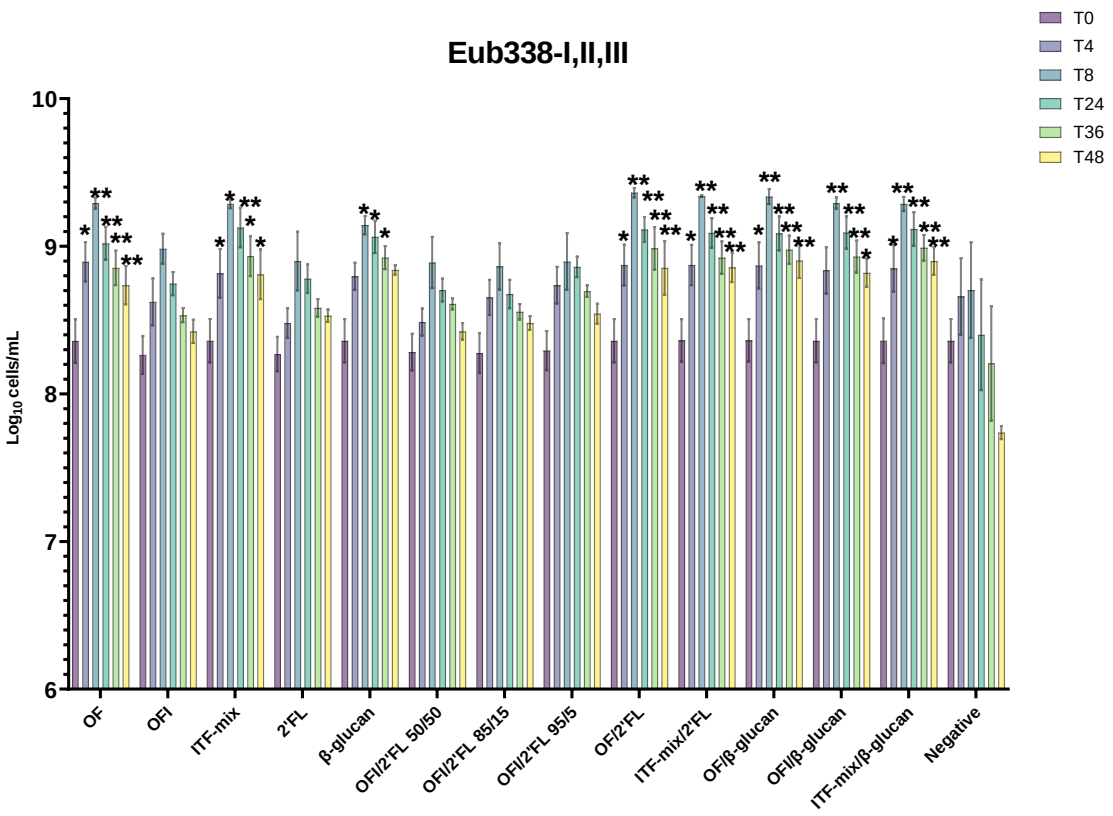

Figure 2.5.1. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL) using probes: total bacteria (Eub338 I-II-III) Mean and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling. **Abbreviations:** OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Significant increases in total bacterial counts (Eub338-II,II,III) were observed with all substrates tested ($Q \leq 0.05$) with exception of 2'FL singular, OFI and all combinations of OFI/2'FL. Highest total bacterial counts across all substrates were observed at 8 h fermentation. 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. Smallest increases in total bacteria at 8 h were recorded in vessels utilising 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 (Figure 2.5.1).

The largest significant changes in bacterial numbers observed throughout the course of fermentation were recorded in *Bifidobacterium* (Bif164) counts (Figure 2.5.2) The bifidogenic

effect varied across the different prebiotic fermentations. At 8h 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 utilising 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 levels of acetate recorded in respective vessels (Figure 2.1).

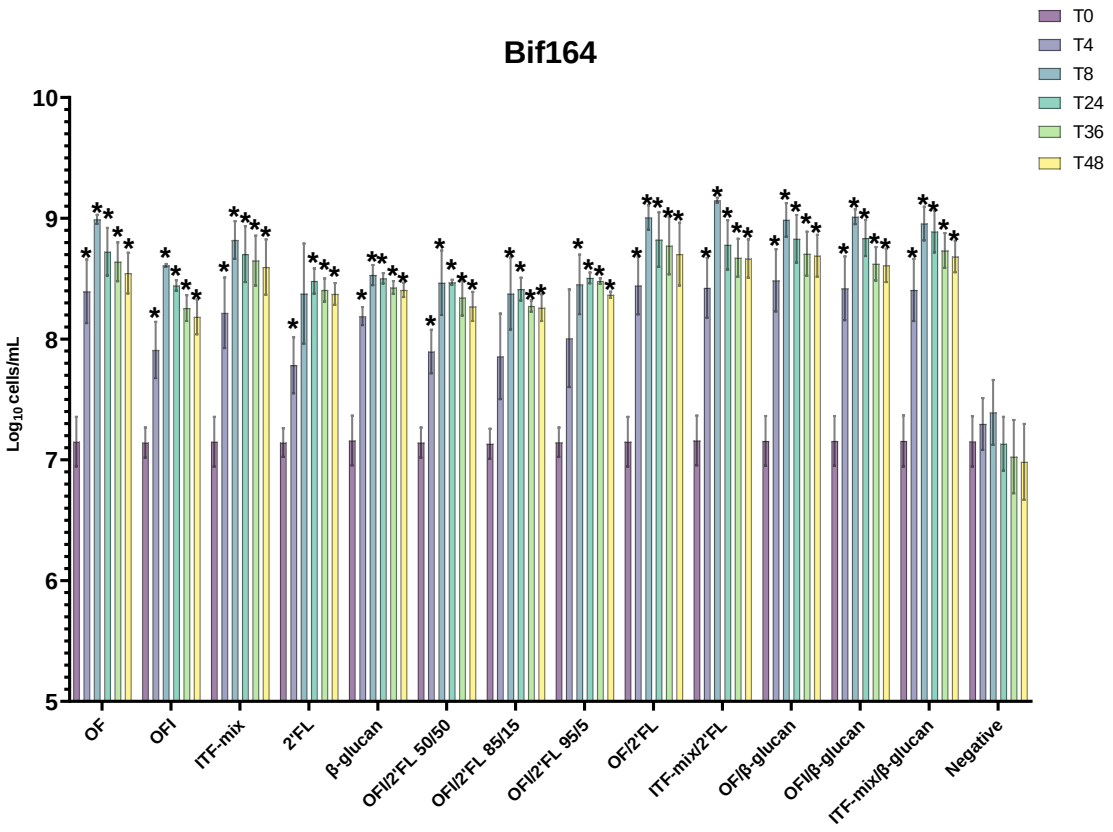

Figure 2.5.2. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL) using probes: *Bifidobacterium* spp. Mean and standard error. * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling. **Abbreviations:** OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

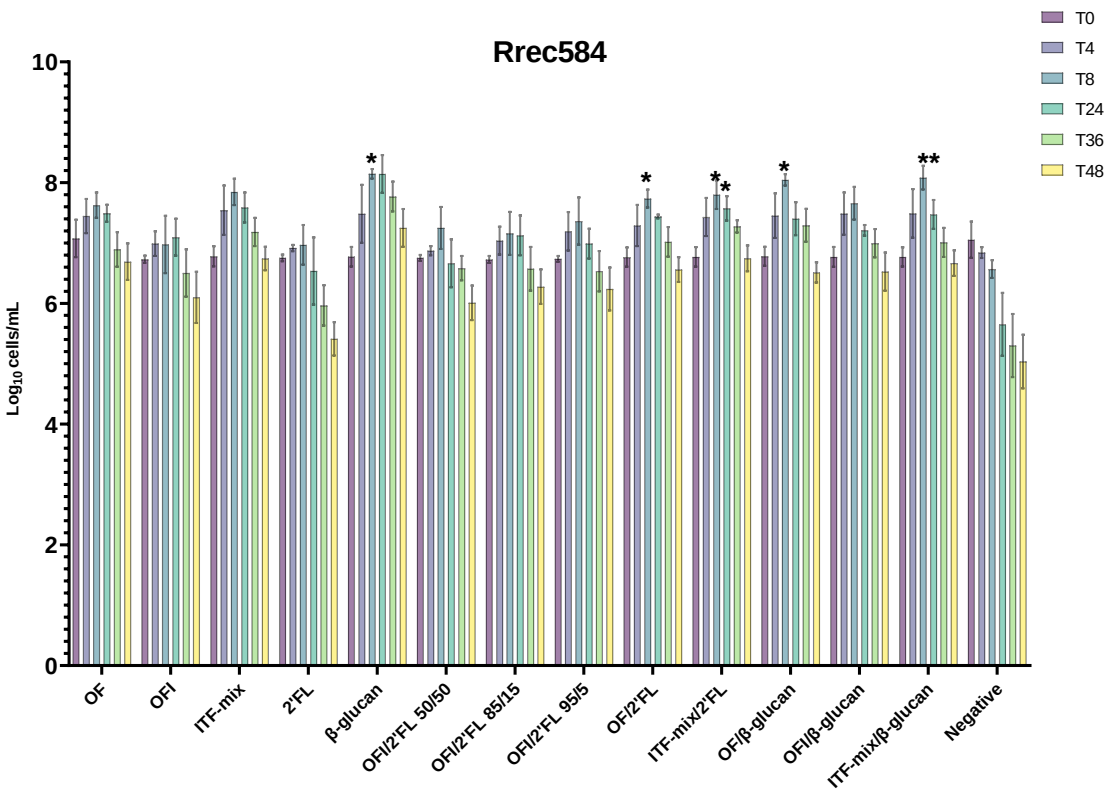

Figure 2.5.3. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL) using probe: *Roseburia* (Rrec584). Mean and standard error. * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling. **Abbreviations:** OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

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 propionate and butyrate producing bacterial groups, including *Roseburia* spp. (Rrec584), *Clostridium* cluster IX (Prop853) and *Faecalibacterium prausnitzii* (Fprau655) (Figures 2.5.3, 2.5.5 and 2.5.6). 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 *Rosburia* spp. and *Clostridium* cluster IX 0.90 ± 0.10 (SE) log_{10} cells/mL at 8 h fermentation (all $Q \leq 0.05$) respectively. Whereas at 8 h fermentation only OF, ITF-mix and β-glucan singular recorded significant increases in *Faecalibacterium prausnitzii* at 0.90 ± 0.11 (SE) log_{10} cells/mL (all $Q \leq$ 0.05) (Figure 2.5.6 and Appendix 2.3). Intriguingly, there were also large increases in *Bacteroides-Prevotella* counts (Bac303) (1.27, 1.31 1.32 and 1.48 log_{10} cells/mL) observed at 8h

fermentation in β-glucan and ITF/β-glucan containing treatments. However, these were not statistically significant from 0 h sampling (all *Q* = >0.05) (Appendix 2.3 and 2.4). The lack of significance likely resulted from the high variability in responses between donors at subsequent time points. These results coinciding with the noticeable increases in propionate and butyrate seen in respective vessels, explicitly those possessing β-glucan as treatments.

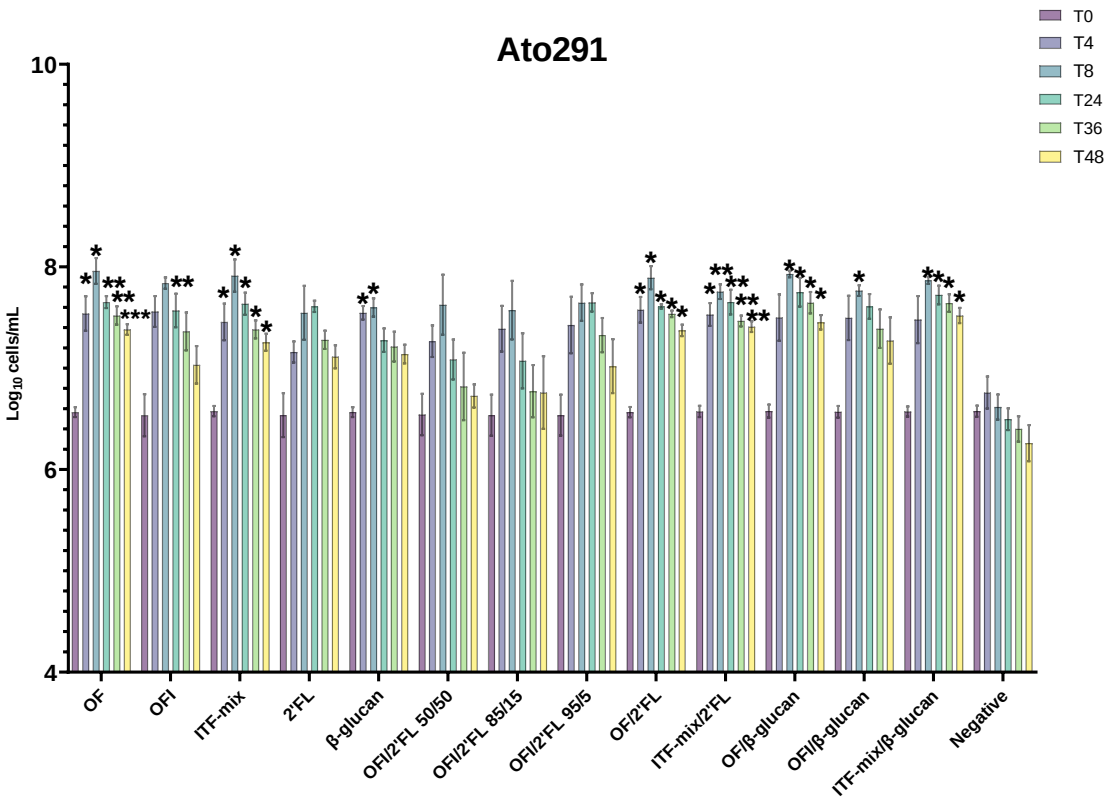

Figure 2.5.4. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL) using probe: *Atopobium* cluster (Ato291). mean and standard error. * (*Q* ≤ 0.05), ** (*Q* ≤ 0.01), and *** (*Q* ≤ 0.001) indicates significance compared with 0 h sampling. **Abbreviations:** OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

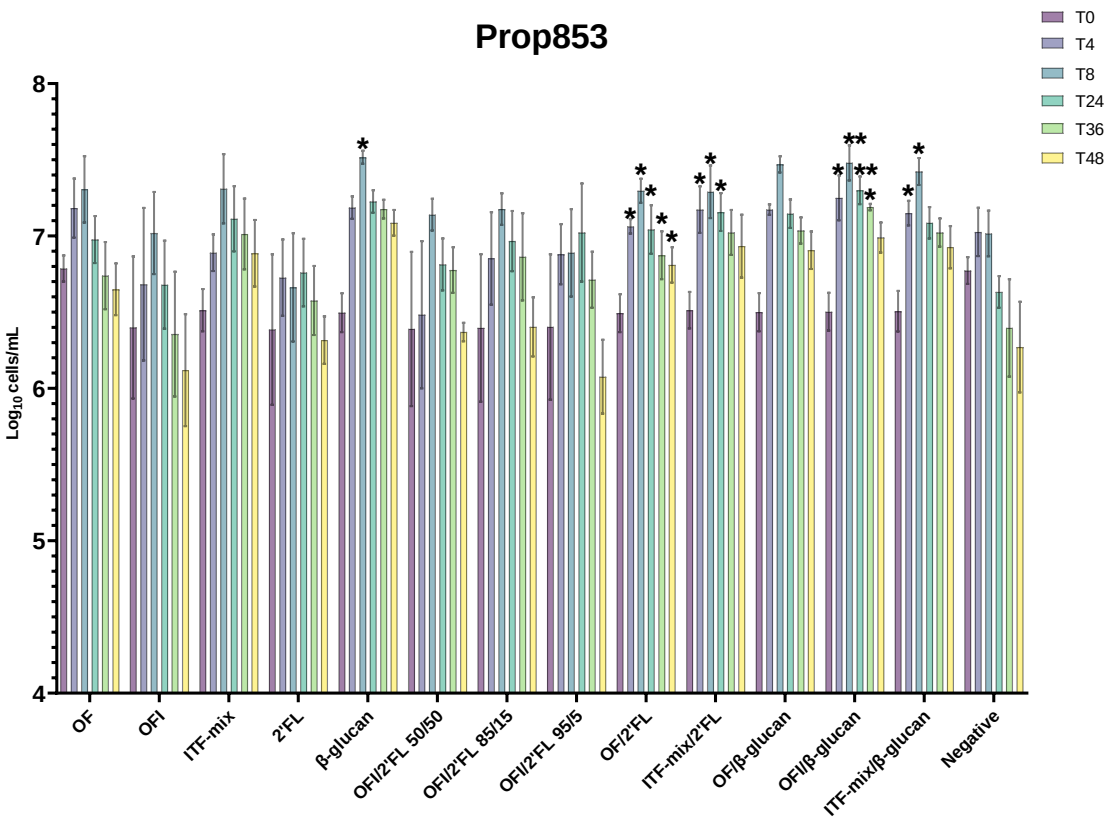

Figure 2.5.5. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL) using probe: *Clostridium* cluster *IX* (Prop853). Mean and standard error. * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling. **Abbreviations:** OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

There were also significant differences in numbers of *Lactobacillus/Enterococcus* (Lab158), *Clostridium coccoides-Eubacterium rectale* group (Erec482) and *Atopobium* cluster (Ato291) detected in several substrates across a number of different timepoints (Figure 2.5.4 and Appendix 2.3). Lastly, only transient changes were observed in *Desulfovibrio* spp. (DSV687) and *Clostridium histolyticum* (Chis150) across all substrates tested.

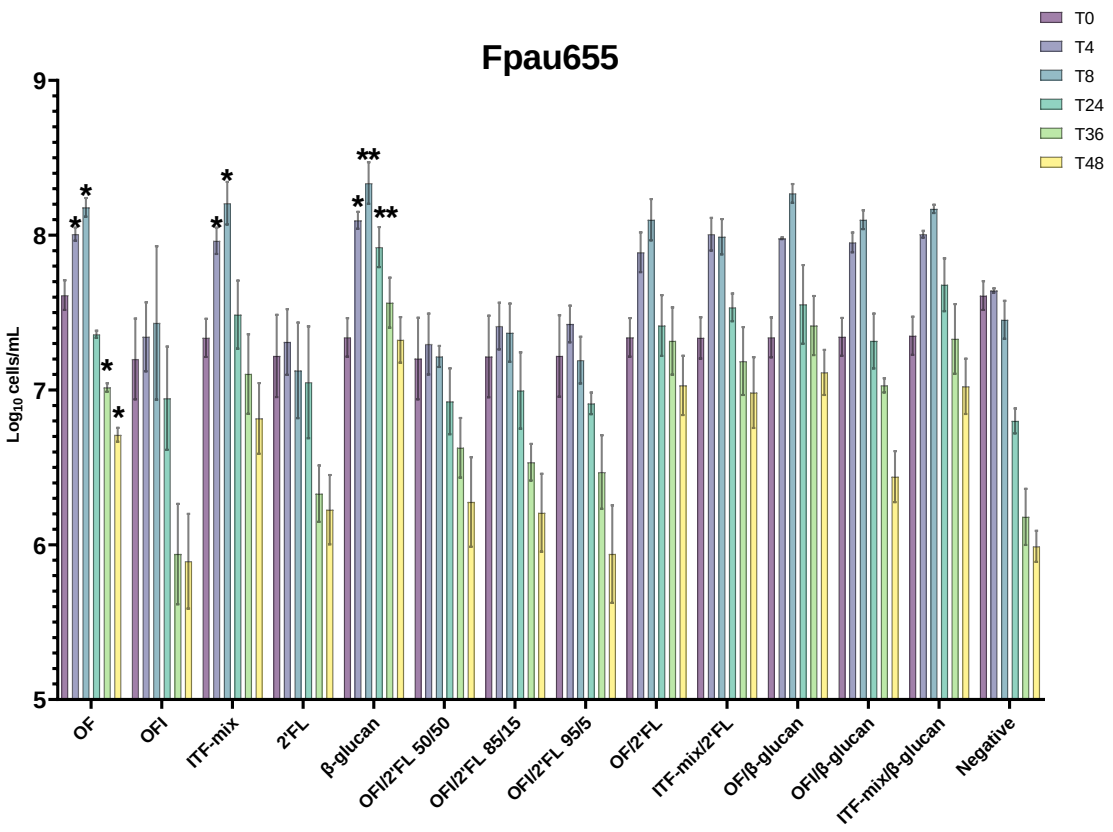

Figure 2.5.6. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL using probe: *Faecalibacterium prausnitzii* (Fprau655). Mean and standard error. * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling. **Abbreviations:** OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

2.2.3 Quantitative Microbiome Profiling (QMP)

Figure 2.6 reports the significant differences in 16S rRNA sequencing results, converted to quantitative microbiome profiling (QMP) data, at 0 and 8 h fermentation across all 14 different treatment conditions. Overall QMP and Individual *Bifidobacterium* QMP values are presented Appendix 2.5 and 2.6).

At genus level, the most notable changes in microbial numbers were seen in *Bifidobacterium*, with significant increases in numbers in several vessels by 8 h. These included OF/2'FL, ITF-mix/2'FL, and ITF-mix/β-glucan. Additionally, several treatments displayed trends towards

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) (Figure 2.6 and Appendix 2.6). No significant differences were detected in any other genus.

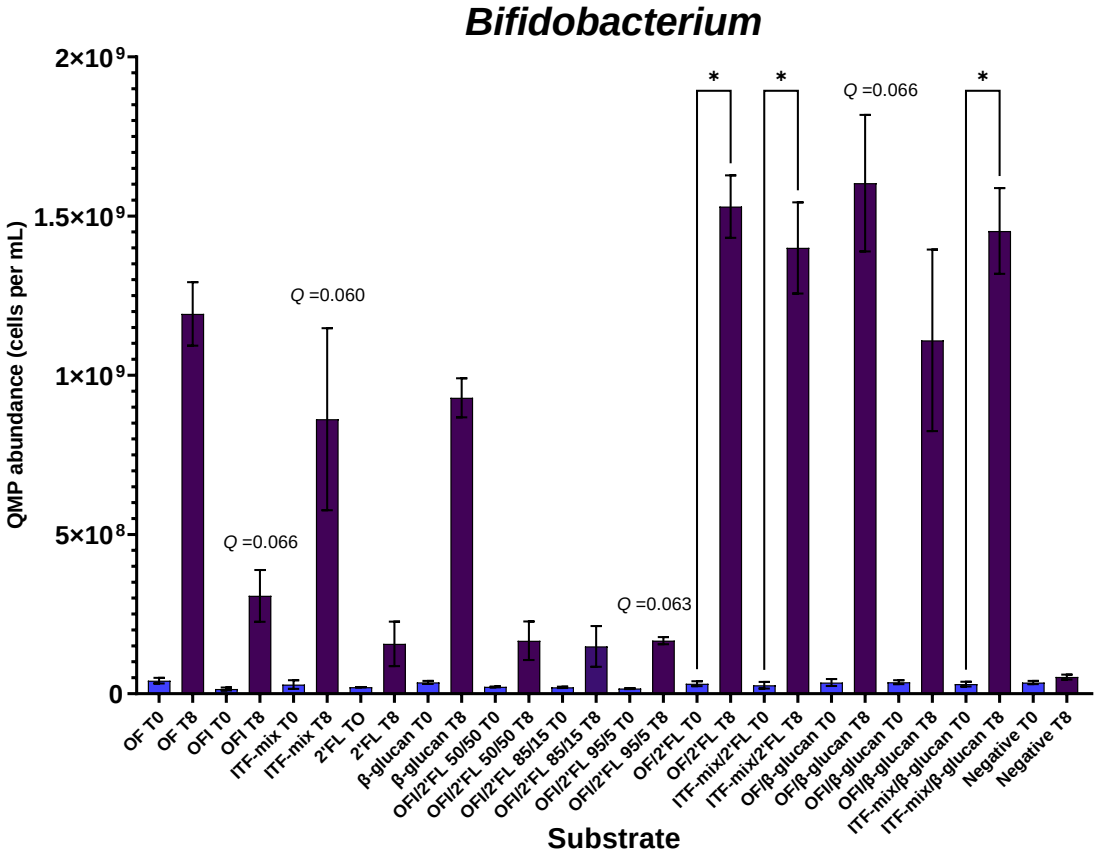

Figure 2.6. QMP of 16S rRNA analysis of batch culture effluents (*Bifidobacterium*) collected at 0 and 8 h fermentation. Mean and standard error (SE) * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling. **Abbreviations:** OF = oligofructose; OFI = oligofructose inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

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 utilising 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, OF/β-glucan and ITF-mix/β-glucan treatments with an average increase of 3.3E+07 ± 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 (Appendix 2.5). In contrast, there were decreases in numbers of *Alistipes* at 8 h on all substrates with the exception of OF. Similarly, numbers of *Clostridium* cluster IVXA + IVXB decreased across all substrates with the exception of ITF-mix, with numbers of *Dorea* decreasing across all substrates by 8 h fermentation mark. Finally, numbers of *Lactobacillus* and *Lactococcus* remained virtually unchanged except on sole β-glucan, OFI/β-glucan and ITF-mix/β-glucan treatments, which demonstrated a slight trend toward increases in *Lactobacillus* respectively.

2.3 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 utilise a wide range of low molecular weight carbohydrates (Riviere *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 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 in *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 utilising 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 (Pompei *et al.*, 2008; Gibson *et al.*, 1995) further confirming the selectivity of ITF towards *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 growth of bifidobacteria compared to OF and OF/inulin mixture in healthy adult donors. Similarly those of Li *et al*., (2012), who 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 of *Bifidobacterium, Lactobacillus, Bacteroides 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 (Dejean *et al.*, 2020; Falony *et al.*, 2009). However, the results of this study indicate that *Bifidobacterium* was able to outcompete *Bacteroides/Prevotella* for substrates.

There were significant increases in 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 (Appendix 2.3). 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 utilisation genes (Scott *et al*., 2015; Hillman *et al*., 2020). While in contrast, several strains of *Roseburia* including *R. inulinivorans* A2-194 and *R. hominis* L1-83 don't appear to be able to grow in the presence of OF, the ability of *F. prausnitzii* to utilise ITF and β-glucan is somewhat inconsistent (Harris *et al*., 2019) as is seen in this study. Yet previous studies conducted by (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 growth of *Roseburia* in this study may be in part due to their ability to utilise 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 of *Bifidobacterium* (Kim *et al*., 2020; Riviere *et al*.,

2016) suggesting that the increases in *F. prausnitzii* and *Roseburia* seen in this study may have resulted from the utilisation 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) who 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* are able to produce propionate from lactate (Gonzalez-Garcia *et al*., 2017; Hosseini *et al*., 2011). There was also a moderate increase in *Bacteroides* recorded in these vessels, and several strains within this genus encode the necessary methylmalonyl-CoA decarboxylase (mmdA) gene to utilise 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 eco-system (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 (Appendix 2.7). 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).

In this study 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 (Fehlbaum *et al*., 2018; Hughes *et al*., 2008; 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 utilisation (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 (Bui *et al*., 2019; Louis and Flint, 2009). However, while quantitative abundances of these organisms were higher at 8h on OF, β-glucan and ITF/β-glucan treatments (Appendix 7), 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 timepoints with 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, El Aidy and van Dijk, 2017) with the results of this study seemingly supporting this. However, what this means regarding clinical health outcomes is not known, as *Atopobium* currently have no identified role in human health.

It should be noted that the use of *in vitro* models in order to identify changes in microbial diversity do 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 upon prebiotic functionality (Jackson *et al*., 2022). Additionally, the high fraction β-glucan flour did not undergo upper GI tract pre-digestion 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 respective treatment vessels and result should be interpreted as such. The strengths of using *in vitro* models, however, include the ability to test novel substates for potential changes in microbial composition prior to investigation in human intervention trials as well as minimising the heterogenicity seen in in vivo studies.

2.4 Conclusion

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 in order to determine their *in vivo* effects.

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The authors would like to thank Andrea Monteagudo-Mera and Carlos Poveda for their help in the initial batch culture work.

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.

Ethics Statement

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

Author contribution

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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Oligofructose alone and in combination with 2'fucosyllactose induces specific changes in microbial composition and γ-aminobutyric acid production compared to sole 2'fucosyllactose supplementation: an *in vitro* study.

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Abstract

Aims

In this study, we explored the effects that the prebiotic oligofructose and prebiotic candidate 2'fucosyllactose, individually and in combination had on microbial load and neurotransmitter and organic acid production.

Methods and results

The effects of oligofructose, 2'fucosyllactose in singular and combination on microbial load, organic acid and neurotransmitter production were investigated using anaerobic pH-controlled *in vitro* batch culture fermentations over 48 h maintained at pH 5.4-5.6. Changes in microbial load were assessed by fluorescence *in situ* hybridisation and organic acid and neurotransmitter production via gas chromatography and liquid chromatography triple-quadruple mass spectrometry respectively. All substrates generated changes in microbial load, the majority achieving peak load at 24 h fermentation with the largest changes in profile documented in *Bifidobacterium* (all $P \leq 0.05$). This coincided with significant increases in acetate production observed throughout fermentation (all $P \leq 0.05$). In comparison to 2'fucosyllactose supplementation both individual oligofructose and oligofructose/2'fusocyllactose fermentations induced substantially greater significant increases in GABA production as well as acetate, propionate, butyrate, and succinate production, all of which were maintained until the end of fermentation (all $P \leq 0.05$).

Conclusion

Oligofructose and combinations of oligofructose and 2'fucosyllactose have the potential to induce meaningful changes in microbial composition, organic acid and GABA production.

Significance and importance of study

Our results indicate that combining oligofructose with 2'fucosyllactose could provide a novel approach to offsetting the heterogenicity seen in response to 2'fucosyllactose on its own and should be studied *in vivo* to determine their real world effects.

3.1 Introduction

In recent years there has been significant growth in interest in the bi-directional relationship existing between the gut and the brain – a term coined the gut-brain axis (Appleton, 2018). There is a increasing body of evidence suggesting that the composition of the gut microbiota plays a key role in influencing mood state including emotional regulation, cognitive performance and mental health, particularly anxiety and depression (Evrensel and Ceylan, 2015).

Within the gut, several genera, species and strains of bacteria can produce a number of different metabolites associated with cognitive and mental state including neurotransmitters such as γ-aminobutyric acid (GABA), serotonin and dopamine as well as organic acids - acetate, propionate, butyrate, lactate and succinate (Cryan *et al.*, 2020; Silva, Bernardi and Frozza, 2020).

Prevalent GABA producers in the gut include several species and strains of *Bifidobacterium* and *Lactobacillus* and to a lesser extent *Bacteroides* (Strandwitz, 2018; Strandwitz *et al.*, 2019). GABA can be produced via several pathways: firstly, by the conversion of arginine to ornithine to putrescine, and GABA, thereafter, resulting in the generation of succinate and finally propionate (Otaru *et al.*, 2021). Secondly it can be produced by the decarboxylation of glutamate. GABA serves as an acid-resistance mechanism to survive the low pH of the intestinal environment (Strandwitz *et al.*, 2019). Serotonin is produced via the metabolism of tryptophan by various microorganisms including *Lactococcus*, *Enterococcus* and *Streptococcus* (Kaur, Bose and Mande, 2019). Tryptophan can also enter the kynurenine pathway resulting in the generation of either kynurenic or quinolinic acid (Gao *et al.*, 2020). The latter being a metabolite associated with psychiatric and neurodegenerative disorders (Lugo-Huitron *et al.*,

2013). Organic acids such as acetate and lactate are predominately produced by *Bifidobacterium* via saccharolytic fermentation and can act as endocrine signalling molecules (Silva, Bernardi and Frozza, 2020). Butyrate produced by bacteria including *Bacteroides* and *Roseburia*, either directly or as a result of cross-feeding on acetate and lactate, can regulate the expression of GABA receptors (Nankova *et al.*, 2014). Furthermore, organic acids are also associated with regulating the expression of tryptophan-5-hydroxylase 1 and tyrosine hydroxylase, enzymes involved in the rate-limiting step in the biosynthesis of several neurotransmitters by enteroendocrine cells (Dalile *et al.*, 2019; Reigstad *et al.*, 2015).

Most of the glutamate and tryptophan, like many other amino acids, are absorbed and metabolised in the small intestine. Yet, it is estimated on average that between 7-10 %, or 6-18 g/day, of dietary protein reaches the large intestine intact (Yao, Muir and Gibson, 2016). This roughly equates to 3.4-6.3 mmol/kg^{-1} total free amino acids entering the proximal and distal region of the colon. Glutamate is one of most abundant amino acids present in food, making up between 8-10% of all dietary protein consumed (van der Wielen, Moughan and Mensink, 2017). Given the sharp rise in high quality protein diets and glutamate rich foods it is estimated that daily consumption of glutamate ranges from 5-15 (12 average) g/day (Beyreuther *et al.*, 2007; Tennant, 2018). This is only rising in Asian countries due to increased consumption of free monosodium glutamate. Based on the assumption that 7-10 % of total dietary protein reaches the colon intact, and an average glutamate intake of 12 g/day, it can be speculated that somewhere in the region 0.7-1.2 g of total dietary glutamate reaches the colon. Daily tryptophan consumption is estimated at approximately 900-1000 (950 average) mg per day and is rising due to the increase in high protein/low carbohydrate diets (Richard *et al.*, 2009). This roughly equates to between 66.5-95 mg dietary tryptophan reaching the colon.

The majority of research on increasing neurotransmitter production via manipulation of the microbiota primarily revolves around several strains of probiotics. This makes sense given the remarkable variability in neurotransmitter production seen in microorganisms, even those within the same genera (Strandwitz *et al.*, 2019; Kaur, Bose and Mande, 2019). However, other means of targeted manipulation of the gut microbiota exist, including prebiotics and potential prebiotic oligosaccharide candidates. The most substantiated of all prebiotics are oligofructose (OF) and inulin which belong to a class of non-digestible carbohydrates referred to as inulin-type fructans (ITF) (Mensink *et al.*, 2015). To date, the ability of ITF to stimulate changes in gut microbiota composition has been substantially demonstrated, both *in vivo* and *in vitro*, across a wide array of dosages (Wang and Gibson, 1993; Kolida, Meyer and Gibson, 2007; Vandeputte *et al.*, 2017). Other oligosaccharides are under consideration as prebiotics include human milk oligosaccharides (HMOs), a group of structurally diverse and complex unconjugated glycans present in human breast milk (Ninonuevo *et al.*, 2006). The most common of these is 2'fuscosyllactose (2'FL), the first HMO to be produced on an industrial scale (Sprenger *et al.*, 2017) and currently under investigation as a novel food ingredient and as a means of treating IBS and cognitive mental disorders (Al-Khafaji *et al.*, 2020; Sans Morales *et al.*, 2022). In comparison to ITF, the data on the ability of 2'FL to stimulate changes in microbial composition are mixed due to a limited number of clinical studies (Elison *et al.*, 2016; Iribarren *et al.*, 2021; Ryan *et al.*, 2021; Suligoj *et al.*, 2020).

Much remains unknown on whether supplementation with prebiotics/prebiotic candidates is enough to stimulate physiologically relevant changes in neurotransmitter production. This is in part due to the large heterogeneity existing between individual gut microbiotas and the need for an individual's microbiota to possesses the required microorganisms. As a result, in this study we investigated whether it is possible for the prebiotic OF and prebiotic candidate 2'FL, singular and in combination, to stimulate increases in neurotransmitter and organic acid

production using pH-controlled *in vitro* batch culture fermentation and a media enriched with glutamate and tryptophan over 48 h as a secondary pre-screening tool for a human intervention trial.

3.2 Material and methods

3.2.1 Materials

3.2.1.1 Prebiotic

The ITF used was oligofructose (Orafti® P95, DP 3-9, average DP 4; BENEO-Orafti, Tienen, Belgium).

3.2.1.2 Prebiotic candidate

2'Fucosyllactose is an 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 and was supplied by BENEO-Orafti, (Tienen, Belgium).

3.2.1.3 Reagents

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

3.2.2 *In vitro* batch culture fermentation

3.2.2.1 Faecal sample preparation

Ethical approval of collecting faecal samples from healthy volunteers was obtained from University of Reading Research Ethics Committee. Freshly voided faecal samples were obtained from five healthy adults aged between 18 and 40, who had not taken antibiotics for at least four months prior to the experiment, had no history of gastrointestinal disorders, were not regular consumers of prebiotics or probiotics and did not follow any restrictive diet. Faecal samples were diluted 1 in 10 (w/v) using 0.1 mol l^{-1}, pH 7.4 anaerobically prepared phosphate buffered saline (PBS, Oxoid, Hampshire, UK). Faecal samples were then homogenised in a stomacher (Seward, stomacher 80, Worthing, UK) for 120 seconds at 260 paddle-beats per min. 5 mL of faecal slurry was immediately used to inoculate each batch culture vessel.

3.2.2.2 Glutamate and tryptophan enriched basal batch culture nutrient medium

To make 1 litre 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, 11 g l-monosodium glutamate, 2.2 g tryptophan, and 4 mL resazurin (pH7) were added into 1 litre of deionised water. 45 mL of medium was placed into glass jars and autoclaved at 121 °C for 15 min.

3.2.2.3 pH-controlled, stirred batch culture fermentation

For each donor one independent batch culture was run. For each, batch culture vessels (4 x 100 mL) were set up and 45 mL of basal nutrient media were 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 and this continued throughout the 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 5.4 and 5.6 was maintained throughout the course of fermentation 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 three different substrates were prepared with one vessel containing each of the following substrates (1% w/v): OF, 2'FL and OF + 2'FL (50/50 w/w). One vessel was set up as the negative control with no added carbohydrate. All vessels were inoculated with 5 mL of a 10% (w/v) faecal slurry (diluted with PBS). A sample (4 mL) was removed from each vessel after 0, 10, 24, and 48 h incubation to ensure enough sample was taken for bacterial, organic acid, and neurotransmitter analysis by fluorescence *in situ* hybridisation-flow cytometry (FISH-FLOW), gas chromatography-flame ionisation detection (GC-FID) and triple quadruple liquid chromatography - mass spectrometry (LC-MS QQQ) respectively.

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

A 750 µL sample of batch culture fermentation effluent was centrifuged at 11337 × g for 5 min. The supernatant was then discarded, and the pellet was then suspended in 375 µL filtered 0.1 mol l^{-1}, pH 7.4 PBS solution. Filtered 4% paraformaldehyde (PFA) at 4 °C (1125 µL) were added and samples were stored at 4 °C for 4 hours. 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 3.1.

Probe	Sequence (5' to 3')	Targeted groups	Reference
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	(Wallner, Amann and Beisker, 1993)
Eub338 I	GCTGCCTCCCGTAGGAGT	Most bacteria	(Amann *et al.*, 1990)
Eub338 I-II	GCAGCCACCCGTAGGTGT	*Planctomycetales*	(Daims *et al.*, 1999)
Eub338 I-II-III	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)

Table 3.1: Name, sequence, and target group of oligonucleotide probe used in this study for FISH of bacterial enumeration

75 µL of fixed samples were mixed with 500 µL filtered cold (4°C) 0.1 mol l^{-1}, pH 7.4 PBS and then centrifuged at 11337 × 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 M 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 11337 × g for 3 min. Supernatants were discarded, and pellets washed with 500 µL filtered cold PBS by aspiration to disperse the pellet. Samples were then centrifuged at 11337 × g for 3 min and supernatants discarded.

Pellets were resuspended in 150 µL of hybridization buffer, aspirated using a pipette and gently vortexed. Samples were centrifuged at 11337 × g for 3 min and supernatants discarded. Pellets were resuspended in 1 mL of hybridisation buffer. Aliquots (50 µL) of samples were placed in labelled 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 hours in the dark.

Following incubation, 125 µL of hybridization buffer were added to each tube and gently vortexed. Samples were then centrifuged at 11337 × 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 11337 × g for 3 minutes. 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. Fluorescence measures were performed on an BD Accuri™ C6 Plus (BD, Erembodegem, Brussels) measuring at 488 nm and 640 nm. A threshold of 9000 in forward scatter (FSC-A) and 3000 in side scatter (SSC-A) was 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, limit of collection was 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.

3.2.4 Organic acids by gas chromatography-flame ionisation detection (GC-FID)

Samples (1.5 mL) of batch culture fluid were collected and centrifuged at 11337 × g for 10 min. Supernatants were transferred to 1.5 mL Eppendorf tubes and stored at -80 °C until analysis could be conducted. Sample extractions were performed according to Richardson *et al.* (1989) with modifications. Briefly, 1 mL of sample was transferred into a labelled 100 mm ×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 x g for 10 min. The resulting diethyl ether (upper) layer of each sample was transferred to clean 100 mL 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 hours to allow samples to completely derivatise.

An Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS 30m × 0.25mm column with a 0.25µm coating (crosslinked (5%-phenyl)-methylpolysiloxane, Hewlett Packard, UK) was used for analysis of SCFA. Temperatures of injector and detector were 275 °C, with the column temperature programmed from 63 °C to 190 °C at 15 °C min^{-1} followed by 190 °C for 3 min^{-1}. 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 organic acids was achieved by calibration with acetic, propionic, butyric, lactate and succinate acids in concentrations between 12.5 and 100 mmol l^{-1}. Mean metabolite concentrations were expressed as mmol l^{-1}.

3.2.5 Neurotransmitter production by LC-MS QQQ

Samples (500 uL) of batch culture effluents were diluted to 1/100 and 1/1000 in LC-MS grade water and 1 mL was pipetted into HPLC screw top vials. For quantification of neurotransmitters a Shimadzu QQQ equipped with a Discovery HS F5 HPLC Column (3 mm particle size, L x I.D. 15 cm x 2.1 mm) maintained at 40°C was used. The mobile phase comprised of solvent A (0.1% v/v formic acid) and solvent B (acetonitrile containing 0.1% formic acid) at a flow rate of 0.25 mL min^{-1}. The gradient elution program was as follows: 2 to 5 min solvent B from 0 to 25%, 5 to 6 min solvent B from 25 to 95%, then holding for 2 min, 8 to 9 min from 95 to 0% and then until 15 min.

A LC/MS-8050 triple quadrupole (QQQ) detector was operated in the multiple reaction monitoring (MRM) mode using the polarity-switching electrospray ionisation (ESI) mode. Dry gas temperature was set at 200°C with a flow of 10 l min^{-1}. Injected sample volume was 4 µL.

For the analysis of neurotransmitters, LC/MS Method Package for Primary Metabolites (Shimadzu Corporation, Kyoto, Japan) was used. The MRM transition for GABA was 104.10 > 87.05 m/z, tryptophan 205.10 > 188.15 m/z, serotonin 177.10 > 160.10 m/z, dopamine 154.10 > 91.05 m/z, kynurenic acid 190.10 > 144.10 m/z, norepinephrine 170.10 > 152.15 m/z and epinephrine 184.10 > 166.10 m/z. Quantification of neurotransmitters was achieved via generation of a linear calibration curve ranging from 1.00 ng ml^{-1} to 1000 ng ml^{-1} based on the detected signal proportional to concentration of the analyte. Good linearity of fit was considered as an R^2 of greater than 0.99.

3.2.6 Statistical Analysis

Statistical Package for Social Science version 27 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Changes in bacteriology, neurotransmitter production and SCFA were analysed using a general linear model (GLM) to assess repeat measures. Post-hoc comparisons were also performed in order to determine any significant differences between interventions at 48 h in organic acid and neurotransmitter production. All post-hoc pairwise comparisons were corrected for type 1 errors using Bonferroni adjustment within each GLM. All tests were two tailed and *P* values were considered significant at $P \leq 0.05$ and are displayed by specified *P* values.

3.3 Results

3.3.1 Neurotransmitter production

Figures 3.1.1, 3.1.2, 3.1.3 report the neurotransmitter concentration for GABA, serotonin and tryptophan over the course of the 48 h fermentation. Norepinephrine, epinephrine and

kynurenic acid were below the limit of detection and were therefore excluded from analysis. Mean and specific donor data is presented in Appendix 3.1 and 3.2.

The largest changes seen in neurotransmitter production across all substrates tested were recorded for GABA. The extent of change and fermentation patterns varied substantially across the substrates tested. Of all substrates tested, OF induced the largest increases in GABA at 48 h going from 3605.01 ± 1347.35 (SE) ng/mL to 836187.28 ± 303310 (SE) ng/mL (832582.27 mean difference) (P = 0.004). In contrast, 2'FL induced the smallest average increases in GABA at 25,5763.59 ng/mL above baseline at 24 h (Appendix 3.1).

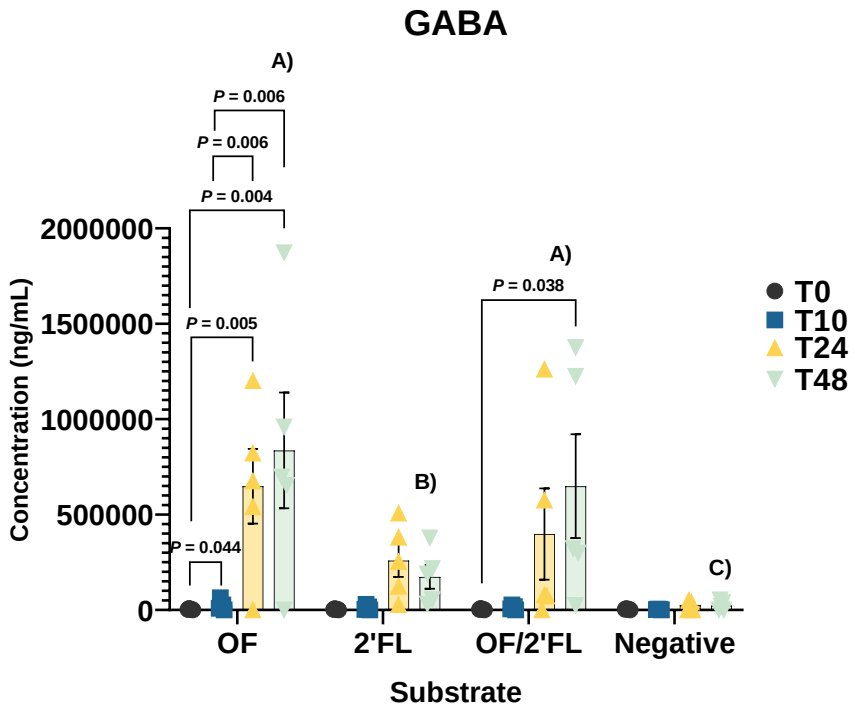

Figure 3.1.1. LC-MS analysis of neurotransmitter concentrations - GABA in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing mean (n = 5) and standard error (SE) of the data with individual volunteer data points. Results that are statistically significant within respective treatments are displayed by specified P values. Significant differences between treatments at 48 h are indicated by differing letters ($P \leq 0.05$). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Figures 3.1.2 and 3.1.3 report the data for tryptophan and serotonin. Both tryptophan and serotonin concentrations remained virtually unchanged throughout the course of fermentation. The only significant increase in tryptophan production was detected in the OF treatment vessel at 24 h fermentation (P = 0.004). Similarly, the only significant increases in serotonin production were detected on OF 0-24 h (P = 0.016) and 10-24 h (P = 0.015) fermentation (Appendix 3.1).

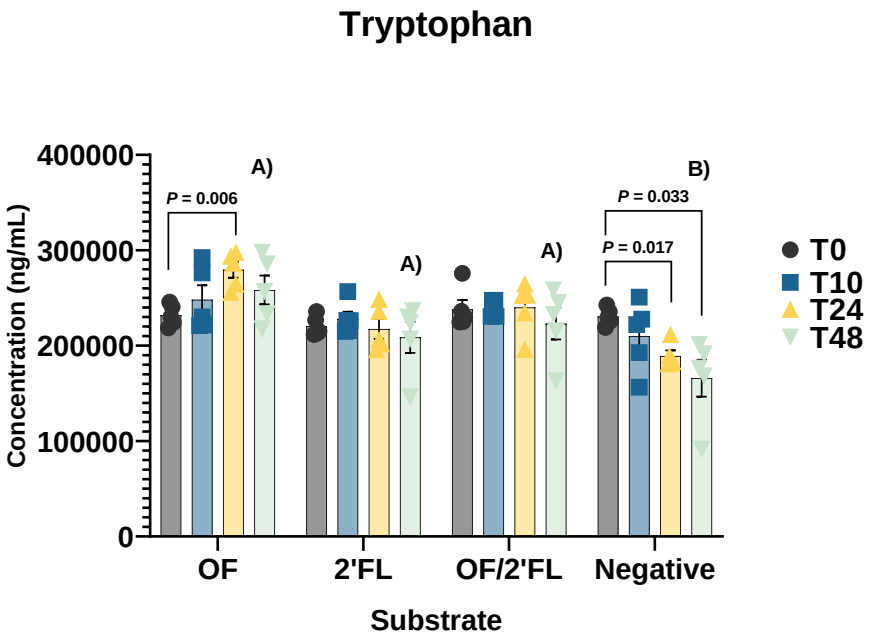

Figure 3.1.2. LC-MS analysis of neurotransmitter concentrations – tryptophan in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing mean (n = 5) and standard error (SE) of the data with individual volunteer data points. Results that are statistically significant within respective treatments are displayed by specified P values. Significant differences between treatments at 48 h are indicated by differing letters ($P \leq 0.05$). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

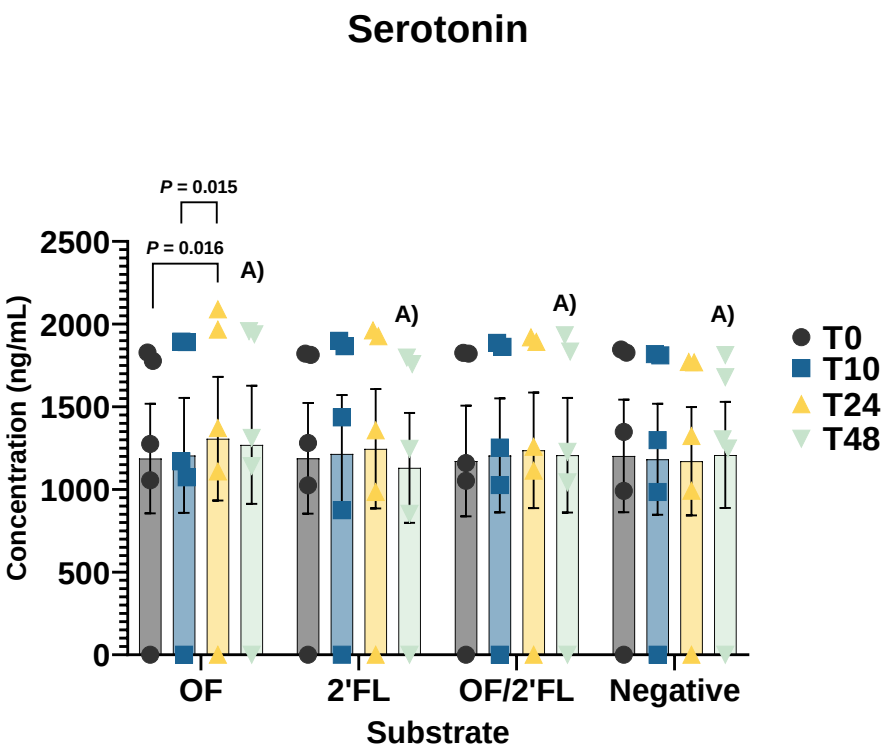

Figure 3.1.3. LC-MS analysis of neurotransmitter concentrations – serotonin in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing mean (*n* = 5) and standard error (SE) of the data with individual volunteer data points. Results that are statistically significant within respective treatments are displayed by specified *P* values. Significant differences between treatments at 48 h are indicated by differing letters ($P \leq 0.05$). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Finally, no significant differences were detected in dopamine concentrations throughout the entire 48h fermentation across any of the substrates tested (Figure 3.1.2 and Appendix 3.1).

3.3.2 Organic acids

Figures 3.2.1, 3.2.2, 3.2.3, 3.2.4, 3.2.5 and 3.2.6 report the concentrations of acetate, propionate, butyrate, lactate, succinate and total organic acids throughout the course of fermentation. Mean and specific donor data is reported in Appendix 3.3 and 3.4.

In this pH-controlled *in vitro* batch culture fermentation acetate was the most abundant organic acid detected representing between 53.9-69.2 % of total organic acids produced at the

end of fermentation. Acetate concentrations were highest in all treatments tested at 48 h and were all statistically significant compared with 0 h (Figure 3.2.1 and Appendix 3.3). Increases in acetate production varied substantially between treatments with OF producing the largest acetogenic effect, averaging an increase of 91.94 ± 3.41 (SE) mmol l^{-1} above baseline. Lowest increases in acetate production were recorded on 2'FL averaging an increase of 42.98 ± 3.94 (SE) mmol l^{-1} above baseline. Increases in acetate in both OF and OF/2'FL treatments being statistically greater than sole 2'FL at 48 h (both $P \leq 0.001$) (Appendix 3.3).

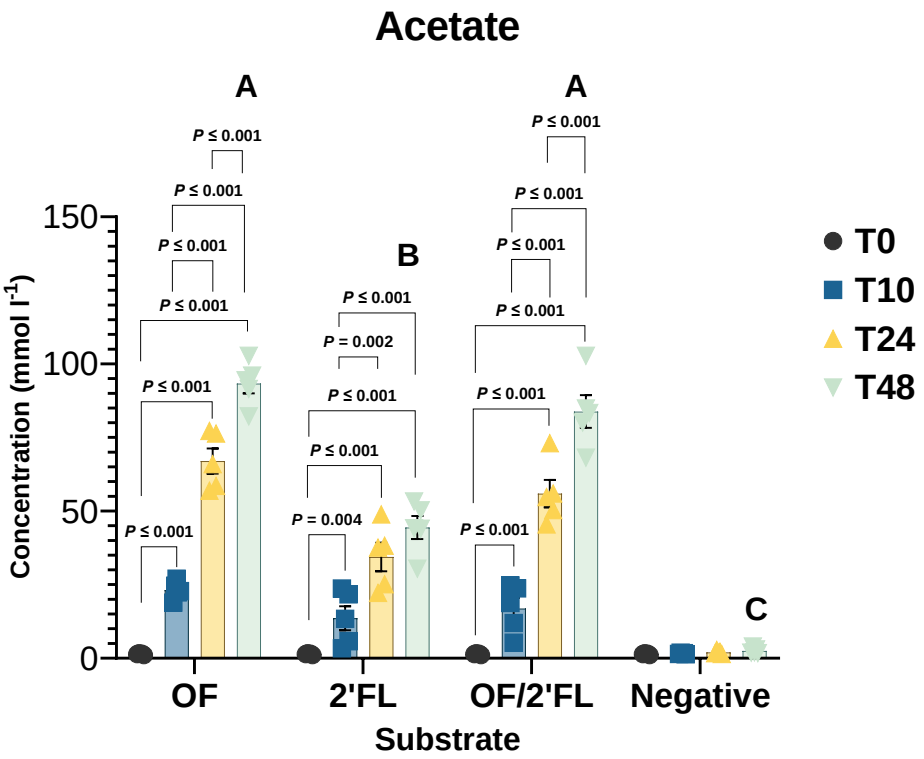

Figure 3.2.1. GC-FID analysis of organic acid concentrations – acetate in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing (n = 5) of the data (all points). Concentration reported in (mmol l^{-1}) mean and standard error (SE). Results that are statistically significant within respective treatments are displayed by specified P values. Significant differences between treatments at 48 h are indicated by differing letters ($P \leq 0.05$). **Abbreviations**: OF = oligofructose; 2'FL = 2'fucosyllactose

Propionate production accounted for between 18.30-26.7 % of total organic acids with all substrates inducing significant increases in propionate at 48 h compared with 0 h (Figure 3.2.2). Most notable propiogenic substrate was OF, averaging an 33.82 ± 1.99 (SE) mmol l^{-1} increase above 0 h. The combination of OF/2'FL produced similar results inducing an average 31.21 ± 1.34 (SE) mmol l^{-1} increase above 0 h sampling (Appendix 3.3). Lowest increases in propionate were seen in the 2'FL treatment vessel at just 10.85 ± 1.66 (SE) mmol l^{-1} above baseline. The increases in propionate recorded by both OF and OF/2'FL combination being statistically different from sole 2'FL at 48 h (both $P \leq 0.001$) (Figure 3.2.2 and Appendix 3.4).

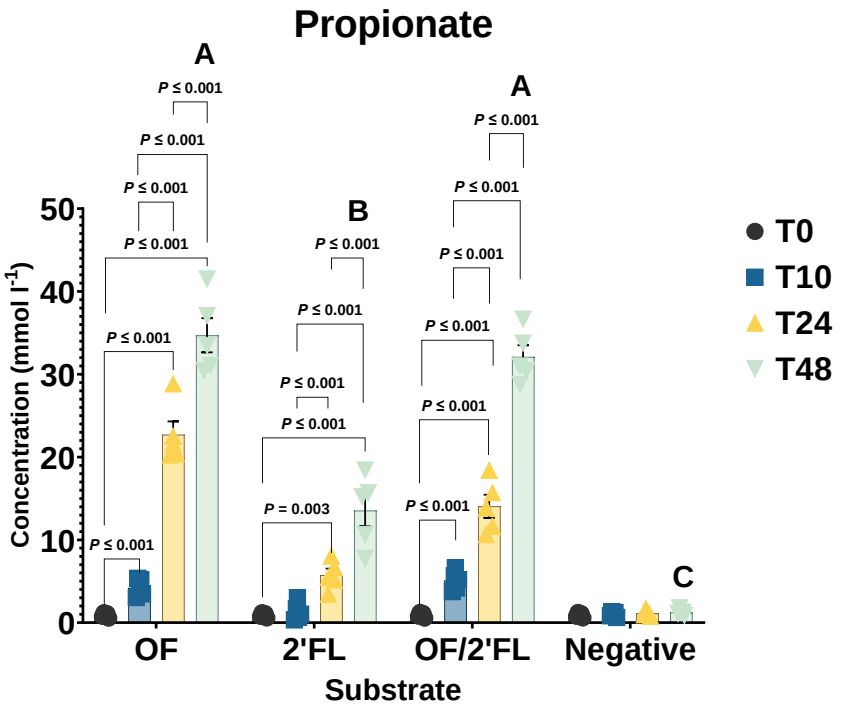

Figure 3.2.2. GC-FID analysis of organic acid concentrations – propionate in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing (n = 5) of the data (all points). Concentration reported in (mmol l^{-1}) mean and standard error. Results that are statistically significant within respective treatments are displayed by specified P values. Significant differences between treatments at 48 h are indicated by differing letters ($P \leq 0.05$). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

All substrates tested resulted in significant increases in butyrate production, yet substantial differences in butyrate production were seen between substrates. Most noticeable butyrogenic substrates were OF and OF/2'FL with an average 22.22 ± 1.44 (SE) mmol l^{-1} and

18.65 ± 1.26 (SE) mmol l^{-1} increase above respective baseline samples. The increases seen in butyrate production in both OF and OF/2'fl treatments were statistically greater compared to 2'FL alone at 48 h (both $P \leq 0.001$) (Figure 3.2.3 and Appendix 3.4). The increases in both propionate and butyrate correlating with those seen in *Bacteroides* throughout the course of fermentation.

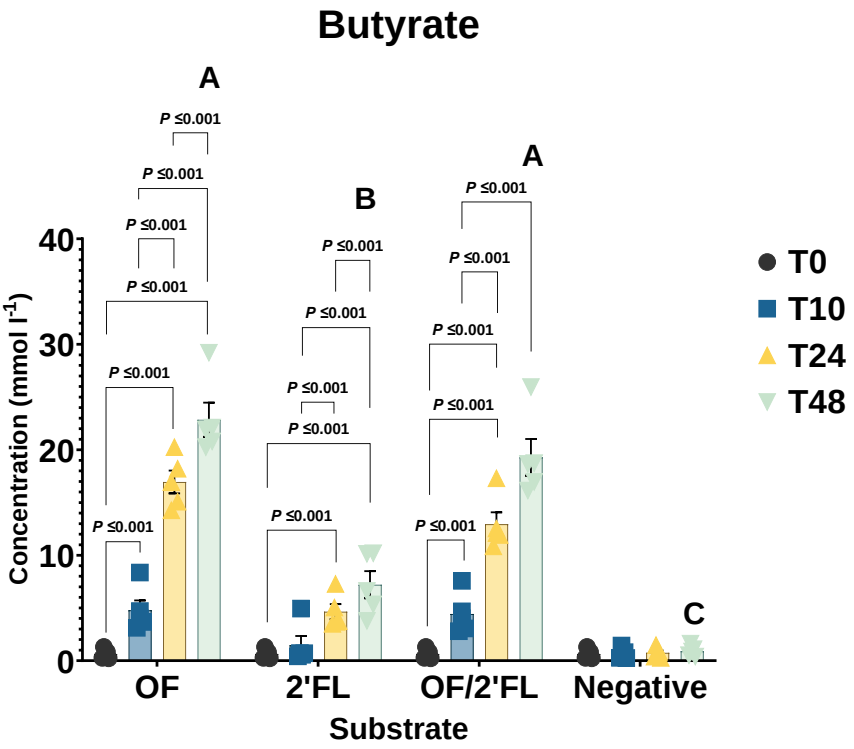

Figure 3.2.3. GC-FID analysis of organic acid concentrations – butyrate in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing (n = 5) of the data (all points). Concentration reported in (mmol l^{-1}) mean and standard error (SE). Results that are statistically significant within respective treatments are displayed by specified P values. Significant differences between treatments at 48 h are indicated by differing letters ($P \leq 0.05$). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

With respect to lactate, all substrates resulted in significant increases in lactate at 10 h fermentation mark (Figure 3.2.4). Thereafter, lactate concentrations declined in all substrates. No significant differences were detected between substrates at 48 h (Fig. 3.2.4 and Appendix 3.4).

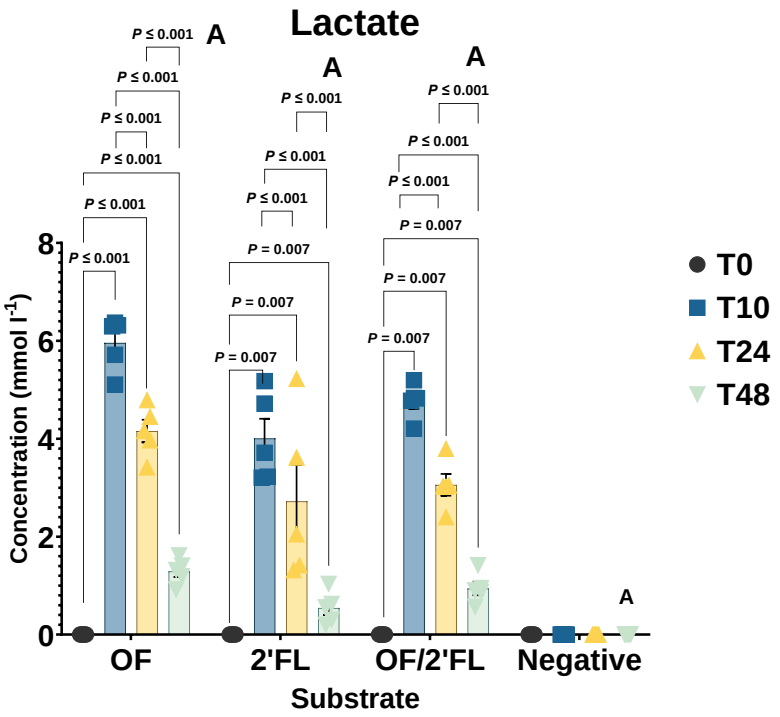

Figure 3.2.4. GC-FID analysis of organic acid concentrations – lactate in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing (*n* = 5) of the data (all points). Concentration reported in (mmol l^{-1}) mean and standard error (SE). Results that are statistically significant within respective treatments are displayed by specified *P* values. Significant differences between treatments at 48 h are indicated by differing letters ($P \leq 0.05$). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

All substrates tested resulted in significant increases in succinate (Figure 3.2.5). Yet, increases in succinate production varied substantially between substrates with OF and combination of OF/2'FL inducing significant increases in succinate production at 10 h (OF $P \leq 0.001$) and (OF/2'FL $P = 0.003$), 24 h (both $P \leq 0.001$) and 48 h (both $P \leq 0.001$) respectively (Figure 3.2.5 and Appendix 3.4). In contrast, significant increases in succinate production in the 2'FL treatment vessel were only detected at 48 h ($P = 0.003$). Furthermore, the increases detected in succinate concentrations at 48 h fermentation in both the OF and OF/2'FL treatment vessels were statistically different from 2'FL alone (both $P \leq 0.001$) (Figure 3.2.5).

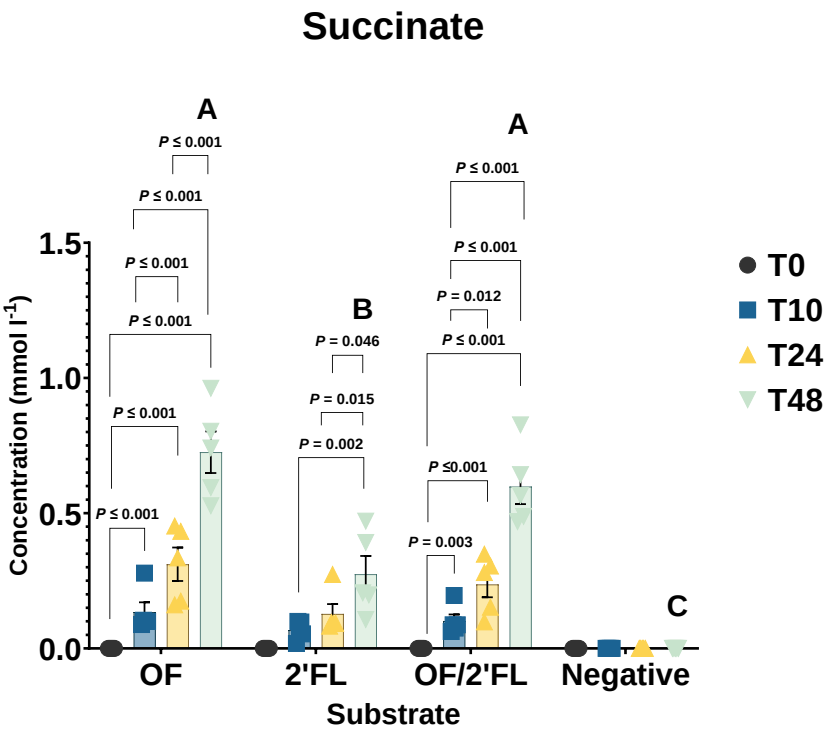

Figure. 3.2.5. GC-FID analysis of organic acid concentrations – succinate in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing (*n* = 5) of the data. Concentration reported in (mmol l^{-1}) mean and standard error (SE). Results that are statistically significant within respective treatments are displayed by specified *P* values. Significant differences between treatments at 48 h are indicated by differing letters ($P \leq 0.05$).
Abbreviations: OF = oligofructose; 2'FL = 2'fucosyllactose

Finally, regarding total organic acids, all substrates resulted in significant increases in total organic acids at the end of fermentation. OF induced the largest increases at 150.018 ± 5.38 (SE) mmol l^{-1} at 48 h, and was statistically greater compared to 2'FL alone ($P \leq 0.001$), but not OF/2'FL. Smallest increases in total organic acids were recorded on 2'FL at 61.24 ± 4.02 (SE) mmol l^{-1} above 0 h (Figure 3.2.6 and Appendix 3.4).

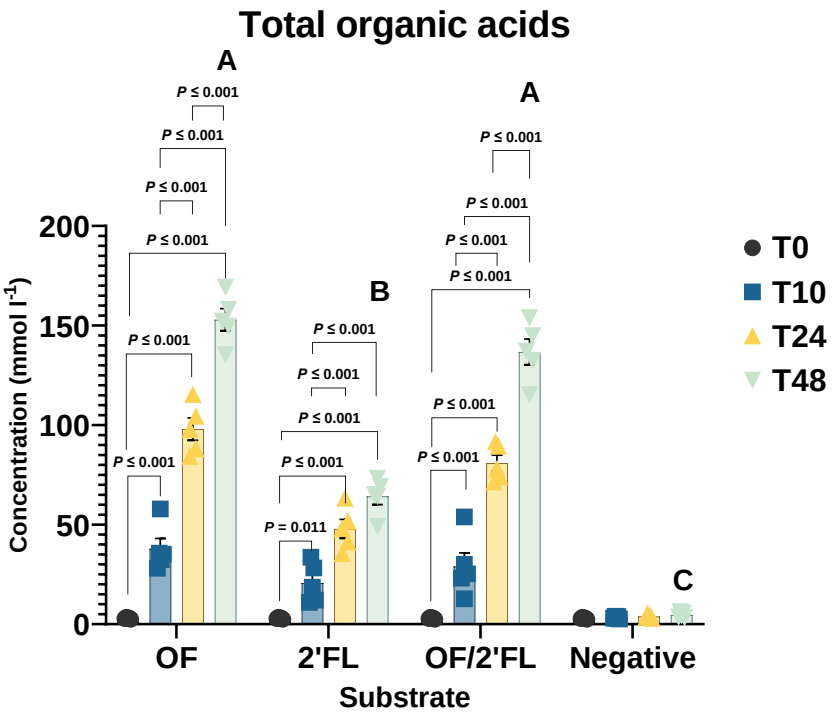

Figure. 3.2.6. GC-FID analysis of organic acid concentrations – Total organic acids in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing (*n* = 5) of the data. Concentration reported in (mmol l^{-1}) mean and standard error (SE). Results that are statistically significant within respective treatments are displayed by specified *P* values. Significant differences between treatments at 48 h are indicated by differing letters ($P \leq 0.05$). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

3.3.3 Bacterial Enumeration

In order to determine shifts in bacterial populations, four 16S-rRNA fluorescence *in situ* hybridisation probes were used to identify changes in numbers of total bacteria and three specifically targeted microbial groups. Results of significant bacterial group counts during the batch culture fermentation are shown in Fig. 3.3.1, 3.3.2, Fig. 3.3.3 and 3.3.4. Mean and donor specific data is reported in Appendix 3.5 and 3.6.

Significant increases in total bacterial counts (Eub338-II, II, III) were observed across all substrates with largest increases in microbial loads peaking at 24 h (Figure 3.3.1). OF induced the largest increases in total bacteria counts averaging 0.72 $\log_{10}$ ± 0.14 cells/mL above 0 h ($P \leq$

0.001). Smallest increases in total bacterial counts were induced on 2'FL and OF/2'FL combination at 0.52 $\log_{10}$ ± 0.12 cells/mL and 0.55 $\log_{10}$ ± 0.13 cells/mL above 0 h. Both of these being statistically significant from respective baseline samples (Figure 4.3.1 and Appendix 3.5).

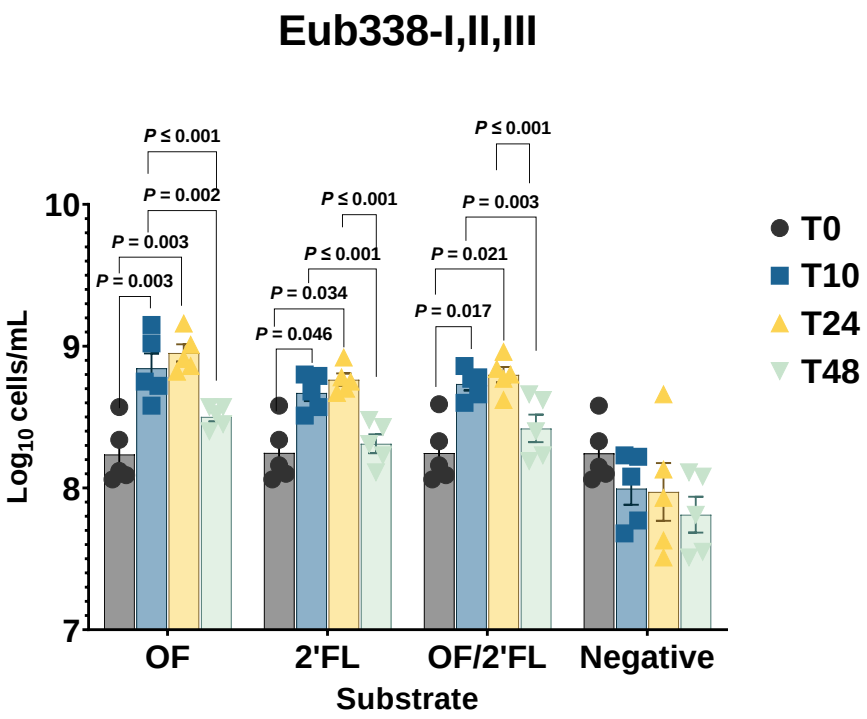

Figure. 3.3.1. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL) using probes: total bacteria (Eub338 I-II-III) at 0, 10, 24 and 48 h. Mean and standard error (SE) (all data points (*n* = 5)). Results that are statistically significant within respective treatments are displayed by specified *P* values**. Abbreviations**: OF = oligofructose; 2'FL = 2'fucosyllactose

Largest changes in microbial numbers were recorded in *Bifidobacterium* (Bif164) counts with all three substrates tested recording significant increases across the course of fermentation. Yet, increases in Bif164 counts varied between the substrates tested with OF inducing the largest average increases in Bif164 counts at 1.49 $\log_{10}$ ± 0.13 cells/mL (SE) ($P \leq 0.001$) at 24 h. Both 2'FL and the combination of OF/2'FL inducing similar increases of Bif164 counts at 1.22 $\log_{10}$ ± 0.11 and 1.26 $\log_{10}$ ± 0.11 (SE) cells/mL (Figure 3.3.2 and Appendix 3.5). The changes

seen in Bif164 counts correlating with those documented in acetate and GABA production throughout the course of fermentation.

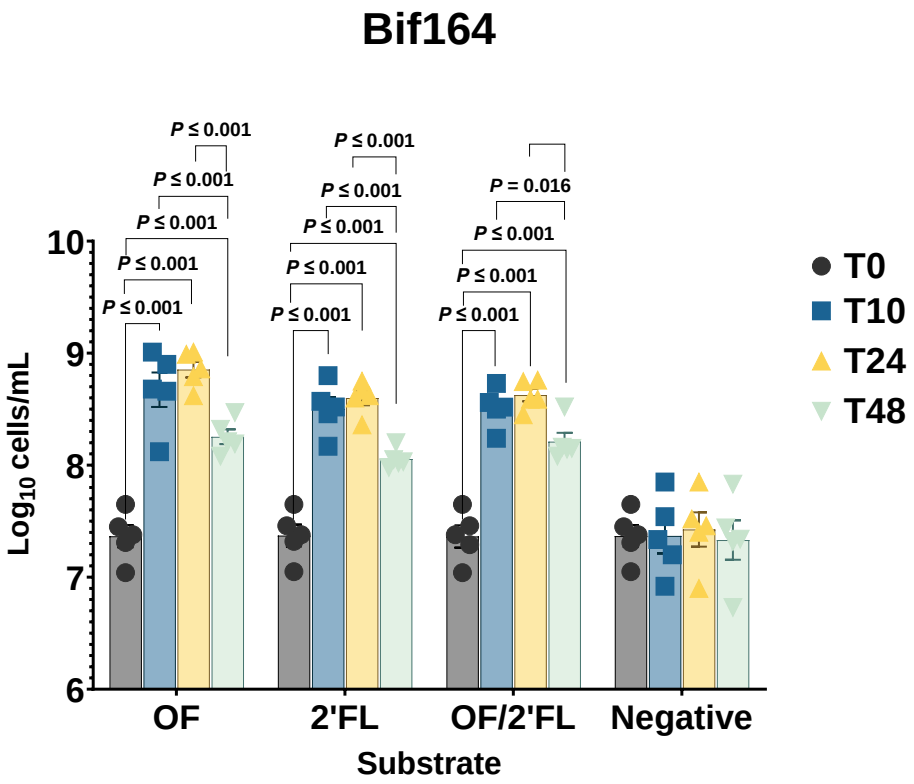

Figure. 3.3.2. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL) using probes: *Bifidobacterium* spp. (Bif164) at 0, 10, 24 and 48 h. Mean and standard error (SE) (all data points (*n* = 5)). Results that are statistically significant within respective treatments are displayed by specified *P* values**. Abbreviations**: OF = oligofructose; 2'FL = 2'fucosyllactose

All substrates resulted in significant increases in Bac303 counts. Significant differences were detected amongst treatments, with OF inducing largest increases in Bac303 at 24 h at 1.64 log_{10} ± 0.23 cells/mL (SE) and remained significant until the end of fermentation (all $P \leq 0.001$). In contrast, 2'FL induced the smallest increases in Bac303 counts at 0.99 log_{10} ± 0.25 cells/mL (SE), both 8 h and 24 h samples recording significant differences compared to 0 h (Figure 3.3.3 and Appendix 3.5).

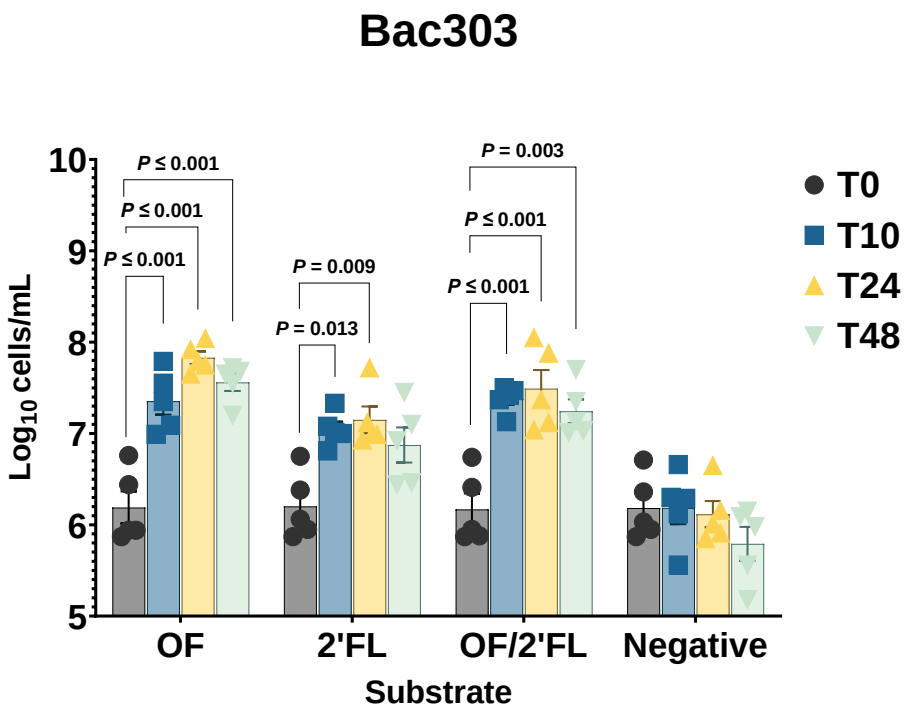

Figure. 3.3.3. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL) using probes: Most *Bacteroidaceae* and *Prevotellaceae,* some *Porphyromonadaceae* (Bac303) at 0, 10, 24 and 48 h. Mean and standard error (SE) (all data points (*n* = 5)). Results that are statistically significant within respective treatments are displayed by specified *P* values. **Abbreviations**: OF = oligofructose; 2'FL = 2'fucosyllactose

Lastly significant differences in Lab158 counts were detected across all substrates tested (Fig. 3.3.4). Largest changes in Lab158 counts were recorded on OF with 0.8 log_{10} ± 0.14 cells/mL (SE) increase at 8 h ($P \leq 0.001$), 2'FL alone giving rise to similar average increases at 0.77 log_{10} ± 0.17 cells/mL (SE) above 0 h ($P \leq 0.001$). Smallest increases in Lab158 counts were in the OF/2'FL treatment vessel at just 0.42 log_{10} ± 0.21 cells/mL (SE) at 24 h fermentation (Figure 3.3.4 and Appendix 3.5).

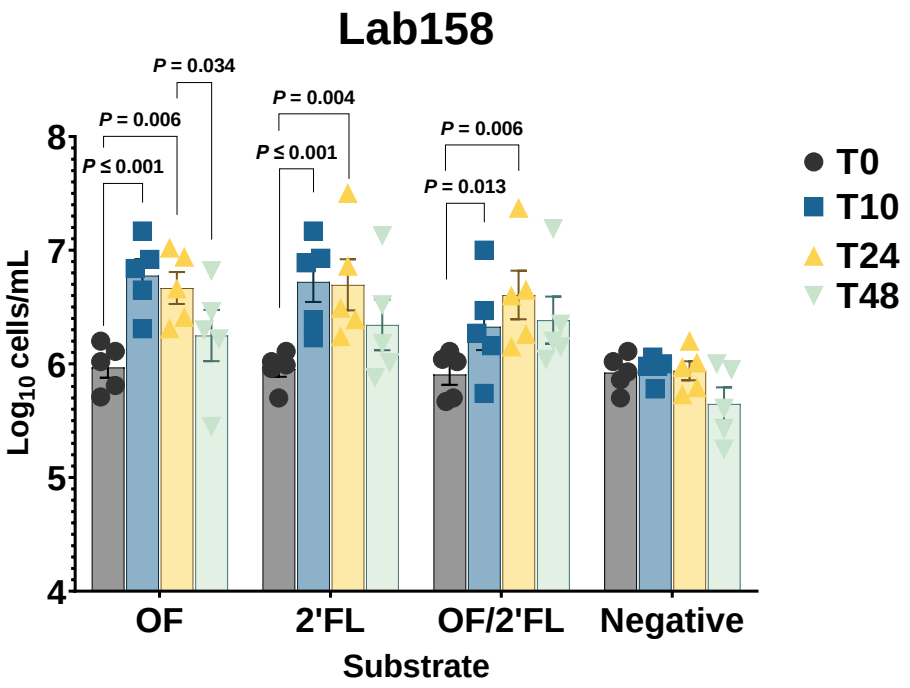

Figure. 3.3.4. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL) using probes: *Lactobacillus/Enterococcus* (Lab158) at 0, 10, 24 and 48 h. Mean and standard error (SE) (all data points (*n* = 5)). Results that are statistically significant within respective treatments are displayed by specified *P* values**. Abbreviations**: OF = oligofructose; 2'FL = 2'fucosyllactose

3.4 Discussion

In this *in vitro* batch culture fermentation, we aimed to determine if the prebiotic OF and prebiotic candidate 2'FL, alone and in combination, could induce meaningful changes in microbial load, organic acid production and physiologically relevant levels of neuroactive metabolites.

All substrates tested, singular and in combination, were able to stimulate large changes in *Bifidobacterium* spp. achieving peak load at 24h fermentation. The largest increases in *Bifidobacterium* were recorded in vessels utilising OF. The response of *Bifidobacterium* to OF is unsurprising and is consistent with those reported in several previous *in vitro* (Pompei *et al.*, 2008; Wang and Gibson, 1993) and *in vivo* studies (Kolida and Gibson, 2007; Vandeputte *et al.*, 2017). In contrast, a high degree of heterogeneity amongst donors was detected in response

to 2'FL treatment. These findings being similar to those recorded by (Ryan *et al.*, 2021; Yu, Chen and Newburg, 2013; Suligoj *et al.*, 2020) indicating a large ressponder/non-responder status exists to 2'FL supplementation. The changes documented in Bif164 counts correlated with increases seen in both acetate and GABA production throughout the course of fermentation.

Similarly, the largest changes in *Bacteroides*/*Prevotella* were seen on OF and combinations of OF/2'FL (Figure 3.3.3). *Bacteroides* possess the largest number of loci for degradation and assimilation of a wide variety of oligosaccharides and polysaccharides, including ITF and HMOs (Flint *et al.*, 2012). However, the ability of *Bacteroides* to utilise specific HMOs, much like the situation in bifidobacteria, is highly species and even strain specific (Jackson, Wijeyesekera and Rastall, 2022). The results of this study suggest that in this instance bifidobacteria were able to outcompete *Bacteroides* with lowest increases on 2'FL supplementation. Interestingly, in the treatment vessel containing both OF/2'FL changes in numbers of *Bacteroides* more closely resembled the changes seen in the OF vessel (Figure 3.3.3 and Appendix 3.5). This implies that combining OF with 2'FL could be a potentially effective means of overcoming the limitations associated with responder/non-responder status to 2'FL supplementation.

Similar increases in Lab158 counts were seen across all three treatments. Yet, upon closer inspection of the data within the 2'FL and OF/2'FL combination treatments several of the volunteers documented substantial increase in Lab158 counts compared to sole OF (Figure 3.3.4 and Appendix 3.6). This is interesting given that it has been previously documented that *Lactobacillus* are not readily able to utilise intact HMOs to any real extent (Jackson, Wijeyesekera and Rastall, 2022). Taking this into consideration it has been demonstrated that *Lactobacillus* are able to proliferate from cross-feeding on lactose and fucose from the extracellular degradation of HMOs by bifidobacteria (Zuniga, Monedero and Yebra, 2018). The

results of this study support these findings with large increases in bifidobacteria also being detected within respective individuals (Appendix 3.6). This further adds to the evidence that the mutualistic behaviour existing between microorganisms found within the gut is a critical character in helping to shape a diverse and flexible ecosystem (Jackson, Wijeyesekera and Rastall, 2022).

Changes in neurotransmitter production were monitored throughout the course of fermentation and were highly heterogenous amongst the individual donors and substrates. The largest increases in neurotransmitters were seen in GABA production and were highest on OF and OF/2'FL peaking at 48 h, whereas smaller increases were detected on 2'FL and peaked at 24 h respectively. It being recently documented that rates of GABA synthesis can be dramatically influenced by the type of carbohydrates used during fermentation (Strandwitz *et al.*, 2019; Cataldo *et al.*, 2020).

Within the gut several microorganisms including bifidobacteria, *Bacteroides* and *Lactobacillus* have developed several mechanisms for producing GABA. GABA can be produced via the decarboxylation of l-glutamate or via the conversion of arginine (Otaru *et al.*, 2021; Strandwitz *et al.*, 2019). As we did not add arginine to our basal media this implies the majority of GABA production likely occurred via the decarboxylation of l-glutamate. Yet, GABA production can also occur via the utilisation of acetate and lactate as intermediates in the citric acid cycle via conversion to acetyl-CoA. Acetyl-Co-A is then converted to citrate, 2-oxoglutarate, glutamate and subsequently GABA (Rowlands, Klugmann and Rae, 2017). Consequently, given that there were significant increases in both acetate and lactate seen throughout the course of fermentation, specifically on OF and OF/2'FL, the additional increases seen in GABA may be from utilisation of both acetate and lactate via the gut microbiota. These results may be of interest as GABA aids in the regulation of mood state and cognitive performance via

modulating the production of other neurotransmitters with GABA concentrations often correlating with levels of depression and several other mood state disorders (Al-Khafaji *et al.*, 2020; Brady *et al.*, 2013).

Small increases in both tryptophan and serotonin production were detected only on OF at 24 h (Figures 3.1.1 and 3.1.2 and Appendix 3.1). The increases seen in both serotonin and tryptophan is novel suggesting modulation of the gut microbiota by prebiotics could potentially result in small increases of bacterially derived serotonin and tryptophan. However, these increases do not likely reflect those seen *in vivo* and are relatively low compared to increases seen in GABA production. Additionally, dietary tryptophan can enter multiple pathways including being converted to serotonin, as well as entering the kynurenine pathway, subsequently being converted to either kynurenic or quinolinic acid (Kaur, Bose and Mande, 2019; Gao *et al.*, 2020). Yet, in our study kynurenic acid was under the limit of detection indicating the regulation of neurotransmitters by the gut microbiota goes far beyond what can be seen using simple *in vitro* batch culture fermentation. This is evidenced by lack of changes seen in serotonin production and ultimately is unsurprising as the conversion of tryptophan to serotonin occurs in the presence of enterochromaffin cells and serotonergic neurons (Reigstad *et al.*, 2015).

Taking this into consideration, concentrations of acetate, propionate and butyrate, lactate and succinate all increased across the course of fermentation with OF and OF/2'FL outperforming 2'FL alone (Figures 3.2.1, 3.2.2, 3.2.3, 3.2.4 and 3.2.5). Organic acids are speculated to play pivotal roles in the cross-talking between the brain and the gut, and while concentrations of organic acids in the brain is suspected to be low, unlike neurotransmitters, acetate appears to be able to cross the blood brain barrier along with propionate appearing to play a key role in regulating blood brain barrier permeability (Braniste *et al.*, 2014; Hoyles *et al.*, 2018). Thus,

one way of increasing GABA production in the brain may be via improving acetate production using prebiotics as it has been shown utilizing ^{13}C-labelled inulin that ^{13}C-labelled acetate was able to cross the blood brain barrier (Frost *et al.*, 2014).

Acetate, primarily produced by *Bifidobacterium*, can reduce inflammation by regulating the expression of pro-inflammatory cytokines IL-6, TNF-α and IL-1β (Soliman *et al.*, 2012; Underwood *et al.*, 2015). Acetate and lactate can also aid in suppression of satiety via stimulation of leptin production in adipocytes (Zaibi *et al.*, 2010). Propionate, which was also significantly increased on OF, and to a lesser extent on the OF/2'FL can act as a precursor to gluconeogenesis (den Besten *et al.*, 2013; Soty *et al.*, 2015), along with protecting against blood brain barrier lipopolysaccharide mediated disruption (Hoyles *et al.*, 2018). Additionally, butyrate, which was significantly increased at the end of fermentation, particularly on OF and OF/2'FL (Figure 3.2.3) not only acts as a major energy source for colonocytes but is speculated to aid in regulation of GABA receptors and enterochromaffin cells in mice (Reigstad *et al.*, 2015). It has also been shown to decrease histone acetylation in piglets and alters the expression of brain-derived neurotrophic factor and glial-derived neurotrophic factor in mice and rats (Kien *et al.*, 2008; Moris and Vega, 2003; Intlekofer *et al.*, 2013; Savignac *et al.*, 2013).

Finally, increases in succinate production were detected throughout the course of fermentation and were highest on OF and OF/2'FL (Figure 3.2.5 and Appendix 3.3. and 3.4). The increases in succinate were within normal physiological ranges seen in healthy adults (Connors, Dawe and Van Limbergen, 2019) and coincide with increases seen in numbers of *Bacteroides* throughout the course of fermentation. These results, at face value, seemingly make sense given that the majority of succinate producers within the gut belong to the *Bacteroidota* (*Bacteroidetes*) phylum, and accumulation of succinate does not routinely occur *in vivo* as it is rapidly converted to propionate upon production (Louis and Flint, 2017;

Reichardt *et al.*, 2014). Much like its successor propionate, succinate plays an essential role in the gut-brain communication via regulating satiety, acting as a critical link in gluconeogenesis (de Vadder *et al.*, 2014; de Vadder and Mithieux, 2018). Overall, the results of this study suggest that OF and combinations of OF/2'FL could likely aid in the regulation of organic acid and neurotransmitter production, specifically GABA, via targeted manipulation of the gut microbiota.

Our study is not, however, without limitation. It should be noted that the use of *in vitro* fermentation models to identify changes in microbial composition and resulting metabolites does not necessary capture changes seen *in vivo*. Furthermore, it is important to note that while we did see significant increases in the production of several neurotransmitters evidence supporting the ability of gut-derived neurotransmitters to cross the blood brain barrier is not well established (Strandwitz, 2018) and findings should be interpreted as such. However, *in vitro* models do allow for the testing of novel substrate combinations as pre-screening tools for assessing potential changes in microbial composition and metabolite production prior to conducting human intervention trials and minimising the heterogeneity seen *in vivo*.

3.5 Conclusion

Overall, the results of this *in vitro* batch culture fermentation study imply that OF and combinations of OF/2'FL can result in notable changes in microbial composition, GABA and organic acid production, the latter being maintained until the end of fermentation (all $P \leq 0.05$). In contrast, the ability of 2'FL to alter microbial composition, GABA and SCFA production appears to be extremely limited *in vitro* except in the presence of OF. These results suggest that the prebiotic OF and combination of OF/2'FL should be taken forward to a human intervention trial to determine their *in vivo* effects.

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Inulin-type fructans and 2'fucosyllactose alter both microbial composition and appear to alleviate stress-induced mood state in a working population: A double-blind randomised placebo controlled trial.

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Abbreviations

Prebiotics; gut microbiota, oligosaccharides, gut-brain axis; mood

Abstract

Background

There is increasing interest in the bi-directional relationship that exists between the gut and brain known as the gut-brain axis and its effects on mental health disorders including anxiety and depression. One way to potentially improve anxiety and depression may be via targeted manipulation of the gut microbiota using prebiotics and candidates such as oligofructose and 2'fuscosyllactose. Yet, while the ability of oligofructose to manipulate microbial composition is well documented, data regarding the ability of 2'fucosyllactose to alter microbial composition in adults is extremely limited.

Methods

We conducted a 5-week, 4-arm, parallel, double-blind, randomised, placebo-controlled trial in 92 healthy adults with mild-to-moderate levels of anxiety and depression. Subjects were randomised to oligofructose 8 g/day (plus 2 g/day maltodextrin); maltodextrin 10 g/day; oligofructose 8 g/day plus 2'fucosyllactose (2 g/day) or 2'fucosyllactose 2 g/day plus (8 g/day maltodextrin). Changes in microbial load (FLOW-FISH) and composition (16s rRNA sequencing) were the primary outcomes. Secondary outcomes included gastrointestinal sensations, bowel habits (frequency and consistency) and mood state parameters.

Results

There were significantly greater increases in several bacterial taxa including *Bifidobacterium*, *Bacteroides*, *Roseburia* and *Faecalibacterium prausnitzii* in both the oligofructose and oligofructose/2'fucosyllactose interventions (all $P \leq 0.05$). Changes in bacterial taxa were highly heterogenous upon 2'fuscoyllactose supplementation suggesting a strong

responder/non responder status exists. Significant improvements in Becks Depression Inventory, State Trait Anxiety Inventory Y1 and Y2, and Positive and Negative Affect Schedule scores and cortisol awakening response were detected across oligofructose, and 2'fucosyllactose and oligofructose/2'fucosyllactose combination groups (all $P \leq 0.05$). Both oligofructose and the oligofructose/2'fuscosyllactose combined interventions outperformed both sole 2'fucosyllactose and maltodextrin in improvements in several mood state parameters (all $P \leq 0.05$).

Conclusion

The results of this study indicate that prebiotics and prebiotic candidates, namely oligofructose and combinations of oligofructose/2'fucosyllactose, can beneficially alter microbial composition along with improving mood state parameters. Future work is needed to understand key microbial differences between responders/non responders to 2'fucosyllactose supplementation.

Trial registration: ClinicalTrials.gov: NCT05212545

4.1 Introduction

Modulation of the gut microbiota is one of the most promising areas with respect to potentially improving health outcomes. Yet, the cause-and-effect relationships between modulation of gut microbiota and specific health outcomes still remain unclear (Umu, Rudi and Diep, 2017). Diet is the major driver of gut microbiota composition and one way to modulate the composition of the gut microbiota is through the use of functional foods such as prebiotics. Prebiotics, "A substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson *et al.*, 2017), include oligofructose (OF) and inulin which belong to a class of linear dispersed non-digestible carbohydrates referred to as inulin-type

fructans (ITF) (Mensink *et al.*, 2015). ITF are the most well substantiated of all prebiotics with their ability to manipulate microbial composition being demonstrated across a wide array of dosages (Costabile *et al.*, 2010; Ramnani *et al.*, 2010; Kolida, Meyer and Gibson, 2007).

In addition to ITF, the International Scientific Association for Probiotics and Prebiotics (ISAPP) classes several oligosaccharides as prebiotic candidates including human milk oligosaccharides (HMOs) (Gibson *et al.*, 2017). HMOs are unconjugated glycans present in breastmilk that are the first carbohydrates encountered by an infant directly after birth (Biddulph *et al.*, 2021). Currently several HMOs are produced on a commercial scale including 3'sialyllactose (3'SL), 6'sialyllactose (6'SL), Lacto-N-tetarose (LNT), 3'fucosyllactose (3'FL), 2'fucosyllactose (2'FL) and lacto-N-neo-tetraose (LNnT), the most common of these being 2'FL. Yet, the data regarding the ability of the microbiota to utilise HMOs in adults remains largely unknown due to the limited number of clinical studies undertaken to date (Ryan *et al.*, 2021; Suligoj *et al.*, 2020; Iribarren *et al.*, 2021; Elison *et al.*, 2016).

Anxiety and depression are the two biggest mental health disorders recorded worldwide costing health services in excess of 1 trillion US$ per year (Dieleman *et al.*, 2016; Chisholm *et al.*, 2016). Therefore, there is a demand to find novel approaches to not only treat the burden of disease, but to also reduce the ever-increasing burden on the health system (Liu, Walsh and Sheehan, 2019). Although the mechanisms by which anxiety and depression are regulated are not well understood (Huang and Wu, 2021), there is increasing interest in the bi-directional relationship that exists between the gut and the brain. The gut-brain axis (Cryan *et al.*, 2020) is involved in neuronal development, brain function and cognitive performance via regulation of neurological, immunological, or endocrine pathways (Morais, Schreiber and Mazmanian, 2021).

Within the gut several genera and species of bacteria can produce a number of different metabolites associated with cognitive state including several neurotransmitters: γ-aminobutyric acid (GABA), serotonin, and dopamine, as well as short chain fatty acids (SCFAs) such as acetate, propionate and butyrate (Cryan *et al.*, 2020; Silva, Bernardi and Frozza, 2020). Predominant GABA producers in the gut include several species of *Bifidobacterium*, *Lactobacillus* and *Bacteroides*, GABA serving as a protective mechanism against the low pH of the intestinal environment (Hoyles *et al.*, 2018). SCFA produced via saccharolytic fermentation play a role in neurotransmitter production via regulating the expression of tryptophan 5-hydroxylase 1 tyrosine hydroxylase, an enzyme involved in the rate-limiting step in the synthesis of serotonin, dopamine, noradrenaline and adrenaline respectively (Dalile *et al.*, 2019; Reigstad *et al.*, 2015). More specifically, acetate and lactate, predominately produced by *Bifidobacterium*, act as endocrine signalling molecules (Silva, Bernardi and Frozza, 2020), along with reducing neuroinflammation via modulation of proinflammatory cytokines (Soliman *et al.*, 2012). Furthermore, acetate and lactate serve as substrates in the production of propionate and butyrate by several bacteria including *Bacteroides*, *Roseburia* and *Faecalibacterium*. Butyrate playing an influential role in the expression of GABA receptors, enterochromaffin cells, and brain-derived neurotrophic factor and glial-derived neurotrophic factor (Moris and Vega, 2003; Reigstad *et al.*, 2015).

To date, in comparison to probiotics, understanding of prebiotic ability to improve mood state is still in its infancy with the majority of studies producing mixed results (Kazemi *et al.*, 2019; Schmidt *et al.*, 2015; Smith, Sutherland and Hewlett, 2015). One area frequently overlooked by many of these studies is microbial composition. In order to gain a greater insight into how prebiotics could impact on mood state, analysing changes in microbial composition is highly important.

As a result, in this double-blind, randomised, placebo-controlled trial, the objective was to investigate the effects that the prebiotic and prebiotic candidate OF and 2'FL singular and in combination had on microbial load and composition as a primary outcome. As secondary outcomes we investigated whether prebiotics and candidates could aid in improvements of Beck Depression Inventory (BDI), State Trait Anxiety Inventory Y1 and Y2 (STAI Y1 and Y2) and Pittsburgh Sleep quality Index (PSQI) scores. We also collected saliva and urine samples to assess changes in cortisol awakening response (CAR) and urinary metabolites in adults with mild-to-moderate levels of anxiety and depression.

4.2 Materials and methods

4.2.1 Volunteers and recruitment

Healthy adults, both males and females, were recruited from the Reading area via previous email lists and by posting on social media. Inclusion criteria were volunteers aged 18-50, BMI ≥ 18.5 and ≤ 30 kg/m^2, no evidence of gastrointestinal disease and possessed mild/moderately elevated levels of stress and anxiety as measured via PHQ-9 (Kroenke, Spitzer and Williams, 2001) and GAD-7 (Spitzer *et al.*, 2006) (PHQ-9 range: 7-15 and GAD-7 range: 8-16). They were free of food allergies and had a stool frequency of at least three bowel movements per week. Exclusion criteria were extreme diets (i.e., ketogenic, vegetarian, vegan, intermittent fasting), antibiotic treatment in the 4 months preceding the study, anaemia, chronic or acute diseases i.e., (pre)-diabetic. Potential volunteers were also excluded if they had been previously or currently diagnosed with neurological or psychiatric disorders or if they undergone surgical resection of any part of the bowel, were current smokers and/or had a history of alcohol or drug misuse or if they were pregnant or lactating. Use of laxatives was also not permitted 4 weeks prior to beginning of the intervention. Use of anti-depressant medication including

selective serotonin receptor inhibitors or amitriptyline was not allowed three months prior to commencing the trial.

4.2.2 Study design and interventions

This was a four-arm parallel, double-blind, randomised, placebo-controlled trial (RCT) lasting 5-weeks, segregated into a 1-week run-in and 4-week intervention phase. 5-weeks was the chosen study length to capture initial day-to-day fluctuations in gastrointestinal habits prior to commencement of intervention, combined with methods previously documented in several mood state and/or gut microbiota supplementation studies (Kazemi *et al.*, 2019; Schmidt *et al.*, 2015; Kelly *et al.*, 2017; Vandeputte *et al.*, 2017a). Eligible volunteers were provided with verbal and written study information and gave written informed consent prior to study entry. During a 1-week run-in period volunteers were asked to complete a daily bowel habit and gastrointestinal sensation diary to establish baseline values. Subsequently they were randomly allocated into one of four groups (n = 24) stratified by initial PHQ-9 and GAD-7 scores and sex using a ratio of approximately 3 : 1 (female : male). Ninety-six adults were divided into Group 1A (19 : 5), Group 1B (17 : 7), Group 2A (17 : 7), and Group 2B (18 : 6).

The ITF used was oligofructose (OF) (Orafti® P95, DP 3-9, average DP 4; BENEO-Orafti, Tienen, Belgium). The other test product was 2'fucosyllactose (2'FL). 2'fucosyllactose is a human milk oligosaccharide (HMO) produced commercially using metabolically engineered organisms. 2'Fucosyllatose (96-98% pure) is a fucosylated HMO composed of L-fucose, D-galactose and D-glucose and was supplied by BENEO-Orafti, (Tienen, Belgium). The comparator (placebo) product was maltodextrin, a readily digestible carbohydrate, which consists of varying chains of D-glucose primarily linked by α-(1,4)-linkages of various chain length. To maintain blinding, interventions were packaged in equal weight sachets (sachets A/B = either 8 g OF or

maltodextrin and sachets 1/2 = either 2 g maltodextrin or 2'FL) by a research assistant not otherwise involved in the study with unique randomisation codes. A random sequence was created utilizing the software RandList Version 1.5. 2 g of 2'FL was the selected dosage based on results of our previous *in vitro* batch culture fermentations (Jackson *et al.*, 2022b), combined with EFSA documentation 258/97 stating that 3 g/day of 2'FL is the maximum intended daily intake from food supplements at which no risk of adverse events should occur (Agostoni *et al.*, 2015).

Stool, urine, and saliva samples were collected from volunteers as the first urine, stool and saliva samples after waking at the start (D0) and the end (D28) of the intervention phase. Volunteers were also asked to complete self-reported mood-state and sleep questionnaires at the start and again at the end of the four-week intervention phase. Details of sample collection and processing and mood-state questionnaires are presented below. No intervention was given until baseline samples and self-reported questionnaires had been completed. Volunteers were instructed to consume both of their assigned sachets once per day for 4 weeks in the morning in water just after or with breakfast resulting in a total daily intervention intake of 10 g. Compliance with consumption of the interventions was assessed by completion of a daily online check-in diary. Participants were considered compliant if they consumed > 95% of the supplied supplements. Volunteers were told to not alter their diet or fluid intake during the trial and were asked to record their dietary intake for 3 consecutive days at the start (Days 0, 1, 2) and end (Days 26, 27, 28) of the intervention phase into specified diary pages via supplied links. Nutrient intakes were calculated using Nutritics v5.83 (Nutritics, 2022).

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.

4.3 Outcome measures

4.3.1 Primary Outcomes

The primary outcome was changes in *Bifidobacterium* counts as measured by fluorescence *in situ* hybridisation flow cytometry. Changes in microbial populations as measured by 16S rRNA sequencing.

4.3.2 Secondary outcomes

4.3.2.1 Beck Depression Inventory

The Beck's Depression Inventory (BDI) is a 21-question self-reported rating inventory in a multiple-choice format (Beck *et al.*, 1961). Within each inventory volunteers are asked to choose from one of four statements that best describes their situation in the past two weeks. Each inventory is scored 0, 1, 2 or 3. 0 represents the normal or least depressive statement and 3 the most depressive statement. An overall score is calculated via summing individual scores for each inventory. Scores range from 0 to 63, lower scores being associated with lower levels of depression.

4.3.2.2 State-Trait Anxiety Inventory

The State-Trait Anxiety Inventory (STAI) is a self-reported questionnaire used for assessing levels of anxiety (Spielberger *et al.*, 1983). The STAI consists of two parts, Y1 (State = now/in the moment) and Y2 (Trait = in general). Each form consists of 20 questions, each question being scored from 1-4 and each form having a range of 20-80. In total the STAI Y1 and Y2 have 40 questions with higher scores indicating higher levels of anxiety.

4.3.2.3 Positive and Negative Affect Schedule-Short FORM (PANAS-SF)

Current mood (i.e., transient affect) was assessed using the Positive and Negative Affect Schedule-Short FORM (PANAS-SF) at D0 and D28 (Watson, Clark and Tellegen, 1988). The PANAS possesses 20 self-reported measures of positive affect (Ten items - PA) and negative (Ten items – NA) that can be used on multiple occasions. Each volunteer rated to the degree which they were currently experiencing each item on a five-point Likert scale. Rating of positive and negative items were summed to give an overall PA and NA score. Scores range from 10 to 50 – the higher the scores indicate higher levels of PA and NA.

4.3.2.4 The Pittsburgh Sleep Quality Index

The Pittsburgh sleep quality index (PSQI) is a self-rating questionnaire consisting of 19 questions plus 5 questions related to either bedpartner or roommate. The PSQI assesses sleep quality across seven different components, each weighted equally from a score of 0-3. Scores from each component are then summed yielding a total PSQI. Scores range from 0 to 21 with higher scores being associated with poor sleep quality (Buysse *et al.*, 1989).

4.3.2.5 Gastrointestinal sensations, bowel consistency and frequency

Bowel habit and gastrointestinal sensation diaries were completed daily throughout the course of both the one-week run-in phase, and four-week intervention phase, in order to assess changes in flatulence, intestinal bloating, abdominal pressure, abdominal pain and feeling of fullness (all none, mild, moderate or 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.

4.3.2.6 Other secondary outcomes

We also collected the first urine sample at waking and saliva samples during the first hour after waking in order to assess changes in urinary metabolites and cortisol awakening response (CAR). Details of sample collection are detailed below.

4.3.3 Sample collection

4.3.3.1 Faecal samples

Volunteers were provided with sterile stool sample pots for D0 and D28 collections. Freshly collected faecal samples were kept in 2.5L Oxoid™ AnaeroJar™ (Oxoid, Hampshire, United Kingdom) with Oxoid™ AnaeroGen™ 2.5L sachet O_2 ≤0.1%; CO_2: 7-15%). Faecal samples were collected from the volunteer's place of residence within 2 hours of voiding. Sample (1.5 g) for metabolic profiling were stored at -80 °C until the study had been completed. An additional 3 g of the same stool sample was diluted 1:10 (w:w) in anaerobic phosphate-buffered saline (PBS, 0.1 mol l^{-1}; pH 7.4), then homogenised using a stomacher (260 paddle beats/min) for 2 min at room temperature. Faecal slurry (20 mL) was 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 was then diluted in 675 µL 0.1 mol l^{-1}, pH 7.4 PBS (1:100 dilution) and 750 µL aliquots were then

stored at -20 °C until cells could be fixed. Aliquots were then centrifuged at 11,337 × g for 5 min and the supernatant was discarded. Pellets were then resuspended in 375 µL of 0.1 mol l^{-1}, pH 7.4 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 (FISH).

4.3.3.2 Urine samples

D0 and D28 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 ^{1}H-NMR could be conducted.

4.3.3.2 Salivary cortisol

Hypothalamic-pituitary-adrenal (HPA) activity was assessed at D0 and D28 of the intervention using salivary cortisol awakening response (CAR). Participants were asked to provide 5 saliva samples in 15 mL falcon tubes. Samples were collected immediately upon waking and subsequently every 15 min until 1 hour post waking (0, 15, 30, 45 and 60 min) in separate falcon tubes. Saliva samples were delivered at the same time as urine and faeces. Saliva samples were allocated in 1.5 mL Eppendorf tubes and stored at – 80 °C until analysis via commercial ELISA (Biotechne®, R&D systems, Oxford, UK) could be completed.

4.3.4 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 4.1. Fluorescence measures were performed by a BD Accuri™ C6 Plus (BD, Erembodegem, Brussels) measuring at 488 nm and 640 nm. A threshold of 9000 in the forward scatter area (FSC-A) and 3000 in the side scatter area (SSC-A) was 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, limit of collection was 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, Amann and Beisker, 1993)
Eub338I	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)

Table 4.1 Name, sequence, and target group of oligonucleotide probes used in bacterial enumeration.

4.3.5 Microbial Profiling

4.3.5.1 Bacterial DNA extraction

1.5 g of fresh stool for metabolic profiling were stored at -80 °C until the study had been completed. Bacterial DNA was extracted from faecal samples using the QIAamp Fast DNA Stool mini kit (QIAGEN) according to the manufacturer's instructions. In short, faecal samples were homogenised and aliquoted 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.

4.3.5.2 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 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, 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 considering

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.*, 2017b).

4.3.6 Metabolic profiling using ^{1}H-NMR spectroscopy

For analysis urine samples were thawed, and a phosphate buffer (pH 7·4 sodium phosphate with 0.2 mol l^{-1} disodium phosphate (Na_2HPO_4), 0.04 mol l^{-1} monosodium phosphate (NaH_2PO_4) in deuterium oxide (99·9 %) was prepared, with 1 mmol l^{-1} 3-(trimethylsilyl) propionic acid-d_4 sodium salt (TSP) and 3 mmol l^{-1} sodium azide in the solution. 400 µL of each urine sample was mixed with 200 µL buffer. 550 µL aliquots of supernatant were dispensed to fill 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 100 ms 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) and nPYc-Toolbox 1.2.7. Further details on the nPYC-Toolbox can be found at https://github.com/phenomecentre/nPYc-Toolbox

4.3.6.1 Chemometric analysis

Processed spectroscopic data were imported to the SIMCA 17.0 software package (Umetrics AB, Umeå, Sweden) to conduct unsupervised and supervised multivariate statistical analysis.

Principal components analysis (PCA) 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. PCA was followed by supervised modelling using orthogonal projections to latent structure discriminant analysis (OPLS-DA) using the NMR spectroscopic data as X variables, and intervention as Y variable, to model pre and post differences and to identify any metabolites contributing to this difference. The models were assessed based on variance explained (R^2Y) and predictive ability (Q^2Y) metrics.

4.3.7 Ethics

The study was given favourable ethical consent by the University of Reading's Research Ethics Committee (21/43) and was conducted in accordance with the Declaration of Helsinki (Appendix 4.1). The study was registered as a clinical trial (**ClinicalTrials.gov ID:** NCT05212545). All participants gave written consent prior to study entry.

4.3.8 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* counts between the four 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 0.7 $\log_{10}$ cells/g wet faecal sample bifidobacteria within subject standard deviation.

Statistical Package for Social Science version 27 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Participant metrics were analysed using a one-way ANOVA to assess differences in categorical data. Mood state parameters (BDI, STAI and PANAS-DF), PSQI and CAR and dietary data were analysed by linear marginal model (LMMs) using an unstructured covariance matrix to model repeat measures. Separate LMMs were performed for each dependant variable in PANAS-SF (PA, NA). Changes in bacteriology (FLOW-FISH and QMP) were analysed using a general linear modelling (GLM) to assess repeat measures. Post-hoc comparisons were also performed in order to determine any significant differences within and between interventions. Baseline values were included as a covariate to assess differences between groups at completion within each LMM and GLM. All comparisons were corrected for type 1 errors using Bonferroni adjustment within each LMM and GLM. Correlations between bacterial taxa and mood state were assessed employing fold change ((post-pre)/pre) using non-parametric Spearman's rank correlation corrected for using false discovery rate (FDR). All tests were two tailed and *P* values ≤ 0.05 were considered statistically significant.

4.4 Results

4.4.1 Participant data

In total, 125 volunteers expressed interest and were screened for eligibility, of whom 96 were randomised (*n* = 24 in each group). Of these, 4 volunteers withdrew from the trial, 92 volunteers completed the trial (62 females and 30 males) (*n* = 23 in each group) and were included in analysis for all primary and secondary outcomes (Figure 4.1).

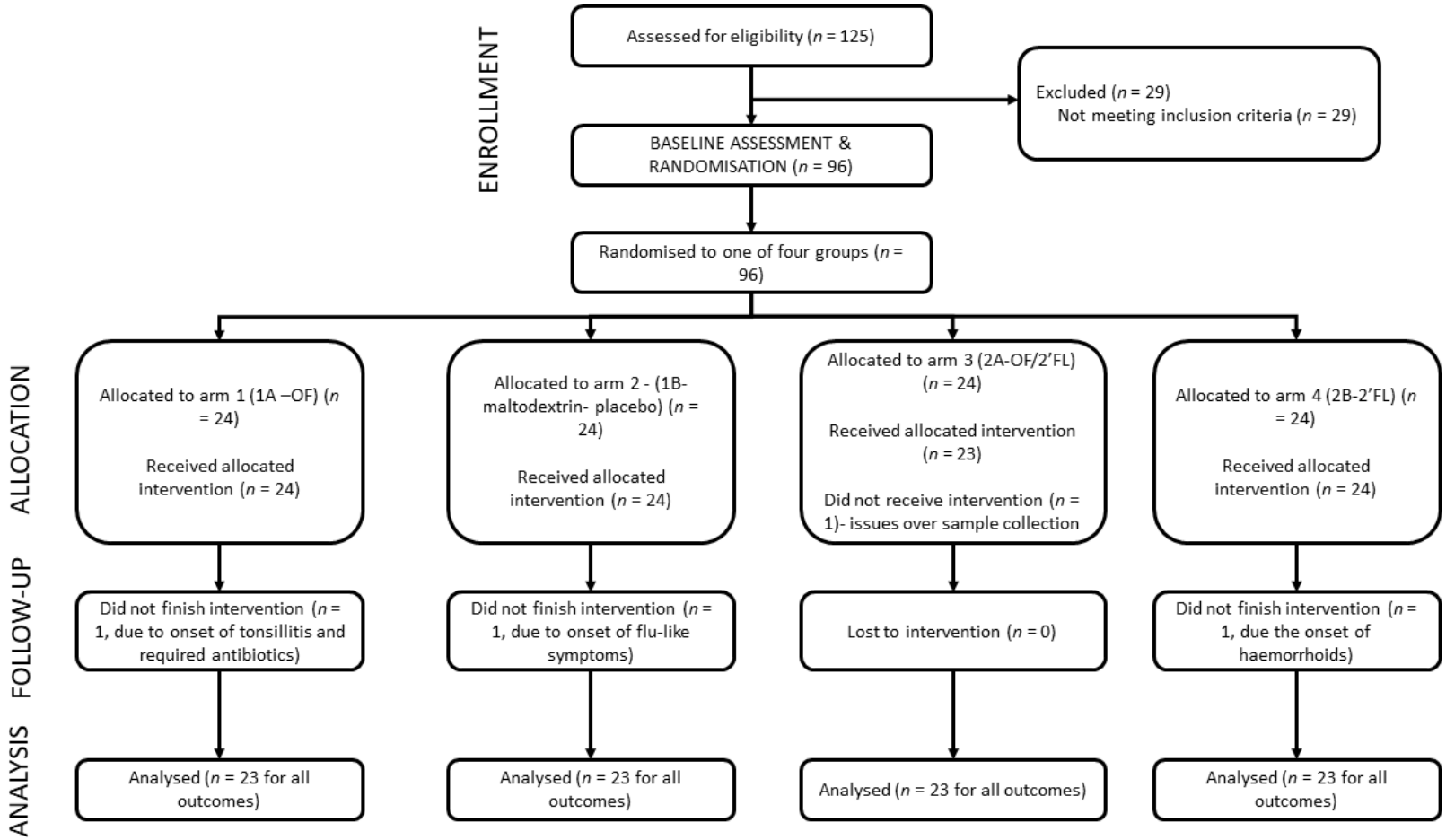

Figure 4.1. CONSORT diagram of participant flow through the intervention. **Abbreviations:** OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

Table 4.2 reports subject characteristics (*n* = 23 per group) (age, height, weight, and BMI) mean and range segregated by intervention. Mean subject age was 28.29 y, weight 66.92 kg, height 168.08 cm and BMI 23.52 kg/m^2. No significant differences in participant metrics were detected between any intervention.

Intervention	OF (*n* = 23)	Maltodextrin (*n* = 23)	OF/2'FL (*n* = 23)	2'FL (*n* = 23)	*P* value Intervention
Age (y)	29.17 (19-50)	29.04 (20-47)	25.39 (19-47)	28.91 (19-46)	*P* = 0.340
Weight (kg)	65.98 (53.34-105.00)	69.23 (50.00-94.00)	67.95 (48.20-100.00)	64.53 (46.00-93.50)	*P* = 0.657
Height (cm)	169.8 (157.00-196.00)	167.7 (145.00-193.00)	168.5 (154.00-187.00)	166.3 (155.00-181.00)	*P* = 0.639
BMI (kg/m^2)	22.79 (18.72-29.07)	24.41 (19.02-28.73)	23.74 (19.84-29.96)	23.17 (18.59-29.73)	*P* = 0.304

Table 4.2. Participant metrics – age, weight, height, and BMI mean and range segregated by intervention. 92 volunteers (*n* = 23 per group). *P* values are the results of using a one-way ANOVA to compare differences in continuous data (orange column). **Abbreviations:** OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

4.4.2 Dietary intake

Nutrient data at baseline 1st week (Days 0, 1 and 2) and final week of (Days 26, 27 and 28) of the intervention are presented in Table 4.3. No significant differences were detected in total energy, protein, carbohydrates, total sugar, fat, saturated fat or dietary fibre between interventions (all $P \geq 0.05$).

	OF (*n* = 23)		Maltodextrin (*n* = 23)		OF/2'FL (*n* = 23)		2'FL (*n* = 23)		*P* value Intervention
	Baseline (D0-D2)	Final Week (D26-28)	Baseline (D0-D2)	Final Week (D26-28)	Baseline (D0-D2)	Final Week (D26-28)	Baseline (D0-D2)	Final Week (D26-28)	
Total energy (kcals)	1896 (106.9)	1855 (97.92)	1787 (89.98)	1811 (91.17)	1722 (113.4)	1819 (116.4)	1831 (144.7)	1786 (104.3)	*P* = 0.384
Protein (g)	84.56 (9.26)	83.14 (9.79)	79.58 (10.47)	85.26 (10.28)	76.66 (5.43)	81.54 (6.33)	75.57 (7.21)	71.91 (6.15)	*P* = 0.445
Fat (g)	67.99 (4.55)	71.57 (4.37)	65.31 (6.29)	69.46 (4.73)	61.3 (5.20)	67.43 (5.56)	64.35 (6.11)	67.54 (5.39)	*P* = 0.987
Saturated fat (g)	21.74 (1.48)	21.39 (1.68)	20.55 (1.57)	22.92 (1.96)	20.08 (1.37)	22.92 (1.93)	22.33 (2.53)	23.32 (2.59)	*P* = 0.569
CHO (g)	222.70 (11.24)	223.80 (12.47)	216.00 (12.62)	210.10 (14.13)	197.50 (15.58)	213.40 (14.44)	218.30 (22.78)	217.70 (14.25)	*P* = 0.136
Total sugar (g)	58.39 (4.32)	56.83 (6.05)	60.58 (5.34)	57.21 (4.58)	51.40 (5.98)	58.58 (7.18)	69.36 (9.52)	65.20 (6.07)	*P* = 0.217
Fibre (g)	20.98 (2.56)	19.67 (2.26)	20.14 (1.66)	17.97 (1.25)	18.60 (1.64)	18.89 (1.36)	18.95 (1.62)	19.20 (1.41)	*P* = 0.382

Table 4.3. Energy and nutrient intake at baseline (D0-D2) and at completion (D26-D28) of intervention phase in 92 volunteers (*n* = 23 per group). Mean and standard error (SE). *P* values are as a result of using an average of run-in (D0-D2) data as a baseline covariate for between group final week (D26-D28) comparisons employing a linear marginal model (orange column). **Abbreviations:** OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose; CHO = Total carbohydrates

4.4.3 Bacterial enumeration by FISH-FLOW

92 volunteers provided stool samples at baseline and end of the intervention. Figure 4.2 and Appendix 4.2 reports changes in total (Eub I-II-III) and *Bifidobacterium* counts observed across the four intervention groups between D0 and D28 of the intervention using FISH-FLOW. Analysis of total bacteria (Eub I-II-III) revealed significant intervention [F (3,92) = 2.652, P = 0.048], day [F (1,92) = 8.52, $P \leq 0.001$], and intervention x day [F (3,92) = 57.84, $P \leq 0.001$)] interactions. Post hoc comparisons revealed significant increases in Eub I-II-III (total bacterial cell count) were observed in OF, OF/2'FL and 2'FL interventions (all $P \leq 0.001$), but not maltodextrin (Figure 4.2A). Largest increases in total bacterial counts were recorded in OF and OF/2'FL treatments going from 10.01 ± 0.07 (SE) to 10.35 ± 0.07 (SE) (0.34 mean difference) and 10.03 ± 0.06 (SE) to 10.25 ± 0.06 (SE) (0.22 mean difference) Log_{10} cells/g wet faeces respectively (Appendix 4.2). OF and OF/2'FL interventions were also significantly different to maltodextrin at D28 – OF ($P \leq 0.001$) and OF/2'FL (P = 0.014) (Figure 4.2A).

Regarding Bif164 (*Bifidobacterium* spp.) counts, there were again significant increases recorded in OF, OF/2'FL and 2'FL, but not maltodextrin intervention (Figure 4.2B). Yet the extent of increase in Bif164 counts varied greatly between interventions with OF and combination of OF/2'FL inducing largest increases going from 8.57 ± 0.13 (SE) to 9.52 ± 0.12 (SE) (0.94 av dif) (OF) and 8.68 ± 0.10 (SE) to 9.13 ± 0.13 (SE) (0.72 mean difference) (OF/2'FL) Log_{10} cells/g wet faeces respectively. Compared to OF and OF/2'FL combination, 2'FL induced less substantial increases in Bif164 counts going from 8.63 ± 0.14 (SE) to 8.93 ± 0.12 (SE) (0.30 mean difference) Log_{10} cells/g wet faeces. There were also differences detected between interventions at D28 with OF, OF/2'FL (both $P \leq 0$.001), and 2'FL (P = 0.021) being significantly different to maltodextrin. Increases in Bif164 (*Bifidobacterium*) recorded in the OF group were also significantly different to 2'FL at D28 (P = 0.015) (Figure 4.2B).

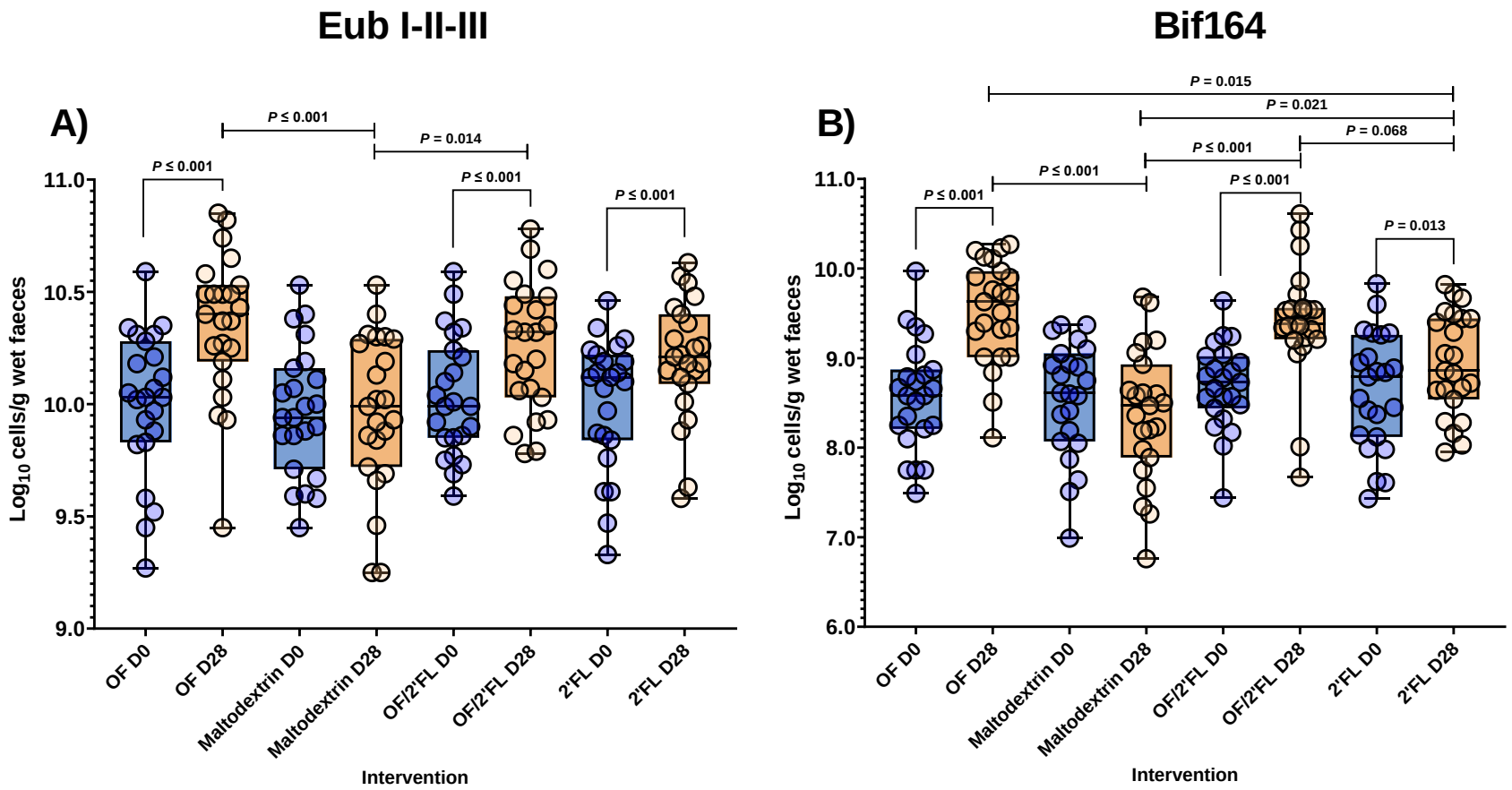

Figure 4.2. Bacterial groups measured by FISH-FLOW (Log_{10} cells/g wet faeces) using probes: Total bacteria (Eub I-II-III) **(A)** and *Bifidobacterium* spp. (Bif164) **(B)**. Box and Whisker plot (min and max) all points, mean and median (*n* = 23 per group). Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values. **Abbreviations:** OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

4.4.4 Microbiota Profiling Analysis - Quantitative Microbiome Profiling (QMP)

Figure 4.3.1 reports the overall quantitative microbiome profiling (QMP) microbial abundance data. Figures 4.3.2, 4.3.3 and 4.3.4 report the most significant changes documented from the QMP data across all four interventions at baseline and completion.

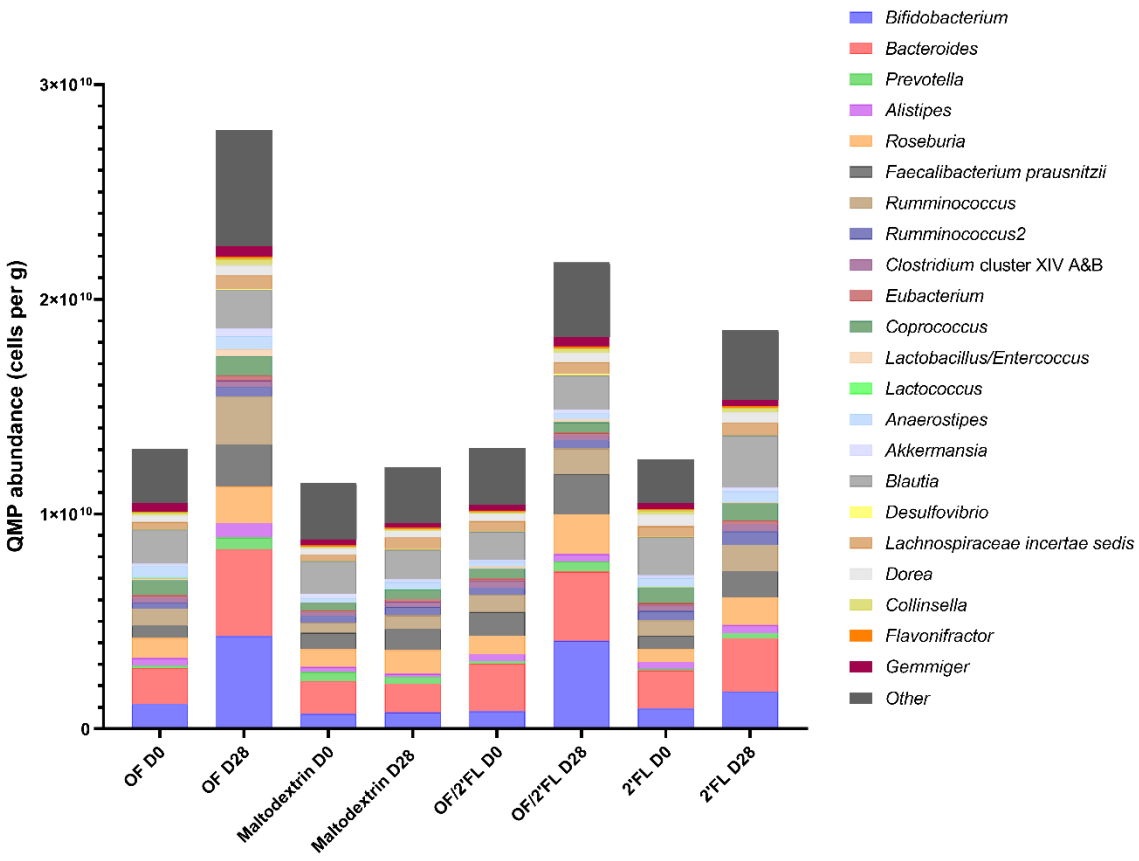

Figure 4.3.1. Quantitative microbiome profiling (QMP) data of overall 16S rRNA sequencing data recorded across all four interventions at D0 and D28. Numbers are expressed as cells per gram of faeces. **Abbreviations**: OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

At phylum level there were several differences detected on completion of the intervention. The magnitude of change varied substantially between interventions. Regarding *Actinomycetota* (*Actinobacteria*), analysis revealed significant day [F (1,91) = 21.989, $P \leq$

0.001], day x intervention [F (3,91) = 4.431, *P* = 0.006] and intervention [F (3,91) = 4.614, *P* = 0.005] interactions (Appendix 4.3). Repeated measures analysis revealed significant increases in *Actinomycetota* only in OF and OF/2'FL interventions with numbers increasing from 1.43 x 10^9 ± 4.06 x 10^8 (SE) to 4.87 x 10^9 ± 9.76 x 10^8 (SE) (3.43 x 10^9 mean difference) and 9.57 x 10^8 ± 1.76 x 10^8 (SE) to 4.41 x 10^9 ± 1.24 x 10^8 (SE) (3.46 x 10^8 mean difference) cells per gram (both *P* ≤ 0.001) respectively. Both these were significantly different to maltodextrin OF (*P* = 0.009), OF/2'FL (*P* = 0.028). This coincided with the significant changes seen in *Bifidobacterium* at genus level (Figure 4.3.2, Appendix 4.2 and Appendix 4.3).

Other notable changes at phylum level occurred in *Bacteroidota* (*Bacteroidetes*) with numbers in the OF intervention increasing significantly from 2.32 x 10^9 ± 4.93 x 10^8 (SE) to 5.64 x 10^9 ± 1.18 x 10^8 (SE) (3.32 x 10^9 mean difference) (*P* ≤ 0.001). This also being significantly different to maltodextrin at completion (*P* = 0.011). Furthermore, *Bacillota* (*Firmicutes*) showed significant day [F (1,91) = 35.77, *P* ≤ 0.001], day x intervention [F (3,91) = 3.50, *P* = 0.019] but not intervention [F (3,91) = 19.73, *P* = 0.124] interactions. Subsequent analysis revealed that significant increases in *Bacillota* were detected in OF (*P* ≤ 0.001), OF/2'FL (*P* = 0.008) and 2'FL (*P* = 0.004) interventions, but not maltodextrin (Appendix 4.3). The changes documented in *Bacillota* in the OF group were statically significant from maltodextrin (*P* = 0.016), but not either OF/2'FL combination (*P* = 0.727) or sole 2'FL (*P* = 0.746).

At genus level, microbial responses varied significantly amongst the interventions with largest changes documented in *Bifidobacterium*, *Bacteroides*, *Prevotella*, *Roseburia*, and *Faecalibacterium prausnitzii*. Regarding *Bifidobacterium,* analysis revealed significant day [F (1,91) = 19.76, *P* ≤ 0.001], day x intervention [F (3,91) = 93.85, *P* = 0.012] and intervention [F (3,91) = 4.70, *P* = 0.008] interactions. Subsequent analysis detailed that both OF and OF/2'FL interventions recorded significant increases in *Bifidobacterium* going from 1.15 x 10^9 ± 3.75 x

10^8 (SE) to 4.28 x 10^9 ± 9.03 x 10^8 (SE) (3.13 x 10^9 mean difference) (OF) and 8.18 x 10^8 ± 1.18 x 10^8 (SE) to 4.05 x 10^9 ± 1.23 x 10^9 (SE) (3.23 x 10^8 mean difference) (OF/2'FL) cells per gram (both $P \leq 0.001$). Both treatments were significantly different to maltodextrin, but not 2'FL (Figure 4.3.2 and Appendix 4.3).

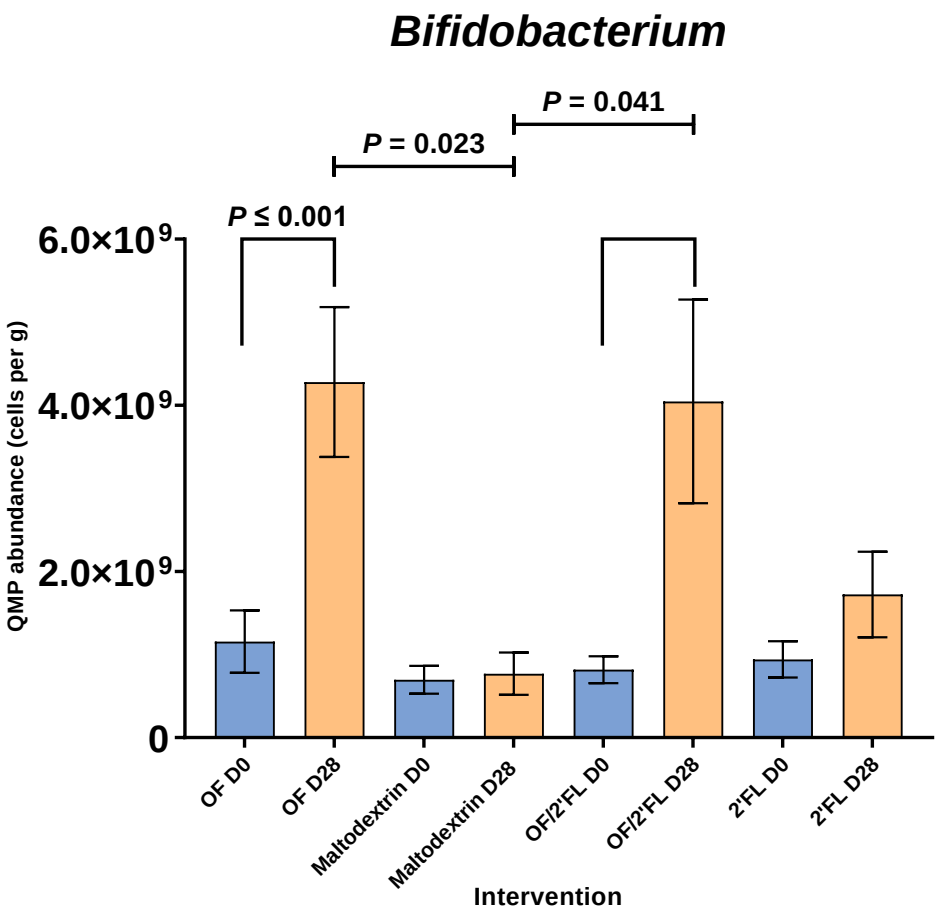

Figure 4.3.2. Quantitative microbiome profiling data of *Bifidobacterium* 16S rRNA sequencing data recorded across all four interventions at D0 and D28. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces. Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values. **Abbreviations:** OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

Significant interactions for *Bacteroides* are documented in Figure 4.3.3A. Analysis revealed significant day [F (1,91) = 7.55, $P = 0.007$], day x intervention [F (3,91) = 3.12, $P = 0.030$], but not intervention [F (3,91) = 21.22, $P \leq 0.001$] interactions. Repeated measures analysis

revealed significant increases in *Bacteroides* in the OF intervention only ($P \leq 0.001$). This result was significantly different to maltodextrin ($P = 0.019$), but not either OF/2'FL ($P = 1.00$) or 2'FL ($P = 0.250$) (Appendix 4.3).

QMP results for *Prevotella* are documented in Figure 4.3.3B with analysis revealing significant day [$F(1,91) = 6.87$, $P = 0.010$], but not either day x intervention [$F(3,91) = 1.69$, $P = 0.173$] or intervention [$F(3,91) = .234$, $P = 0.873$] interactions. Post hoc analysis documenting increases in *Prevotella* in both OF ($P = 0.013$) and OF/2'FL ($P = 0.039$) interventions but not maltodextrin ($P = 0.665$) or 2'FL ($P = 0.278$). No significant differences were detected being interventions at completion (Figure 4.3.3B and Appendix 4.3).

Regarding *Roseburia,* significant day [$F(1,91) = 21.22$, $P \leq 0.001$], but not either day x intervention [$F(3,91) = 0.931$, $P = 0.429$] or intervention [$F(3,91) = 0.931$, $P = 0.498$] interactions were detected. This was confirmed with post hoc analysis revealing significant increases in numbers of *Roseburia* in OF ($P = 0.008$), OF/2'FL ($P = 0.001$) and 2'FL ($P = 0.026$) interventions from baseline (Figure 4.3.4A and Appendix 4.3).

Similarly, with *Faecalibacterium prausnitzii*, only day [$F(1,91) = 24.61$, $P \leq 0.001$] interactions were significant (Figure 4.3.4B). Yet, unlike *Roseburia* there was also a trend towards day x interventions interactions [$F(3,91) = 2.64$, $P = 0.055$]. Repeated measures analysis revealed that OF induced the largest increases in *Faecalibacterium prausnitzii* going from $5.82 \times 10^8 \pm 9.90 \times 10^7$ (SE) to $1.93 \times 10^9 \pm 4.16 \times 10^8$ (SE) (1.35×10^9 mean difference) cells per gram ($P \leq 0.001$). Smaller increases in *Faecalibacterium prausnitzii* were recorded in the OF/2'FL intervention group going from $1.12 \times 10^9 \pm 3.30 \times 10^8$ (SE) to $1.83 \times 10^9 \pm 4.48 \times 10^8$ (SE) (7.11×10^8 mean difference) ($P = 0.015$) as well as the 2'FL intervention going from $6.20 \times 10^8 \pm 1.43 \times 10^8$ (SE) to $1.76 \times 10^9 \pm 1.76 \times 10^8$ (SE) (5.80×10^8 mean difference) cells per gram ($P = 0.047$).

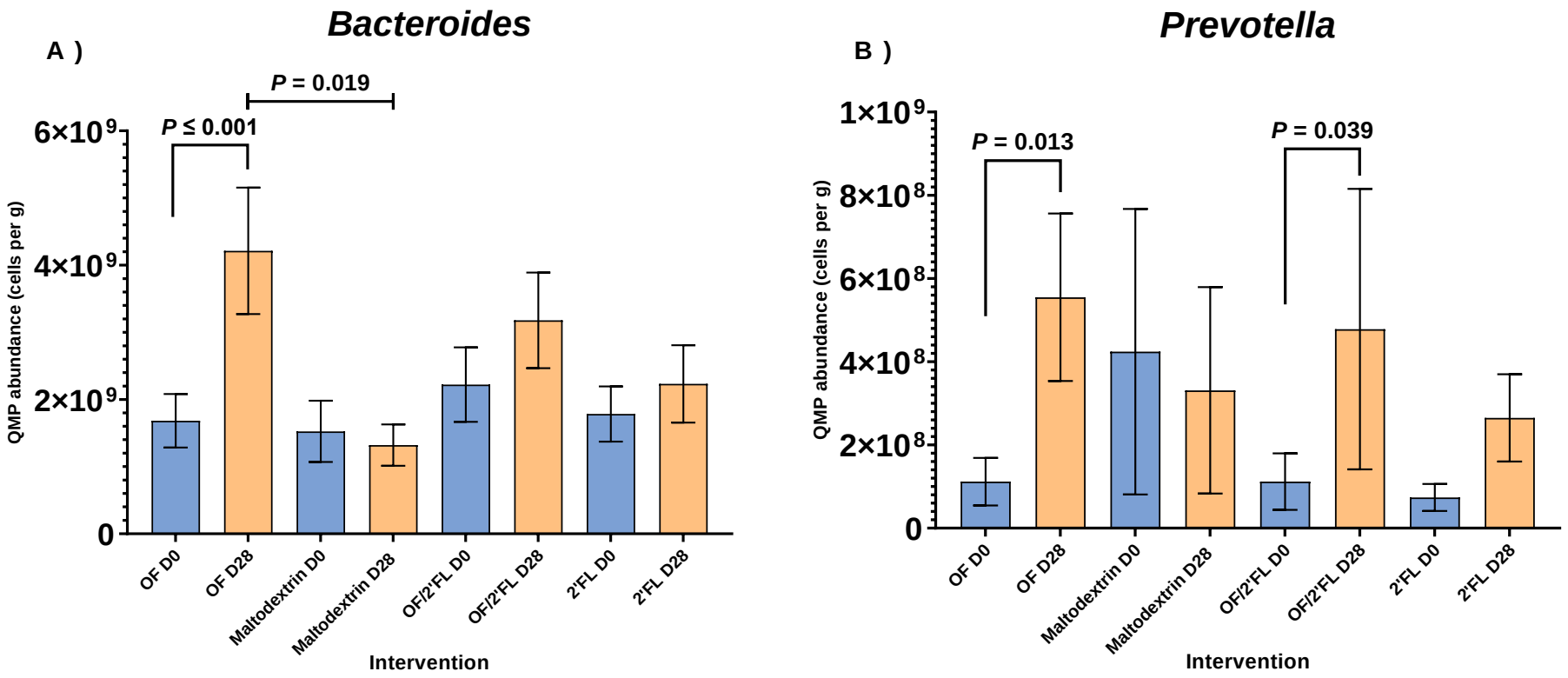

Figure 4.3.3. Quantitative microbiome profiling data of *Bacteroides* **(A)** and *Prevotella* **(B)** 16S rRNA sequencing data recorded across all four interventions at D0 and D28. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces. Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values. **Abbreviations:** OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

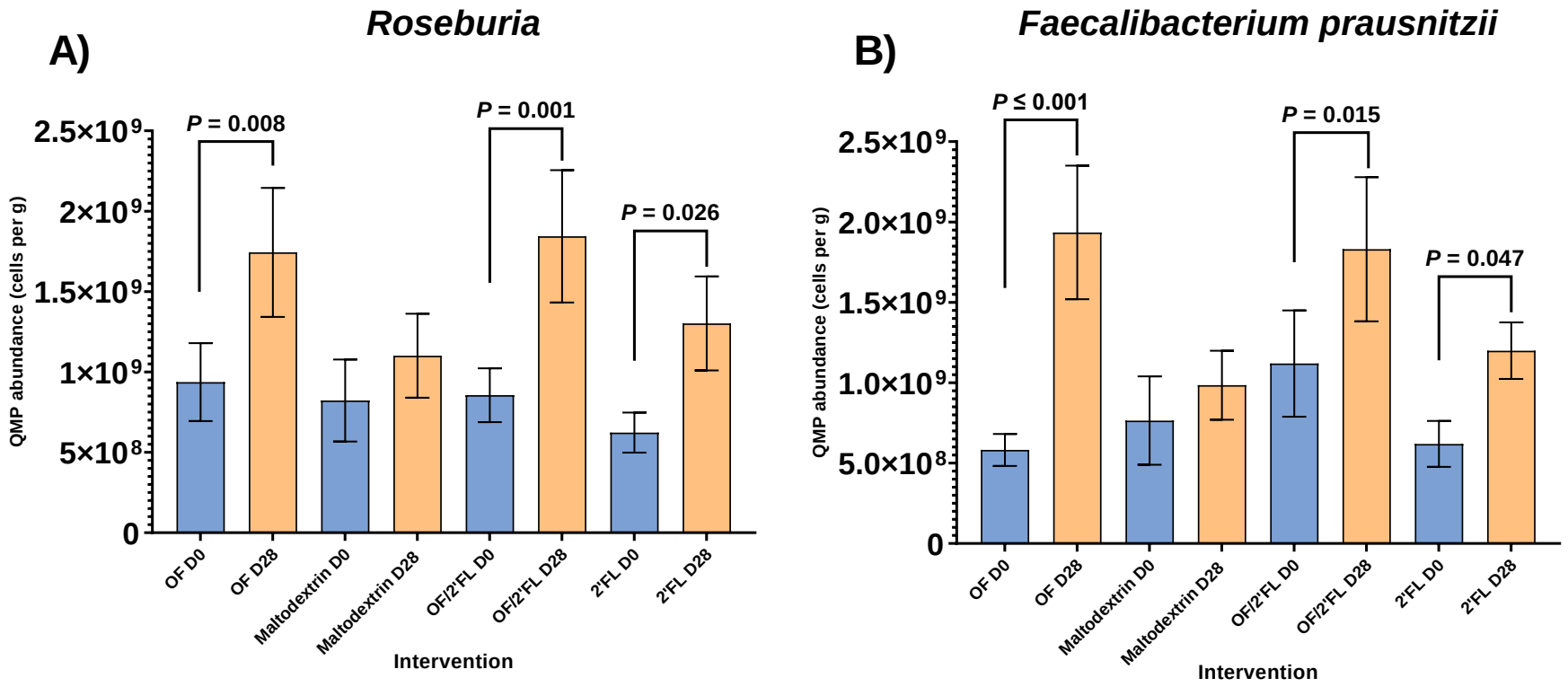

Figure 4.3.4. Quantitative microbiome profiling data of *Roseburia* **(A)** and *Faecalibacterium prausnitzii* **(B)** 16S rRNA sequencing data recorded across all four interventions at D0 and D28. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces. Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values. **Abbreviations**: OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

OF intervention also recorded a number of significant increases in bacterial taxa including *Alistipes* ($P = 0.004$), *Ruminococcus* ($P \leq 0.001$), *Lactobacillus/Enterococcus* ($P = 0.006$), *Akkermansia* ($P = 0.015$), *Desulfovibrio* ($P = 0.021$), *Flavonifractor* ($P = 0.011$) and *Collinsella* ($P = 0.007$). Significant changes in *Collinsella* were also detected in the OF/2'FL intervention ($P = 0.006$) (Appendix 4.3). An increase in *Ruminococcus2* ($P = 0.040$) was the only significant change detected in the 2'FL intervention. There was also a large increase in *Blautia* seen in 2'FL intervention, however, due to the high level of heterogeneity seen between individuals only a trend towards reaching significance was recorded ($P = 0.063$) (Appendix 4.3).

4.4.5 Bowel habit and function

Changes in gastrointestinal (GI) sensations (flatulence, intestinal bloating, abdominal pressure, abdominal pain and feeling of fullness), stool consistency and stool frequency were self-recorded daily throughout both the one-week run-in and 28-day intervention period. Scores of 0, 1, 2, and 3 corresponded to none, mild, moderate, and severe (Ramnani *et al.*, 2010; Costabile *et al.*, 2008; Walton *et al.*, 2012). Data are presented as an average of one-week run-in period and the last week of the intervention phase (D22-D28). Changes in stool consistency measured as per Bristol Stool Form Scale (Lewis and Heaton, 1997) and stool frequency are reported in Figure 4.4.

Significant increases in flatulence ($P = 0.001$) and intestinal bloating ($P = 0.007$) scores were only detected in the OF group on completion, however, these increases were not statistically different from any other intervention on completion – flatulence ($P = 0.404$), intestinal bloating ($P = 0.199$). No other significant differences in GI tolerance (abdominal pressure, abdominal pain or feeling of fullness) were detected either within or between interventions (Appendix 4.4).

Changes in stool consistency (trends towards stool softness) reported significant day [F (1,92) = 6.93, *P* = 0.01], day x intervention [F (3,92) = 3.91, *P* = 0.011] and intervention [F (3,92) = 3.790, *P* = 0.01] interactions. Follow-up analysis revealed significant differences in stool consistency in both OF (*P* = 0.006) and OF/2'FL (*P* = 0.007) interventions and respectively (Figure 4.4A). Additionally, changes in stool consistency in both the OF and OF/2'FL interventions were significantly different to the 2'FL intervention on completion (both *P* = 0.004).

Analysis of changes in stool frequency revealed significant day x intervention [F (3,92) = 3.32, *P* = 0.02] and intervention [F (3,92) = 4.980, *P* = 0.03] interactions. These results were confirmed with post hoc analysis revealing that significantly greater increases in stool frequency in the OF/2'FL intervention at completion ($P \leq 0.001$) (Figure 4.4B). The increase in stool frequency detected at completion in the OF/2'FL intervention was significantly greater compared to both maltodextrin (*P* = 0.005) and 2'FL (*P* = 0.01), but not OF (*P* = 0.253) respectively (Figure 4.4B).

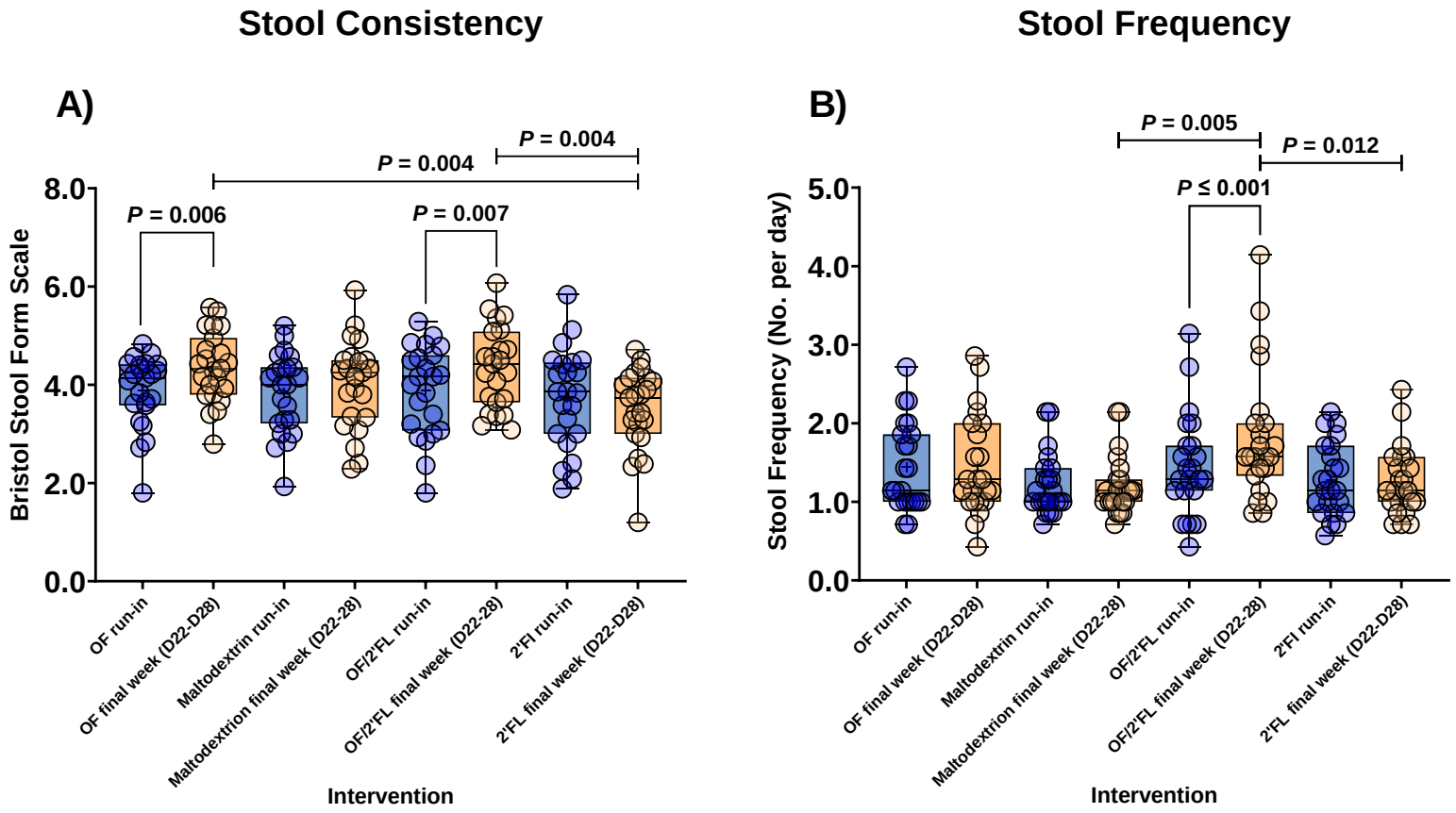

Figure 4.4. Gastrointestinal scores for stool consistency as per the Bristol Stool Form Scale **(A)** and Stool Frequency **(B)** at baseline (run-in) and again at last week of intervention (D22-D28) in 92 volunteers (*n* = 23 per group). Box and Whisker plot (min and max) all points, mean and median. Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values. **Abbreviations:** OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

4.4.6 Mood State

4.4.6.1 Becks Depression Inventory

Figure 4.5.1 displays BDI scores across the four interventions at D0 and D28 with analysis revealing significant intervention [F (3,92) = 3.36, *P* = 0.022], Day [F (1,92) = (147.968), *P* ≤ 0.001], and intervention x day [F (3,92) = 12.396, *P* ≤ 0.001] interactions. Repeated measure comparisons documenting significant reductions in BDI scores in OF, OF/2'FL and 2'FL (all *P* ≤.001), but not maltodextrin (*P* = 0.074). Additional pairwise comparisons revealing both OF and OF/2'FL outperformed maltodextrin (both *P* ≤ 0.001) with reductions in BDI scores in the OF intervention also being significantly different to 2'FL at D28 (*P* = 0.042) (Figure 4.5.1 and Appendix 4.5).

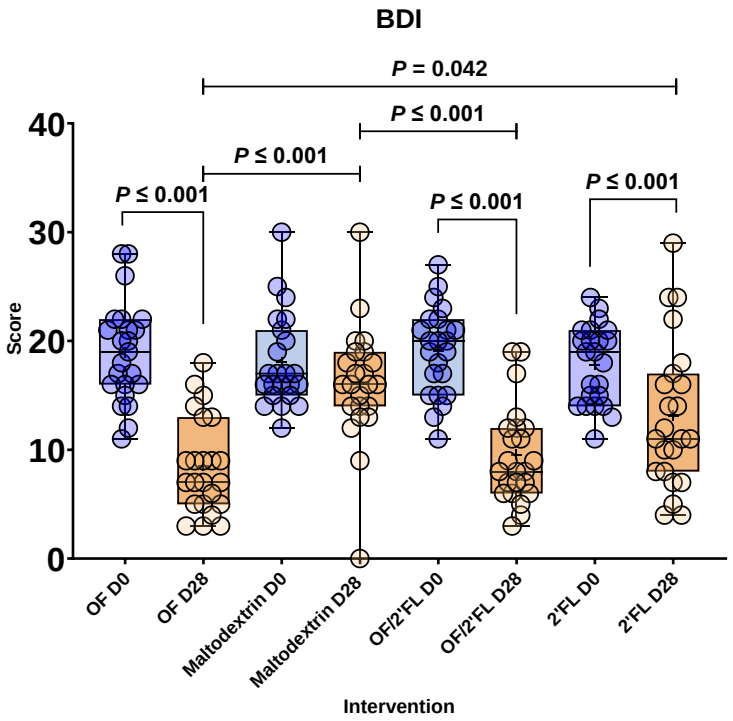

Figure 4.5.1. Becks Depression Inventory (BDI Scores) at D0 and D28 across all four interventions (92 volunteers (*n* = 23 per group)). Box and Whisker plot (min and max) all points, mean and median. Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values. **Abbreviations:** OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

4.4.6.2 State Trait Anxiety Inventory Y1 and Y2

Significant comparisons for STAI Y1 and STAI Y2 are displayed in Figure 4.5.2. Regarding STAI Y1 results analysis revealed significant intervention [F (3,92) = 6.34, $P \leq 0.001$], day [F (1,92) = 91.81, $P \leq 0.001$], and intervention x day [F (3,92) = 6.58, $P \leq 0.001$] interactions (Figure 4.5.2A). Post hoc within subject comparisons revealed significant reductions in STAI Y1 scores in OF, OF/2'FL (both $P \leq 0.001$) and 2'FL ($P = 0.001$). Additionally, pairwise comparisons revealed that both OF and OF/2'FL treatments outperformed maltodextrin in extent of reduction in STAI Y1 scores at D28 (Figure 4.5.2A). There was also a trend towards a difference in STAI Y1 scores at D28 between 2'FL and maltodextrin ($P = 0.058$).

Similarly, analysis of STAI Y2 scores revealed significant intervention [F (2.374) = 92.00, $P = 0.048$], day [F (134.456) = 92.00, $P \leq 0.001$], and intervention x day interactions [F (13.326) = 92.00, $P \leq 0.001$] (Figure 4.5.2B). Within subject pairwise comparisons revealed that OF, OF/2'FL and 2'FL resulted in significant reductions in STAI Y2 scores at D28 (all $P \leq 0.001$), while between subject comparisons documented that both OF and OF/2'FL outperformed maltodextrin (OF $P \leq 0.001$; OF/2'FL $P = 0.002$), but not 2'FL in the extent of reduction in STAI Y2 scores detected on completion (Figure 4.5.2B and Appendix 4.5).

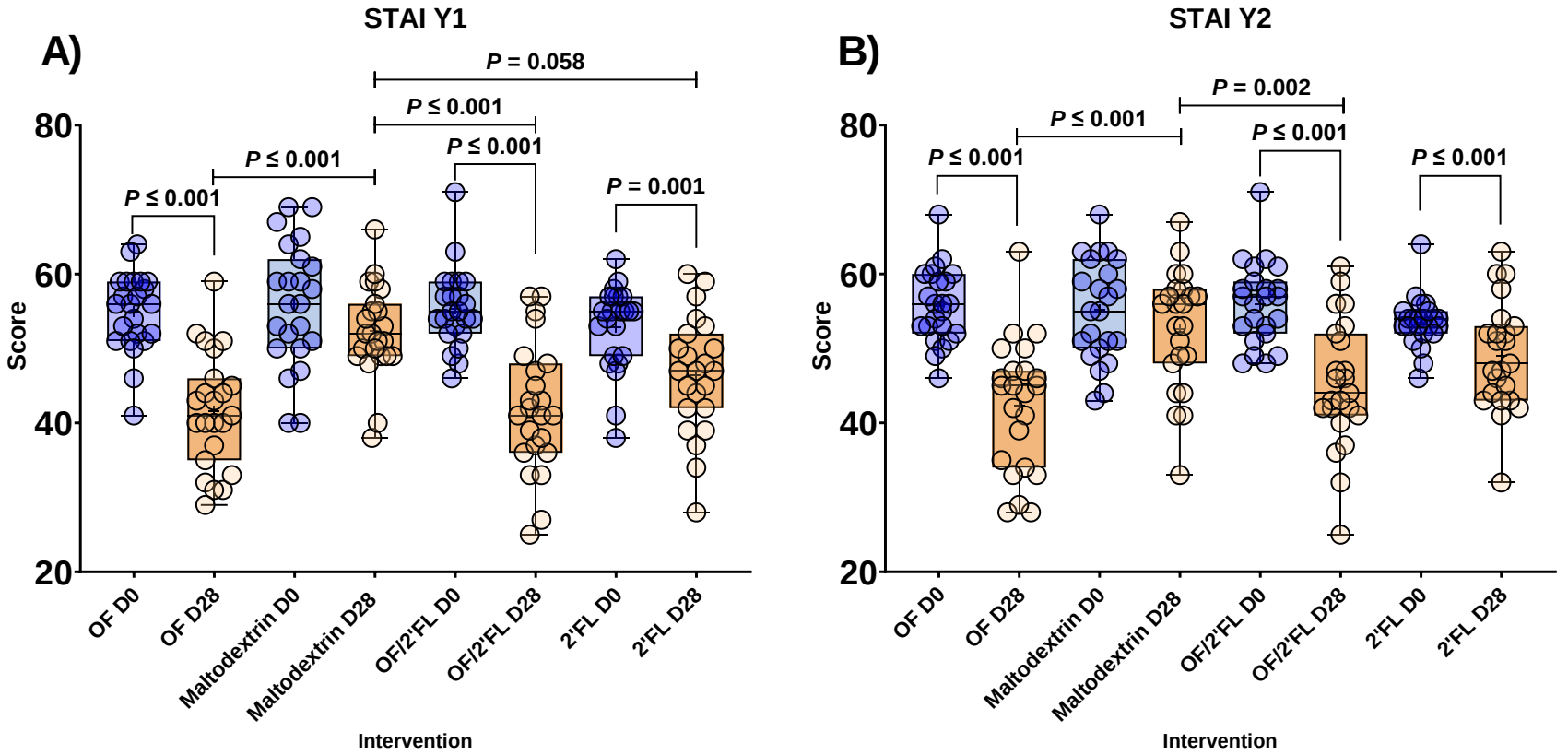

Figure 4.5.2. State Trait Anxiety Inventory (STAI) Y1 (State) **(A)** and Y2 (Trait) **(B)** scores at D0 and D28 across all four interventions. Box and Whisker plot (min and max) all points, mean and median (92 volunteers (*n* = 23 per group)). Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values. **Abbreviations:** OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

4.4.6.3 Positive and negative affect score – short form (PANAS-SF)

Significant Positive affect (PA) and negative affect (NA) scores are reported in Figure 4.5.3. Regarding PA scores, analysis revealed significant intervention [F (1,92) = 4.14, $P = 0.008$], day [F (1,92) = 64.00, $P \leq 0.001$], and intervention x day interactions [F (3,92) = 4.81, $P = 0.009$]. Pairwise comparisons revealed OF, OF/2'FL and 2'FL treatments resulted in significant increases in PA scores at D28 (all $P \leq 0.001$) (Figure 4.5.3A). Between subject comparisons also documented that OF outperformed both maltodextrin ($P \leq 0.001$) and OF/2'FL ($P = 0.030$) but not 2'FL (Appendix 4.5) with 2'FL also outperforming maltodextrin ($P = 0.013$) in the extent of increases in PA scores at D28 (Figure 4.5.3A).

In terms of NA scores significant day [F (1,92) = 7.39, $P \leq 0.001$], intervention x day [F (3,92) = 9.30, $P = 0.009$] but not intervention [F (3,92) = 1.59, $P = 0.198$] interactions were detected. Further analysis revealed OF, OF/2'FL and 2'FL treatments resulted in significant decreases in NA scores at D28 (all $P \leq 0.001$) (Figure 4.5.3B). While between subject comparisons documented that OF ($P = 0.002$) and OF/2'FL ($P = 0.004$) treatments outperformed maltodextrin ($P = 0.002$) but not 2'FL ($P = 1.00$) in the extent of reduction in NA scores. Finally, there was a trend towards differences in reduction of NA scores between 2'FL and maltodextrin detected at D28 ($P = 0.055$) (Figure 4.5.3B).

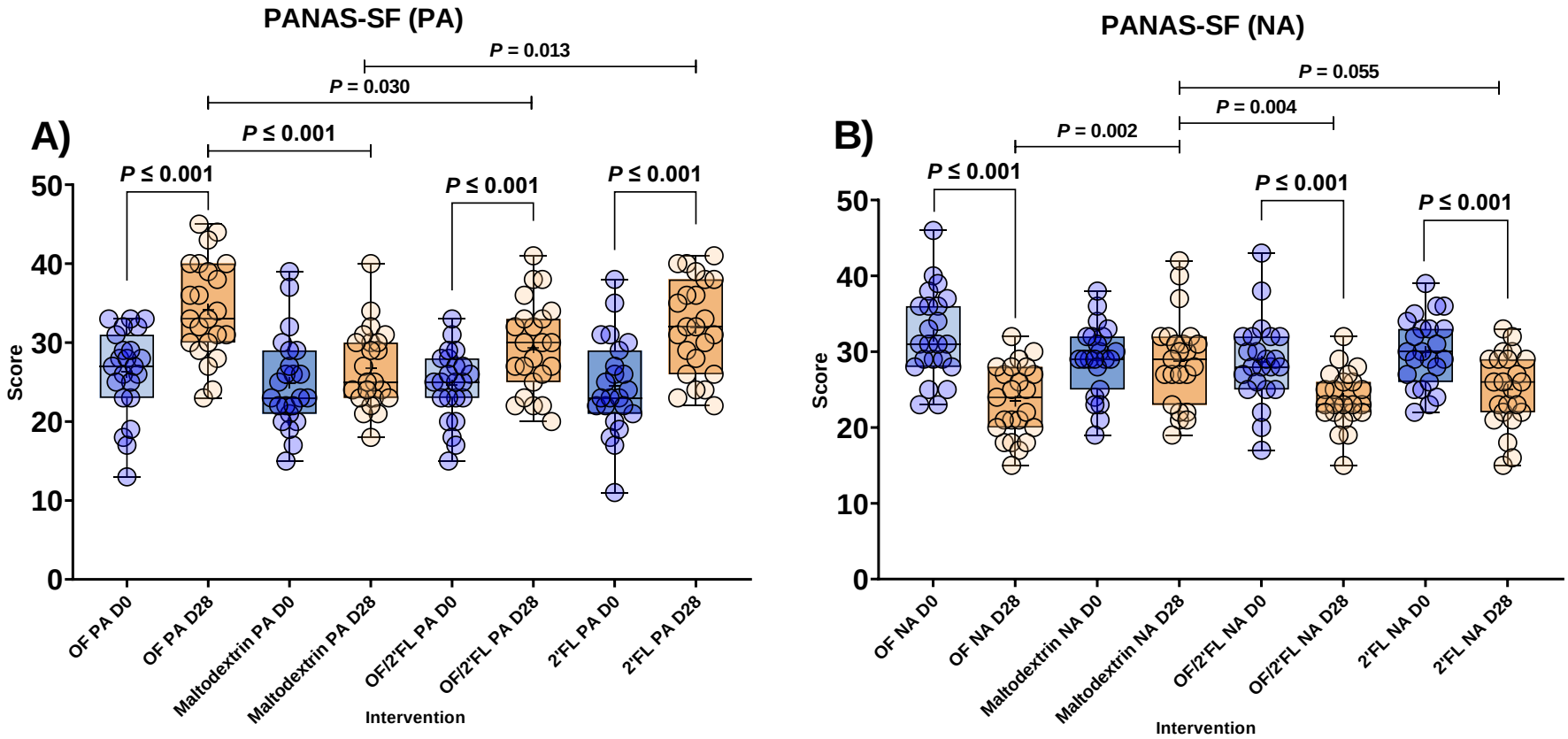

Figure 4.5.3. Positive affect (PA) **(A)** and Negative affects (NA) **(B)** scores at D0 and D28 across all four interventions. Box and whisker plots (min and max) all points, mean and median (92 volunteers (*n* = 23 per group)). Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values. **Abbreviations:** OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

4.4.6.4 Pittsburgh Sleep Quality Index (PSQI)

Statistical analysis of PSQI scores (Figure 4.5.4) revealed significant day [F (1,92) = 43.89, $P \leq 0.001$), but not either intervention [F (3,92) = 1.28, $P = 0.294$) or intervention x day [F (3,92) = 0.41, $P = 0.935$) interactions. These results were confirmed with follow-up analysis denoting that all interventions resulted in significant decreases (improvements in PSQI scores) at D28. Subsequent pairwise comparisons revealing no significant differences in PSQI scores between interventions at completion ($P = 0.239$) (Appendix 4.5).

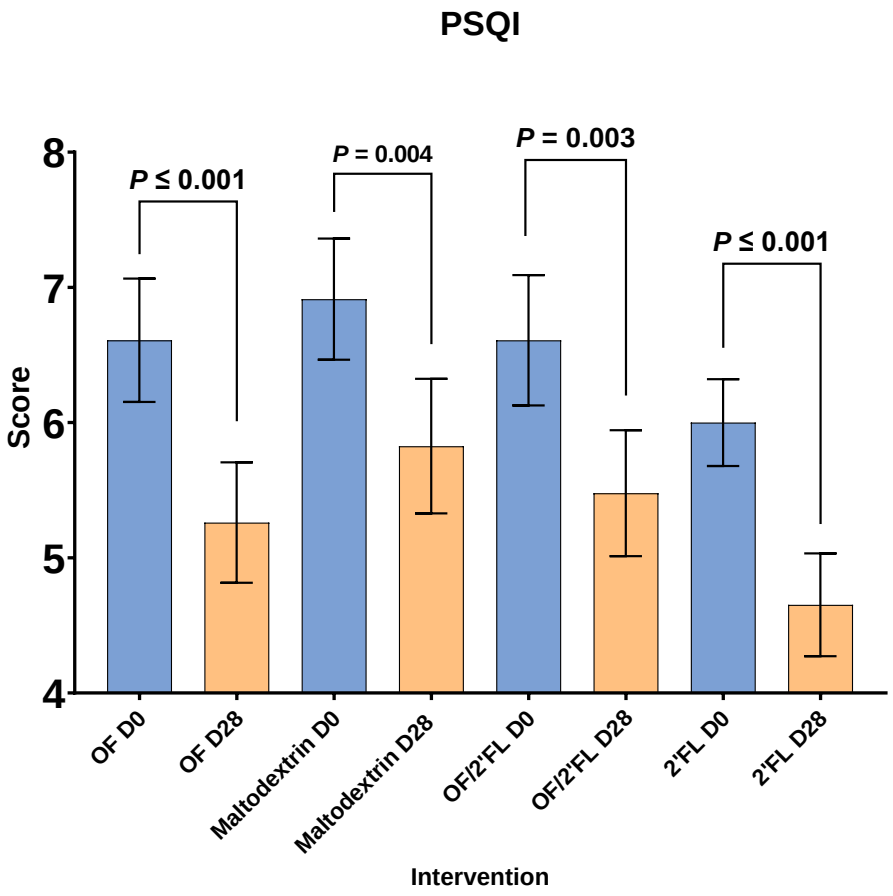

Figure 4.5.4. Pittsburgh Sleep Quality Index (PSQI) scores at D0 and D28 across all four interventions**.** Mean and standard error (SE) (92 volunteers ($n = 23$ per group)). Results that are statistically significant within and between subject (intervention) are displayed by specified *P* values. **Abbreviations**: OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

4.4.6.5 Cortisol Awakening Response (CAR)

Figure 4.5.5 reports the average total cortisol awakening response (CAR) in nmol/L at D0 and D28 across the four interventions. Analysis revealed significant day [F (1,92) = 41.13, $P \leq 0.001$], intervention [F (3,92) = 9.58, ($P \leq 0.001$)] and intervention x day [F (3,92) = 9.86, $P \leq 0.001$)] interactions. Post hoc analysis revealed a significant reduction in CAR in OF ($P \leq 0.001$), OF/2'FL ($P \leq 0.001$) and 2'FL ($P = 0.008$) interventions, but not maltodextrin ($P = 0.30$). Pairwise comparisons at D28 revealing that both OF and OF/2'FL combinations significantly outperformed maltodextrin in reductions of CAR values (both $P \leq 0.001$) (Appendix 4.5).

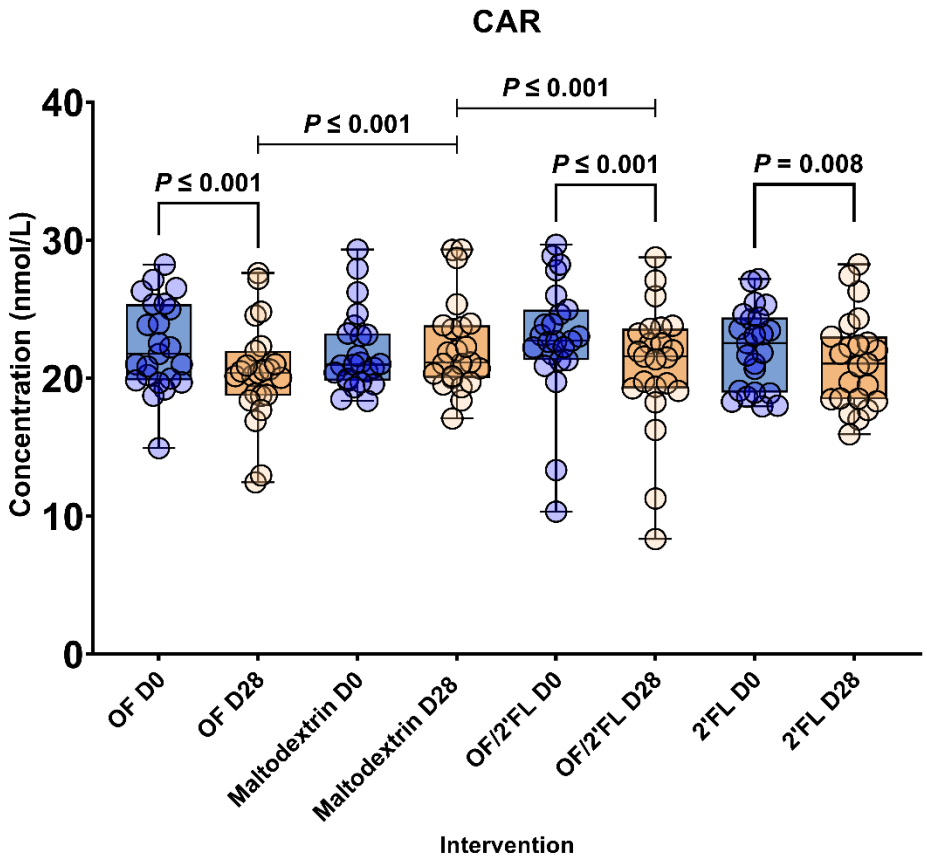

Figure 4.5.5. Total average cortisol awakening response (0, 15, 30, 45 and 60 min) at D0 and D28 across the four interventions. Box and whisker plots (min and max) all points, mean and median (92 volunteers (n = 23 per group)). Results that are statistically significant within and between subject (intervention) are displayed by specified P values. **Abbreviations**: OF = oligofructose; OF/2'FL = oligofructose/2'fucosyllactose; 2'FL = 2'fucosyllactose.

4.4.7 Correlation between bacteriology and mood state

In order to investigate the relationships between changes in gut microbiota taxa and mood state we constructed a correlation matrix using the fold change ((post-pre)/pre)) for the entire cohort of the QMP bacterial taxa and mood state data. The data were then analysed using a non-parametric Spearman's rank correlation corrected for using false discovery rate (FDR) (Figure 4.6.1 and 4.6.2 and Appendix 4.6).

Using a Spearman's rank correlation corrected for using FDR we observed several significant correlations between both taxa-taxa and taxa-mood state. Regarding taxa-taxa interactions *Bifidobacterium* was found to be positively correlated with *Eubacterium* (Spearman's $p = 0.57$, $P = 1.13 \times 10^{-7}$), *Coprococcus* (Spearman's $p = 0.56$, $P = 4.03 \times ^{10-9}$), *Anaerostipes* (Spearman's $p = 0.48$, $P = 9.43\ 10^{-7}$), *Blautia* (Spearman's $p = 0.48$, $P = 1.59 \times 10^{-6}$), *Dorea* (Spearman's $p = 0.54$, $P = 2.34 \times 10^{-8}$), *Collinsella* (Spearman's $p = 0.53$, $P = 4.70 \times 10^{-8}$), *Gemmiger* (Spearman's $p = 0.53$, $P = 6.00 \times 10^{-8}$), and to a lesser extent *Lachnospiraceae incertae sedis* (Spearman's $p = 0.26$, $P = 0.01$). While *Bacteroides* were found to be positively correlated with *Alistipes*, (Spearman's $p = 0.75$, $P = 1.41 \times 10^{-17}$), *Roseburia* (Spearman's $p = 0.32$, $P = 0.002$), *Faecalibacterium Prausnitzii* (Spearman's $p = 0.38$, $P = 0.0002$), and *Flavonifractor* (Spearman's $p = 0.71$, $P = 3.28 \times 10^{-15}$). Interestingly there were also significant correlations found between *Eubacterium* and *Coprococcus* (Spearman's $p = 0.98$, $P = 1.3 \times 10^{-64}$) (Figure 4.6.1 and Appendix 4.6).

Regarding taxa-mood state interactions, significant negative correlations were found between *Bifidobacterium* and BDI (Spearman's $p = -0.37$, $P = 2.91 \times 10^{-4}$), STAI Y1 (Spearman's $p = -0.33$ $P = 0.001$), STAI Y2 (Spearman's $p = -0.42$, $P = 3.12 \times 10^{-5}$), PANAS-SF NA (Spearman's $p = -0.32$, $P = 0.03$) as well as CAR (Spearman's $p = -0.22$, $P = 0.04$). There were also mild correlations

found between *Faecalibacterium prausnitzii,* (Spearman's *p* = -0.20, *P* = 0.02), *Eubacterium* (Spearman's *p* = -0.21, *P* = 0.041), *Anaerostipes* (Spearman's *p* = -0.21, *P* = 0.04), *Blautia* (Spearman's *p* = -0.22, *P* = 0.03), *Lachnospiraceae incertae sedis* (Spearman's *p* = -0.22, *P* = 0.04), *Dorea* (Spearman's *p* = -0.22, *P* = 0.04) and BDI. Additionally, several significant correlations were found between *Eubacterium* (Spearman's *p* = -0.25, *P* = 0.01), *Coprococcus* (Spearman's *p* = -0.23, *P* = 0.03), *Lactobacillus/Enterococcus* (Spearman's *p* = -0.24, *P* = 0.03), *Anaerostipes* (Spearman's *p* = -0.22, *P* = 0.02), *Akkermansia* (Spearman's *p* = -0.21, *P* = 0.046) *Blautia* (Spearman's *p* = -0.27, *P* = 0.007)*,* and *Dorea* (Spearman's *p* = -0.26, *P* = 0.01) with STAI Y1. As well as *Eubacterium* (Spearman's *p* = -0.24, *P* = 0.02), *Coprococcus* (Spearman's *p* = -0.21, *P* = 0.04), *Lactobacillus/Enterococcus* (Spearman's *p* = -0.22, *P* = 0.03), *Blautia* (Spearman's *p* = -0.27, *P* = 0.01)*, Akkermansia* (Spearman's *p* = -0.22, *P* = 0.03), and *Dorea* (Spearman's *p* = -0.25, *P* = 0.02) with STAI Y2 (Figure 4.6.2 and Appendix 4.6).

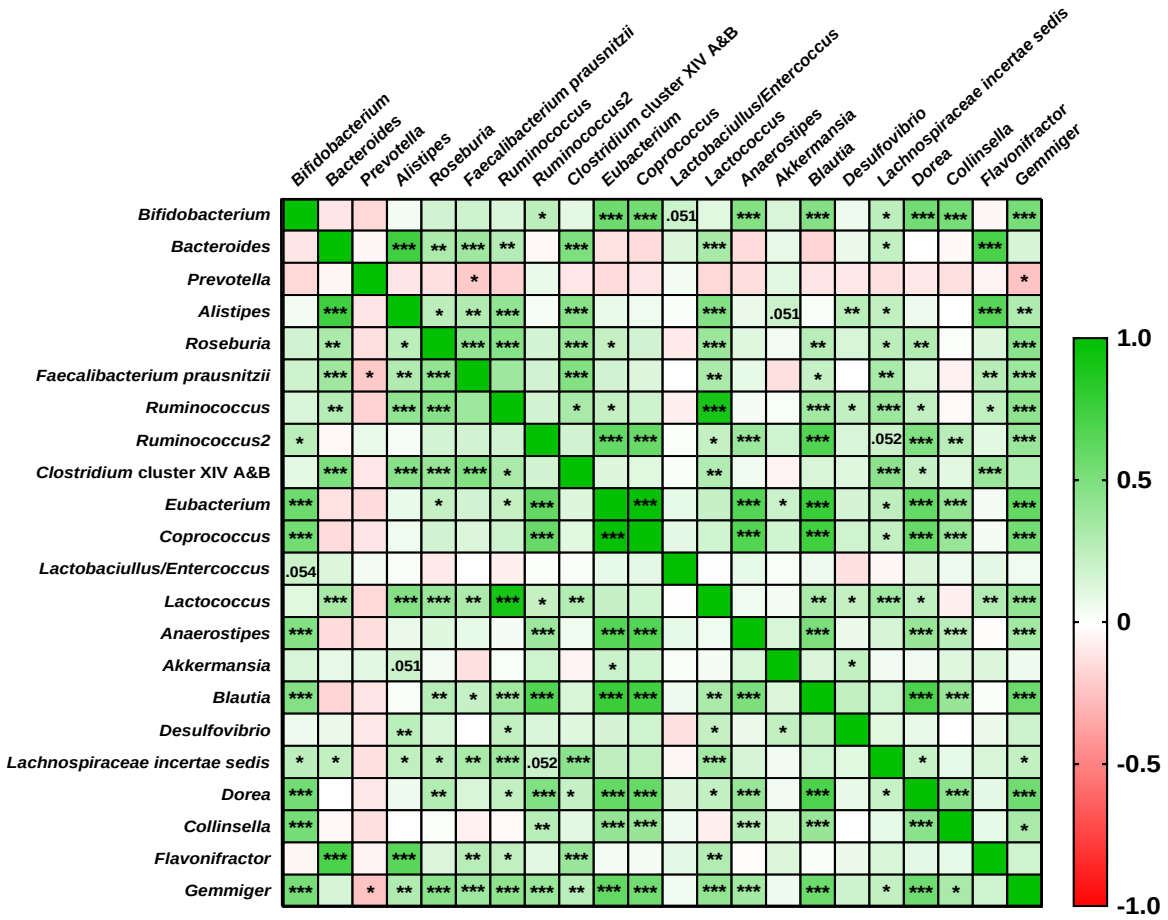

Figure 4.6.1 Bacterial taxa-taxa interactions from the entire cohort. Pairwise correlations between bacterial taxa fold change data were calculated using a Spearman's rank correlation (two sided adjusted for using FDR). Taxa-taxa correlations ranged from -1 to 1 (negative to positive). The depth of the colour represents the strength of the correlation. Adjusted *P* (*Q*) values represent significance at * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

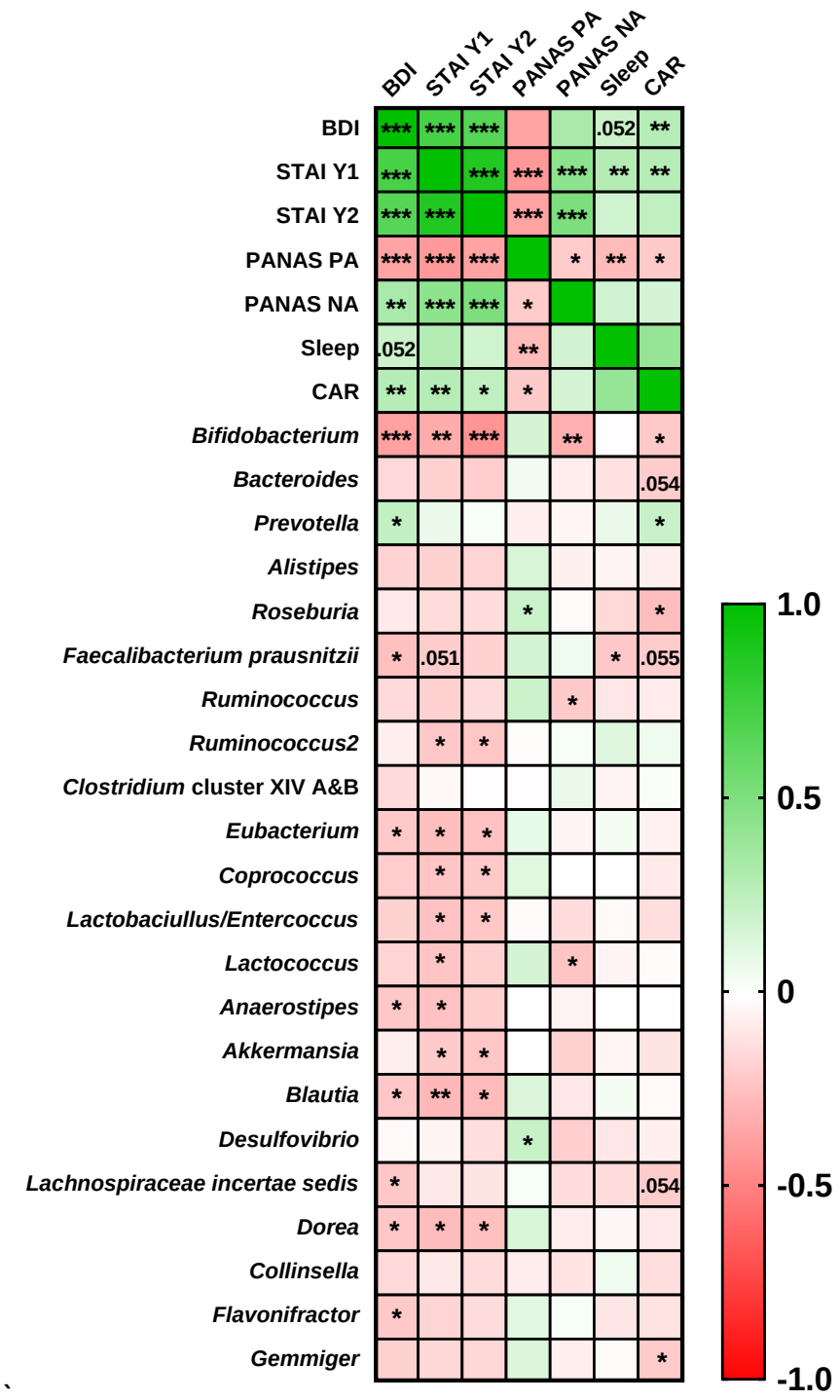

Figure 4.6.2 Bacterial taxa-mood state interactions from the entire cohort. Pairwise correlations between bacterial taxa and mood state fold change data were calculated using a Spearman's rank correlation (two sided adjusted for using FDR). Taxa-mood state correlations ranged from -1 to 1 (negative to positive). The depth of the colour represents the strength of the correlation. Adjusted *P* (*Q*) values represent significance at * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. **Abbreviations:** BDI = Beck's Depression Inventory; STAI Y1 and Y2 = State Trait Anxiety Inventory; PANAS = Positive and Negative Affect Scores – PA = Positive Affect; NA = Negative Affect; CAR = Cortisol Awakening Response; PSQI = Pittsburgh Sleep Quality Index

4.4.8 ^{1}H-NMR spectroscopic profiles

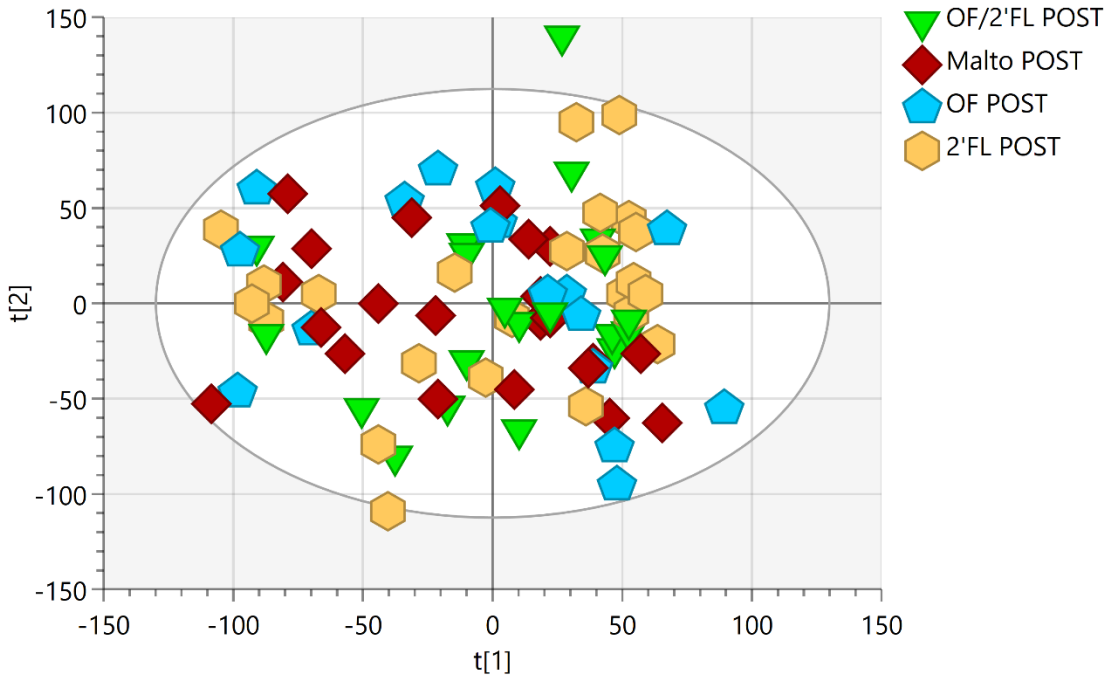

Figure 4.7. Urinary ^{1}H magnetic resonance (^{1}H-NMR) profiles for the entire cohort at completion segregated by intervention. Unsupervised principal components analysis (PCA) scores plot of pre and post intervention urine samples. $R^2Cum = 0.695$, $Q^2Cum = 0.431$.

Metabolic profiles of urine samples across the four interventions were analysed using unsupervised (PCA) methods (first seven components) showing the separation between pre and post intervention ($R^2Cum = 0.695$, $Q^2Cum = 0.431$) (Figure 4.7). Analysis revealed no direct clustering of interventions, yet, both oligofructose and combination of OF/2'FL showed trends towards clustering on completion of the intervention (OF $R^2Cum = 0.614$, $Q^2Cum = 0.279$) and (OF/2'FL $R^2Cum = 0.623$, $Q^2Cum = 0.217$). However, upon performing OPLS-DA analysis the model was unable to differentiate based on assigned pre and post classifications (OF $R^2Y = 0.778$, $Q^2Cum = -0.306$) and (OF/2'FL $R^2Y = 0.676$, $Q^2Cum = -0.312$). Subsequently, no further analysis was carried out.

4.5 Discussion

In this current trial we explored the effects of the prebiotic OF and prebiotic candidate 2'FL alone and in combination on microbial composition, mood state (BDI, STAI Y1, STAI Y2, PANAS-SF), sleep quality and CAR in a healthy adults with mild-to-moderate levels of anxiety and depression. This is the first study to demonstrate that intake of the prebiotic OF and the prebiotic candidate 2'FL alone and in combination can result not only in noticeable differences in microbial modulation, but more importantly substantial improvements in BDI, STAI Y1, STAI Y2, PANAS-SF scores and CAR in adults with mild-to-moderate levels of anxiety and depression.

Large differences in microbial responses were seen between the four different interventions with OF recording largest increases in microbial load in a number of different bacterial groups including *Bifidobacterium*, *Bacteroides*, and *Faecalibacterium prausnitzii*. These results coincide with several previous human intervention studies (Ramirez-Farias *et al.*, 2009; Healey *et al.*, 2018; Costabile *et al.*, 2010) further confirming the evidence of the high level of selectivity of OF towards *Bifidobacterium* and the wider microbiota (Jackson *et al.*, 2022a). Yet, while combining OF with 2'FL did not seemingly induce any complementary effects compared to OF supplementation, OF did seem to offset the lack of changes seen upon consumption of 2'FL in several bacteria including *Bifidobacterium*, *Bacteroides*, *Roseburia* and *Faecalibacterium prausnitzii*.

It is well documented that large interindividual differences in responses exist in terms of bifidobacterial response to HMOs supplementation as the majority of adults do not possess the necessary bifidobacteria required to enzymatically degrade and utilise HMOs (Jackson, Wijeyesekera and Rastall, 2022). Within our 2'FL cohort several volunteers documented larger

increases in *Roseburia*, *Faecalibacterium prausnitzii* and more interestingly *Blautia* in comparison to *Bifidobacterium.* This further suggests that a strong responder/non-responder relationship exists between an individual's gut microbiota and microbial responses to HMOs supplementation.

Concerning increases in *Blautia, Rosburia* and *Faecalibacterium prausnitzii* one could speculate that these might have occurred due to utilisation of 2'FL by *Bifidobacterium*. It was recently reported that increases in *Blautia* can occur in the presence of fucosidase-producing bacteria via utilisation of the extracellularly liberated fucose (Horigome *et al.*, 2022). Increases in both *Roseburia* and *Faecalibacterium prausnitzii* often occur in the presence of bifidobacteria as a result of cross-feeding acetate and lactate (Louis and Flint, 2017; Cheng *et al.*, 2020). Furthermore, increases in *Blautia*, *Roseburia* and *Faecalibacterium prausnitzii* may have occurred as result of 2'FL degradation by *Akkermansia muciniphilia*. Within the gut *Akkermansia muciniphilia* is considered a keystone species for its role in mucin degradation (Derrien *et al.*, 2004). As HMOs share large structural similarities with mucin one could hypothesise that increases in *Blautia*, *Roseburia* and *Faecalibacterium prausnitzii* may have also occurred as a result of proliferation on HMO degradation products in the presence of *Akkermansia muciniphilia*. It being recently documented in pure and co-cultured experiments that *Roseburia* spp. showed little-to-no signs of growth on HMOs, except for in the presence of *Akkermansia muciniphilia* (Pichler *et al*., 2020).

As previously stated, the majority of studies focusing on the effects of prebiotics on cognitive function and mood state fail to analyse changes in the gut microbiota (Schmidt *et al.*, 2015; Smith, Sutherland and Hewlett, 2015; Kazemi *et al.*, 2019). While we acknowledge it is hard to establish the exact mechanisms by which the gut microbiota influence mood state, we observed several significant correlations between bacterial taxa namely *Bifidobacterium*,

Roseburia, *Anaerostipes*, *Blautia* and *Faecalibacterium prausnitzii* and improvements in anxiety, depression, positive and negative affect scores and CAR values (Figure 4.6.2). On this basis one could hypothesise that targeted manipulation of the gut microbiota could have a profound effect on mood state via regulations of neurological, immunological, or endocrine pathways (Silva, Bernardi and Frozza, 2020; Huang and Wu, 2021).

Several strains of bifidobacteria, *Lactobacillus* and *Blautia* are prominent GABA producers (Strandwitz *et al.*, 2019; Duranti *et al.*, 2020) and genera were significantly increased upon consumption of either OF, 2'FL or combination of OF/2'FL (Figure 4.2, 4.3.1 and 4.3.2 and Appendix 4.3). Additionally, higher abundances of *Lachnospiraceae* and *Ruminococcus* have been associated with lower levels of major depressive disorder (Jiang *et al.*, 2015) and these taxa were significantly increased upon consumption of OF (*Lachnospiraceae* ($P = 0.004$) and *Ruminococcus* ($P \leq 0.001$) (Appendix 4.3). Furthermore, higher levels of *Faecalibacterium prausnitzii* have been associated with improved sleep quality and lower levels of generalised anxiety (Evans *et al.*, 2017) and these were also significantly increased upon consumption of OF, OF/2'FL and 2'FL in our cohort.

We did not measure changes in SCFA as faecal samples do not give an accurate measure of metabolite concentrations within the colon, and we were unable to collect blood samples as a result of Covid-19 restrictions. The increases seen in several acetate, propionate and butyrate producing bacteria (*Bifidobacterium*, *Roseburia*, *Lachnospiraceae* and *Faecalibacterium prausnitzii*) on OF, OF/2'FL and 2'FL likely led to increases in SCFA production. Increased SCFA production may have beneficial effects on mood state as SCFA play vital roles in the regulation of neurotransmitter production, reduction in inflammatory responses via modulation of anti- and proinflammatory cytokines (IL-6, TNF-α and IL-1β) and can act as endocrine signalling molecules (Silva, Bernardi and Frozza, 2020; Soliman *et al.*, 2012). Additionally, propionate can

protect against lipopolysaccharide-mediated blood brain barrier disruption (Hoyles *et al.*, 2018), while butyrate has been associated with decreased histone acetylation (Moris and Vega, 2003). Furthermore, lower levels of SCFA have been detected in depressed individuals and non-human primate models, while higher SCFA concentrations improved BDI scores (Skonieczna-Zydecka *et al.*, 2018; Deng *et al.*, 2019). The results of this study add to the evidence that the composition of the gut microbiota and resulting metabolites impacts mood state and that targeted manipulation with prebiotics may be a viable method of reducing depression and anxiety.

CAR values were also significantly reduced across OF, 2'FL and OF/2'FL interventions with OF recording largest decreases. CAR is often thought to be a reflection of the hypothalamic-pituitary-adrenal axis (HPA) and often correlates with levels of stress, anxiety and sleep (Dedovic and Ngiam, 2015). Our results are in accordance with those documented in probiotic interventions in healthy adults (Matsuura *et al.*, 2022) and anxious students (Andersson *et al.*, 2016), but not those documented by (Schmidt *et al.*, 2015), who recorded that β-GOS reduced CAR response but not OF. Discrepancies in findings are likely due to differences in sample sizes, length of intervention and supplement dosages. As CAR, and HPA axis activity are influential markers of health status (Stephens and Wand, 2012) our findings suggest that supplementation with OF and/or 2'FL may provide novel approaches in reducing CAR and therefore influences on overall mood state.

In order to assess changes in stool frequency and stool consistency the validated Bristol Stool Form Scale was used. In our cohort significant increases in stool consistency were detected in OF and OF/2'FL interventions. Both these results are significantly different to the 2'FL intervention (both $P = 0.004$). The softening effect of OF on stool consistency has previously been documented in both healthy and constipated adults (Micka *et al.*, 2017; Watson *et al.*,

2019). Furthermore, in our cohort significant increases in stool frequency were only documented upon consumption of OF/2'FL (P = 0.001). This result is also significantly different to both maltodextrin (P = 0.005) and 2'FL (P = 0.012) interventions. These results are unsurprising given the high daily stool frequency at baseline seen in our cohort as changes in bowel frequency upon inulin consumption are often seen in individuals who are constipated or possess lower stool frequency (François *et al.*, 2014; Isakov *et al.*, 2013; Micka *et al.*, 2017; Vandeputte *et al.*, 2017a). The mechanisms by which improvements in stool consistency and frequency occurred are probably a result of increases in bacterial mass, combined with the effects of SCFA on gut motor hormones and the osmotic properties of SCFA drawing water into digestive tract, softening stools thereby making them easier to pass (Micka *et al.*, 2017).

Similar changes in gastrointestinal sensations including flatulence, intestinal bloating, abdominal pressure, pain and feeling of fullness were detected across four interventions. Significant increases in flatulence and bloating on consumption of OF and have been documented previously (Kleessen *et al.*, 2007; Ramnani *et al.*, 2010; Marteau *et al.*, 2011). These end values were not, however, significantly different to either placebo or OF/2'FL or 2'FL interventions (all $P \geq 0.05$), and the slight increases in flatulence or intestinal bloating did not result in any reported discomfort or discontinuation of the study by any of the volunteers and were rated as mild at most.

Finally, during the trial volunteers were asked not to alter either their dietary intake or lifestyle. Analysis of 3-day food diaries revealed dietary fibre intakes were estimated at 19.3 g/day. These results are in line with the current UK average of 14.9-18 g/day and are significantly lower than the current UK recommendation of 30 g/day (Scientific Advisory Committee on Nutrition, 2015; Gressier and Frost, 2022). As dietary analysis revealed no significant changes in dietary fibre intakes between the start and end of the intervention

phase dietary fibre intakes within both the OF and OF/2'FL group likely increased by 8 g/day from supplementation. This suggests that OF can contribute towards beneficial increases in dietary fibre without significant increases in adverse gastrointestinal reactions occurring.

4.6 Conclusion

In conclusion, this is the first study to demonstrate that consumption of the prebiotic OF and prebiotic candidate 2'FL alone and in combination can result in substantial improvements in BDI, STAI Y1 and Y2, PANAS-SF scores and CAR. We can also conclude that both OF and combinations of OF/2'FL induce changes in microbial composition, especially increasing numbers of *Bifidobacterium*, *Roseburia, Faecalibacterium* and *Prevotella*. The changes recorded in bacterial taxa correlating with those seen in several mood state parameters. In contrast, 2'FL alone was unable to match OF in terms of changes in microbial composition due to the large heterogeneity seen between individuals. Future studies are needed to identify differences between suspected responders and non-responders to 2'FL supplementation.

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A follow-up to the EFFICAD trial – 2'fuscosyllactose – A deeper dive into responder/non-responder status.

Abstract

In a follow-up to the heterogeneity seen in response to 2'fucosyllactose supplementation in the EFFICAD trial (**ClinicalTrials.gov ID:** NCT05212545) we aimed to identify microbial differences that separated non-responders and responders. Non-responders and responders were defined based on differential changes seen in bifidobacterial response. We used a general linear model at both genus and species level to identify potential key differences in microbial response to 2'fucosyllactose supplementation. Genus and species level analysis revealed no differences between non-responders and responders at baseline. Yet, upon completion several significant differences between responders and non-responders were documented. At genus levels significant differences between responders and non-responders were detected with responders documenting significant increases in *Bifidobacterium* (P = 0.008), *Roseburia* (P = 0.03), *Anaerostipes* (P = 0.03), *Blautia* (P = 0.02), *Clostridium* cluster 14 A&B (P = 0.01) and *Gemmiger* (P = 0.01), *Eubacterium* (P = 0.05) and *Coprococcus* (P = 0.05). While, at species level significant differences between responders and non-responders were detected in *B. Longum* subsp. *infantis* (P = 0.03), *B. animalis* subsp. *lactis* (P = 0.009), *Roseburia faecis* (P = 0.03), *Blautia wexlerae* (P = 0.03), *Coprococcus eutactus* (P = 0.03) and *Eubacterium hadrum* (P = 0.04). Accordingly, mood state parameters, but not sleep scores were only significantly improved in the responder group. However, the significant

improvements in mood state parameters detected in the responder group did not significantly differentiate from non-responders (all $P \geq 0.05$). Future work should focus on larger samples sizes and sequencing down to strain level to tease out key microbial differences separating non-responders from responders and association with changes in mood state.

5.1 Introduction

In recent years there has been much focus on a group of unconjugated glycans referred to a human milk oligosaccharides (HMOs) and their effects on health outcomes, not only in infants but in healthy and diseased adults as well (Bode, 2012). In nature HMOs make up the 3rd largest component of breastmilk after lactose and lipids, while commercially they are synthesized by genetically modified *E. coli* and yeasts. To date, over 200 different HMOs have been categorized (Barile and Rastall, 2013). Currently, however, only a small selection of these HMOs are available commercially including 2'fucosyllactose (2'FL), lacto-*N*-neotetraose (LNnT), 6-sialyllactose (6'SL), Lacto-N-tetraose (LNnT), 3-fuscosyllactose (3'FL). The most commonly used HMOs in both commercial and research settings are 2'FL and LNnT.

The efficacy of 2'FL at stimulating changes in the gut microbiota in infants is better understood than its effects in adults. Even so, effects in infants are complex due to several competing and interacting factors including birthing methods, breastfed vs bottle fed and secretor status amongst others (Jackson, Wijeyesekera and Rastall, 2022). Despite this, HMOs show high levels of selectivity towards bifidobacteria, *Lactobacillus* and *Bacteroides.* However, as our recent review revealed (Jackson, Wijeyesekera and Rastall, 2022) there can be marked variability even within bacteria of the same genus. Several species and strains including *B. longum* subsp. *infantis* and *Bacteroides fragilis are particularly able to readily utilise HMOs*. This complexity increases in adults due to diversification of dietary intake and loss of exposure

to routine HMOs consumption. Results of recent studies in both healthy and diseased adults have shown high levels of heterogeneity in response to HMOs supplementation (Ryan *et al.*, 2021; Iribarren *et al.*, 2020). This suggests that a strong responder (RES) and non responder (NR) status exists.

By definition RES are classed as individuals who show a favorable response to an intervention. While, in contrast, NR are classed as those individuals who show no response. In truth, responder/non-responder status can be even further separated by the degree of responsiveness and the parameter used to judge a response to a given intervention (Reid *et al.*, 2010). This is evidenced by the outcomes of the EFFICAD trial with results reporting remarkable heterogeneity in response to 2'FL supplementation, particularly in bifidobacterial response. As a result, in this follow-up analysis we segregated the 2'FL cohort into RES/NR based on changes in bifidobacterial response. The aim was to see if we could identify key differences in microbial compositions that could help give us insight into what drives NR/RES status. An additional aim was to see if differences existed between NR and RES in terms of mood state parameters and cortisol awakening response (CAR).

5.2 Materials and methods

Materials and methods are as per Chapter 4

5.2.1 Defining responders/non-responder status

RES and NR status was based on changes documented in bifidobacterial response using quantitative microbiome profiling data. Volunteers were ranked in order of change seen in bifidobacterial response from low to high. Volunteers were then segregated into two groups.

Individuals documenting no change or decreases in *Bifidobacterium* response were classed as NR. Those individuals documenting increases in *Bifidobacterium* response being classed as RES.

5.2.2 Statistical analysis

Statistical Package for Social Science version 27 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Differences in participant metrics between NR and RES was assessed using independent t-tests. Mood state parameters (BDI, STAI and PANAS-DF), PSQI and CAR and gastrointestinal scores and dietary data were analysed by linear marginal model (LMMs) using an unstructured covariance matrix to model repeat measures. Separate LMMs were performed for each dependant variable in PANAS-SF (PA, NA). Changes in bacterial taxa were analysed using corrected paired T-tests (entire cohort) and general linear model (GLM) upon segregation into non-responders and responders to assess repeated measures. A baseline covariate was employed to determine differences between NR and RES at Day 28. Post-hoc comparisons were also performed in order to determine any significant differences between RES and NR in bacterial genus and species, as well as mood state parameters. All pairwise comparisons were corrected for type 1 errors using Bonferroni adjustment within each LMM and GLM. All tests were two tailed and P values ≤ 0.05 were considered statistically significant.

5.3 Results

5.3.1 Participant data

Table 5.1 reports the participant data (age, height, weight, and BMI) mean and range segregated by overall, NR and RES status. No significant differences were detected in any participant metric between NR and RES.

Intervention	Overall (*n* = 23)	NR (*n* = 11)	RES (*n* = 12)	*P* value NR vs RES
Age (y)	28.91 (19-46)	29.27 (19-46)	28.58 (19-39)	0.84
Weight (kg)	64.53 (46.00-93.50)	65.12 (50.00-93.50)	64.00 (46.00-85.00)	0.84
Height (cm)	166.30 (155.00-181.00)	166.50 (157.00-181.00)	166.17 (155.00-180.00)	0.91
BMI (kg/m^2)	23.17 (18.59-29.73)	23.37 (18.59-29.73)	23.07 (19.15-29.21)	0.88

Table 5.1. Participant metrics – age, weight, height, and BMI mean and min and max. Entire cohort (*n* = 23) and segregated by RES (*n* = 12) and NR (*n* = 11) status. *P* values are the results of a independent t-tests comparing differences in categorical data between RES and NR. **Abbreviations:** NR = non responder; RES = responder

5.3.2 Dietary intake

Nutrient data at Baseline (1st week, days 0, 1 and 2) and final week (days 26, 27 and 28) of the intervention are presented in Table 5.2. No significant differences were detected in total energy, protein, carbohydrates, total sugar, total fat, saturated fat or dietary fibre at D28 between RES and NR (all $P \geq 0.05$).

	Overall (*n* =23)			NR (*n* =11)		RES (*n* =12)		*P* Value NR vs RES (b)
	Baseline (D0-D2)	Final Week (D26-D28)	*P* Value Overall (a)	Baseline (D0-D2)	Final Week (D26-D28)	Baseline (D0-D2)	Final Week (D26-D28)	
Total energy (kcals)	1831 (144.70)	1786 (104.30)	0.575	2063 (273.77)	1881 (210.10)	1618 (193.72)	1700 (59.08)	0.37
Protein (g)	75.57 (7.21)	71.91 (6.15)	0.45	72.11 (12.95)	69.72 (12.03)	78.75 (7.59)	73.91 (4.83)	0.99
Fat (g)	64.35 (6.11)	67.54 (5.39)	0.52	73.78 (55.70)	73.06 (10.56)	55.71 (11.85)	62.48 (3.69)	0.97
Saturated Fat (g)	22.33 (2.52)	23.32 (2.60)	0.62	24.97 (4.91)	25.15 (4.95)	19.91 (1.79)	21.63 (2.21)	0.96
CHO (g)	218.30 (22.78)	217.70 (14.25)	0.961	251.60 (43.47)	225.70 (28.12)	187.87 (15.41)	210.46 (1046)	0.13
Total sugar (g)	69.36 (9.52)	65.20 (6.07)	0.504	84.28 (17.65)	68.63 (11.86)	55.69 (7.22)	62.06 (4.71)	0.28
Fibre (g)	18.95 (1.62)	19.2 (1.41)	0.801	20.90 (2.82)	20.58 (2.49)	17.15 (1.68)	17.93 (1.48)	0.97

Table 5.2. Energy and nutrient intake at baseline (Day 0, 1, 2) and at completion (Day 26, 27 and 28) of the entire 2'FL cohort (*n* = 23) and segregated by RES (*n* = 12) and NR (*n* = 11) status. Mean and standard error (SE). **(a)** *P* values are as a result of paired t tests for baseline vs completion comparisons. **(b)** *P* values are as a result of using an average of D0-D2 data as a baseline covariate for between group (NR vs RES) comparisons at completion employing a linear model. **Abbreviations:** NR = non-responders; RES = responders; CHO = Total carbohydrates.

5.3.3 Bacterial enumeration by FISH

Figure 5.1.1 and 5.1.2 reports changes in total bacterial (Eub I-II-III) counts observed in the overall cohort (Figure 5.1.1) and segregated by RES/NR status (Figure 5.1.2) at D0 and D28 of the intervention. Analysis of the entire 2'FL cohort revealed a significant 0.19 Log_{10} Cells/g wet faeces increase total bacterial (Eub I-II-III) counts (P = 0.001) (Figure 5.1.1). Upon separation into RES/NR status, analysis revealed significant day [F (1,22) = 18,77 , P = ≤ 0.001] and day x responder status [F (1,23) = 12.63, P = 0.002] interactions. This was confirmed with post hoc analysis revealing significant increases in total bacteria (Eub I-II-III) counts in the RES group only (P ≤ 0.001) going from 9.97 ± 0.08 (SE) to 10.30 ± 0.08 (SE) (0.33 mean difference) Log_{10} cells/g wet faeces. The increases detected in in total bacteria (Eub I-II-III) counts in RES being statically significant from NR at completion (P = 0.046) (Figure 5.1.2 and Appendix 5.1).

Regarding Bif164 (*Bifidobacterium* spp.) counts, analysis revealed significant increases across the entire 2'FL cohort going from 8.64 ± 0.14 (SE) to 8.94 ± 0.12 (SE) (0.30 mean difference) (P = 0.001) (Figure 5.2.1). Upon separation into RES/NR status analysis detected significant day [F (1,23) = 22.32, P = ≤ 0.001] and day x responder status [F (1,22) = 56.95, P ≤ 0.001] interactions. Subsequent repeated measures and pairwise comparisons revealing significant increases in Bif164 counts in the RES group only (P ≤ 0.001) along with a trend towards a decline in Bif164 counts in the NR group (P = 0.064). No significant differences were also detected between NR and RES at baseline (P = 0.265), but there was a trend towards significance detected between NR and RES at completion (P = 0.067) (Figure 5.2.2 and Appendix 1).

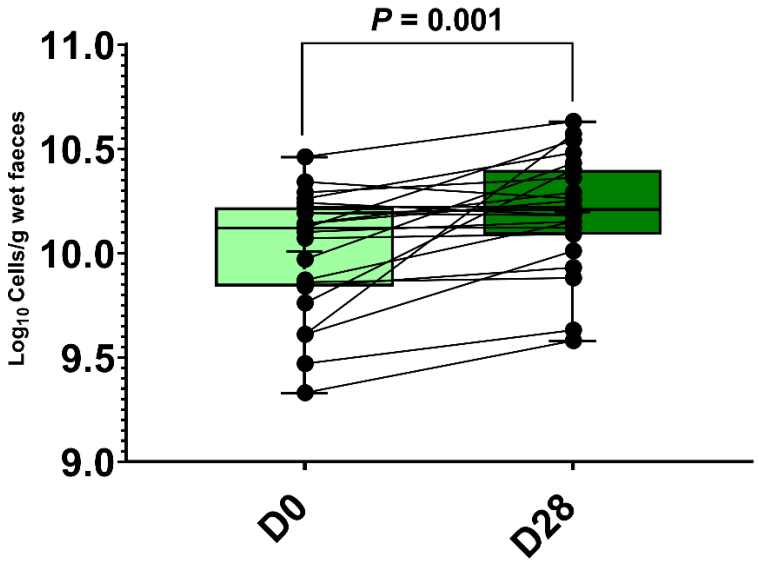

Figure 5.1.1 Bacterial groups measured by FISH-FLOW (Log_{10} cells/g wet faeces) using probes: total bacteria (Eub338 I-II-III) total cohort (*n* = 23). Box and Whisker plot (min and max) all points, mean and median. Results that are statistically significant within group (pre vs post) are displayed by specified *P* values

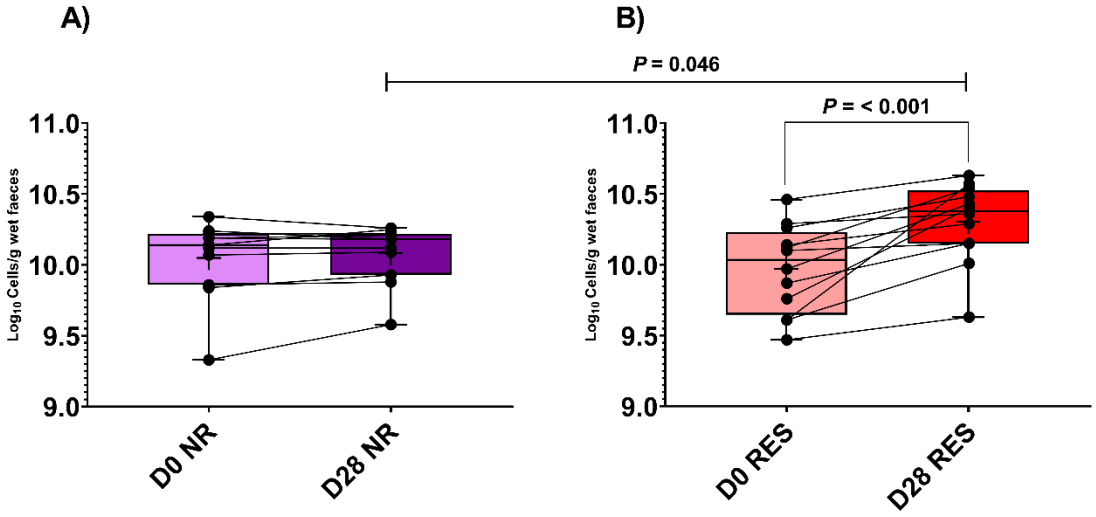

Figure 5.1.2 Bacterial groups measured by FISH-FLOW (Log_{10} cells/g wet faeces) using probes: total bacteria (Eub338 I-II-III) segregated RES (*n* = 12) and NR (*n* = 11) status. Box and Whisker plot (min and max) all points, mean and median. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values.
Abbreviations: NR = non-responder, RES = responder

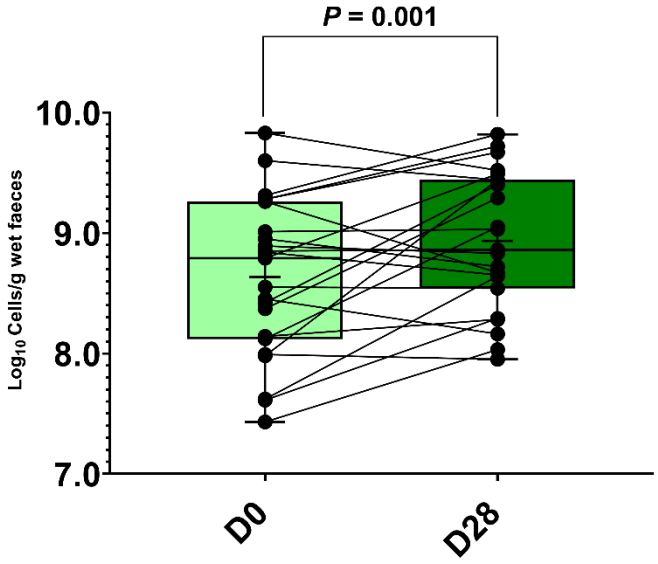

Figure 5.2.1. Bacterial groups measured by FISH-FLOW (Log_{10} cells/g wet faeces) using probes: Bif164 (*Bifidobacterium* spp.) acrototal cohort (*n* = 23). Box and Whisker plot (min and max) all points, mean and median. Results that are statistically significant within group (pre vs post) are displayed by specified *P* values

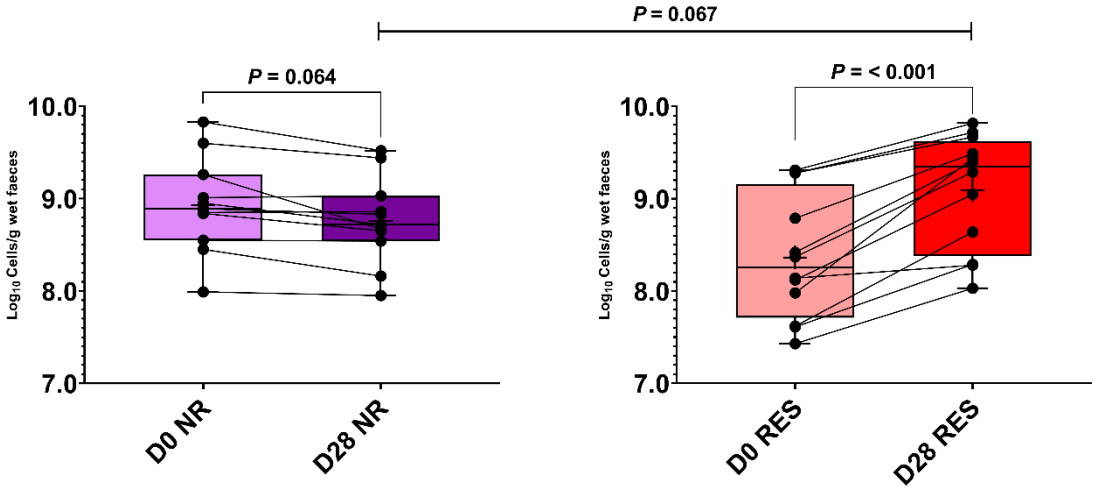

Figure 5.2.2. Bacterial groups measured by FISH-FLOW (Log_{10} cells/g wet faeces) using probes: Bif164 (*Bifidobacterium* spp.) segregated RES (*n* = 12) and NR (*n* = 11) status. Box and Whisker plot (min and max) all points, mean and median. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values.
Abbreviations: NR = non-responder, RES = responder

5.3.4 Microbiota Profiling Analysis

5.3.4.1 Quantitative Microbiome Profiling (QMP)

Figure 5.3.1 reports the QMP data at genus level across the entire 2'FL cohort and segregated by NR/RES status. Figures 5.3.2, 5.3.3 and 5.3.4 reports the most significant changes documented at genus level across the entire 2'FL cohort and segregated by RES/NR status.

Firstly, concerning the entire 2'FL cohort largest changes in microbial composition were recorded in several genera including *Bifidobacterium*, *Blautia* and *Roseburia*. Regarding bifidobacteria, changes in counts varied vastly amongst volunteers with an average increase from $9.41 \times 10^8 \pm 2.01 \times 10^8$ (SE) to $1.72 \times 10^9 \pm 4.76 \times 10^8$ (SE) (7.83×10^8 mean difference) cells per gram being recorded ($P \leq 0.001$) (Figure 5.3.1 and 5.3.2 and Appendix 2). Upon separation into NR/RES status, significant day [$F(1,22) = 6.214$, $P = 0.03$], day x responder status [$F(1,22) = 15.173$, $P = 0.002$] interactions were documented. Increases in bifidobacteria were only detected in the RES group ($P \leq 0.001$), with numbers increasing from $7.03 \times 10^8 \pm 1.92 \times 10^8$ (SE) to $2.63 \times 10^9 \pm 9.68 \times 10^8$ (SE) (2.12×10^9 mean difference) cells per gram. Pairwise comparisons revealed that bifidobacterial counts in the RES group at D28 were statistically significant from the NR group ($P = 0.008$) (Figures 5.3.1, 5.3.2 and 5.3.3).

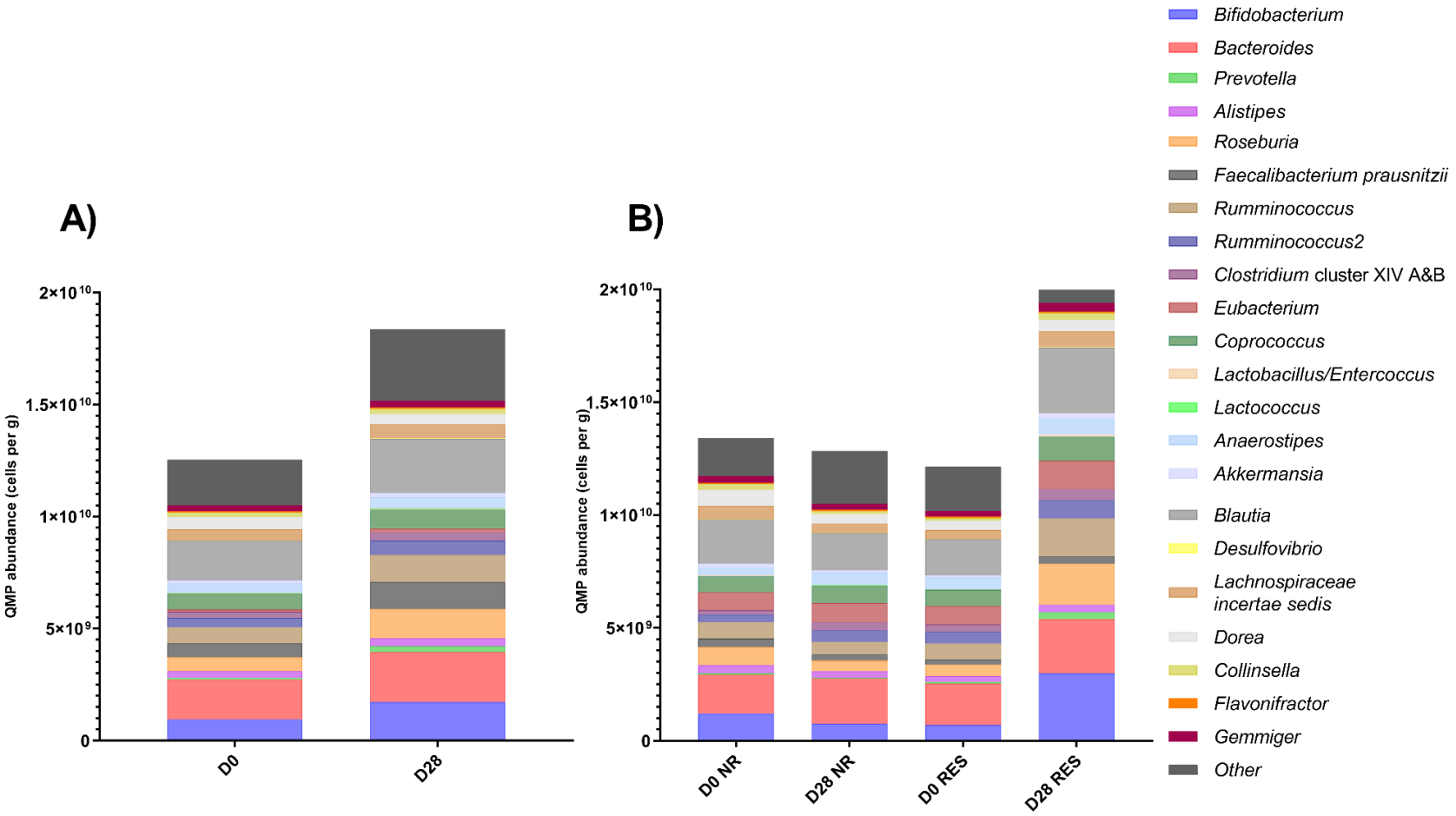

Figure 5.3.1. Quantitative microbiome profiling (QMP) of overall 16S rRNA sequencing data recorded across the entire 2'FL cohort (*n* = 23) and segregated by RES (*n* = 12) and NR status (*n* = 11) at D0 and D28. Numbers are expressed as cells per gram of faeces

Similar to bifidobacteria, large increases in numbers of *Blautia* were documented across the entire 2'FL cohort. However, changes in *Blautia* were highly heterogeneous amongst individuals with numbers increasing from 1.78 x 10^9 ± 2.87 x 10^8 (SE) to 2.42 x 10^9 ± 4.72 x 10^8 (SE) (1.85 x 10^8 mean difference) cells per gram (Figure 5.3.2). This was reflected in the level of significance (*P* = 0.08) (Figure 5.3.2). Upon separation into RES/NR status even greater levels of significance were detected with numbers of *Blautia* in the RES group increasing from 1.61 x 10^9 ± 4.91 x 10^8 (SE) to 2.93 10^9 ± 7.81 x 10^8 (SE) (1.31 x 10^9 mean difference) cells per gram (*P* = 0.01). In contrast, no significant differences in *Blautia* counts were detected in the NR group (*P* = 0.859) (Figure 5.3.3). Number of *Blautia in* RES group were statistically significant from NR at D28 (*P* = 0.02).

Significant increases in numbers of *Roseburia* were also detected across the entire 2'FL cohort (*P* ≤ 0.001) going from 6.23 x 10^8 ± 1.25 x 10^8 (SE) to 1.31 x 10^9 ± 2.92 x 10^8 (SE) (6.79 x 10^8 mean difference) cells per gram (Figure 5.3.2). Upon separation of *Roseburia* into NR vs RES GLM analysis revealed day x responder status [F (1,22) = 5.282,*P* = 0.03 and Day [F (1,22) = 6.591, *P* = 0.02] interactions. Increases detected in *Roseburia*, were driven by changes seen in the RES group only (*P* ≤ 0.001) (Figure 5.3.3 and Appendix 2). The end values documented in *Roseburia* in the RES group were also statistically different from NR (*P* = 0.03).

Several other differences between NR and RES at D28 were detected including *Clostridium* cluster 14A&B (*P* = 0.01) *Gemminger* (*P* = 0.01) and *Anaerostipes* (*P* = 0.038). There were also a number of trends towards differences in bacterial genera between NR and RES identified. These included increases and decreases in RES and NR in *Eubacterium* (*P* = 0.05) and *Coprococcus* (*P* = 0.05) (Figures 5.3.3 and 5.3.4). Finally, there were a number of significant within group bacterial changes including increases in *Ruminococcus* (*P* = 0.01), (*P* = 0.01), *paruanitzii* (*P* = 0.02). *Collinsella* (*P* = 0.01), *Eubacterium* (*P* = 0.01) and *Coprococcus* (*P* = 0.03)

in the RES group only (Figure 5.3.3 and 5.3.4 and Appendix 2). Along with a trend towards increases in *Ruminococcus*2 and *Anaerostipes* (all $P = 0.05$) and *Akkermansia* ($P = 0.06$) in the RES group only. The was also a trend towards a decreases numbers of *Geminger* in the NR group at completion ($P = 0.06$) (Figures 5.3.3 and 5.3.4).

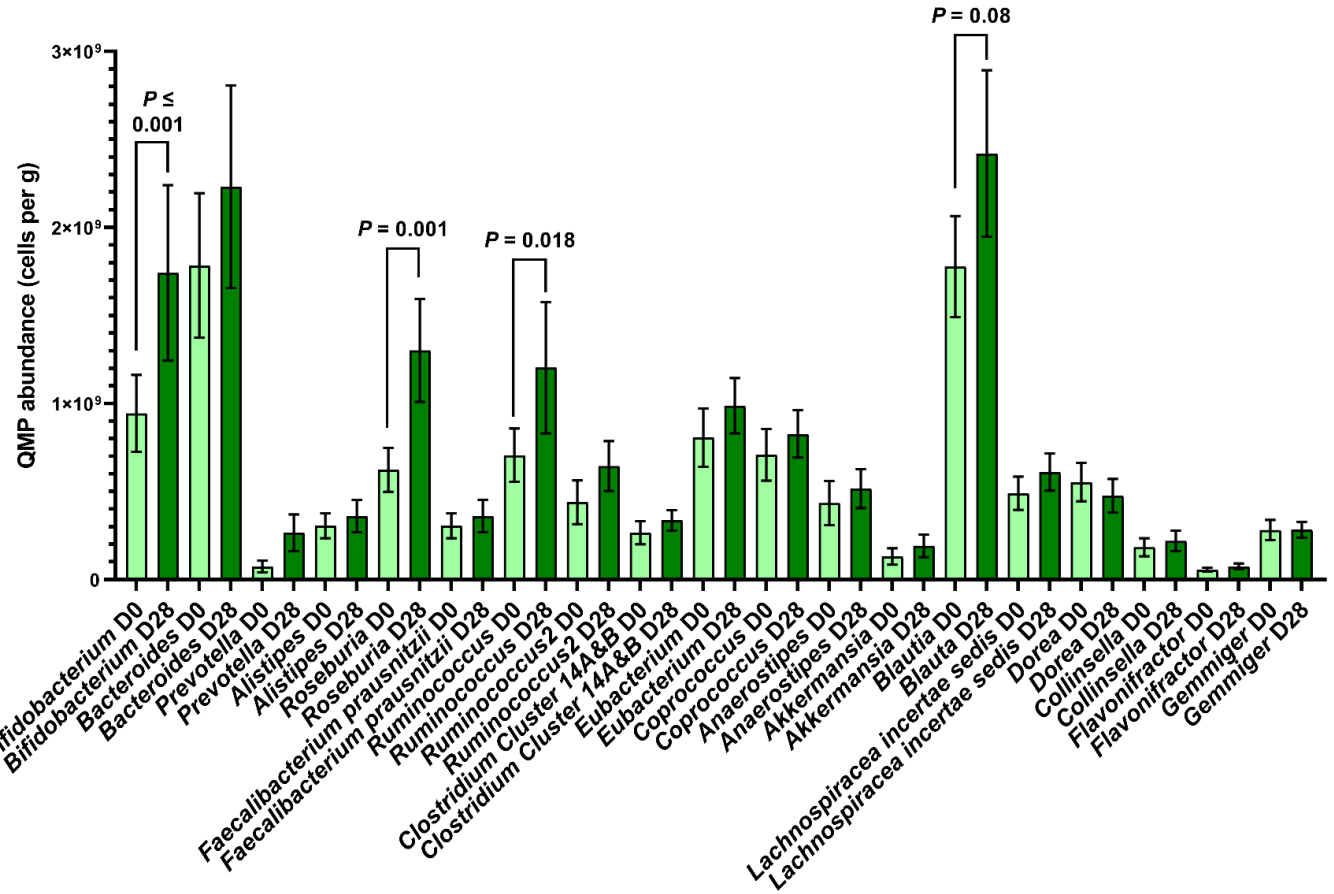

Figure 5.3.2. Quantitative microbiome profiling (QMP) of 16S rRNA sequencing data segregated by genus recorded across the entire 2'FL cohort (*n* = 23) at D0 and D28. Mean and Standard error (SE). Results that are statistically significant are displayed by specified *P* values. Numbers are expressed as cells per gram of faeces

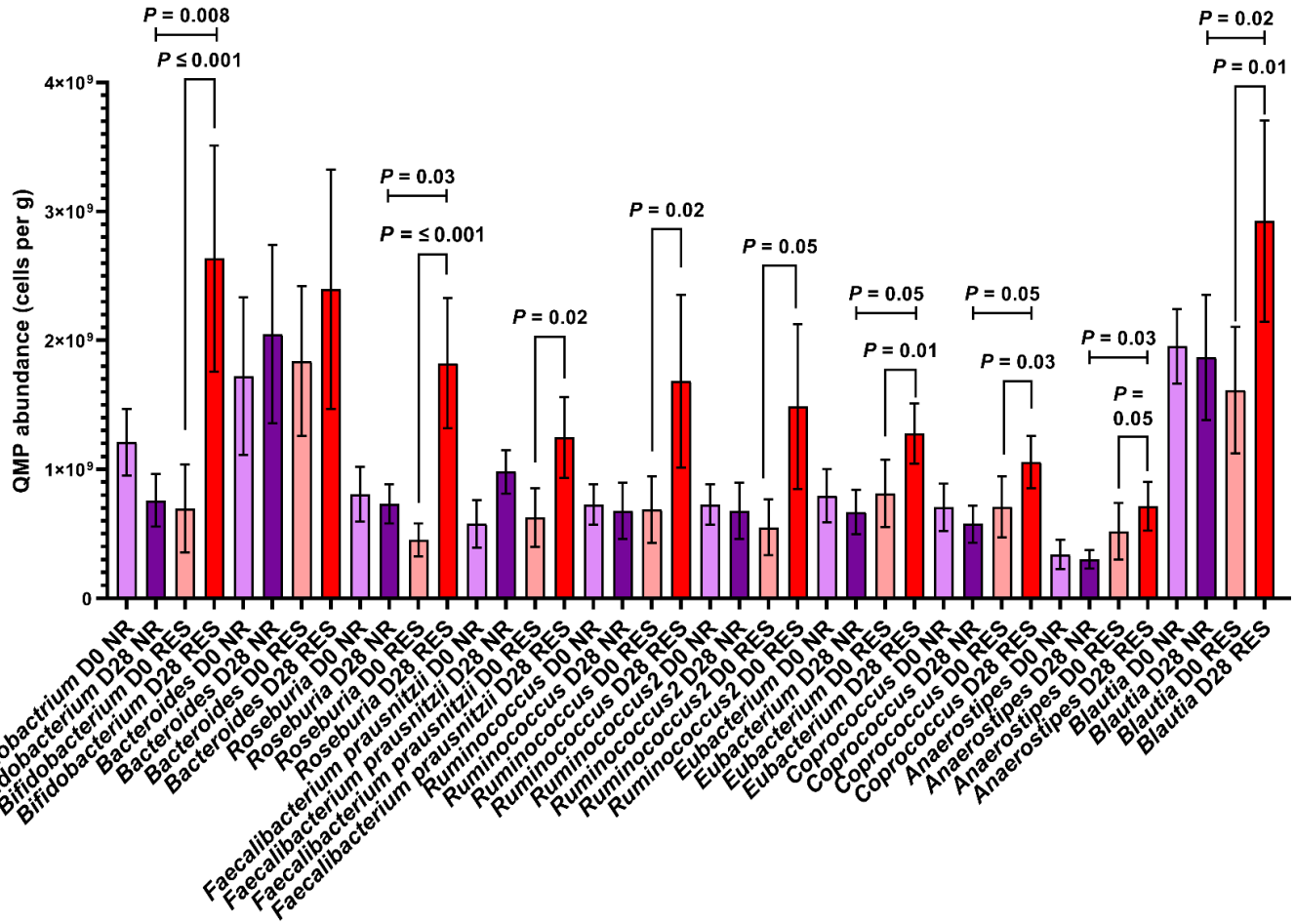

Figure 5.3.3. Quantitative microbiome profiling (QMP) of 16S rRNA sequencing data segregated by genus and RES (*n* = 12) and NR (*n* = 11) status at D0 and D28. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responder, RES = responder

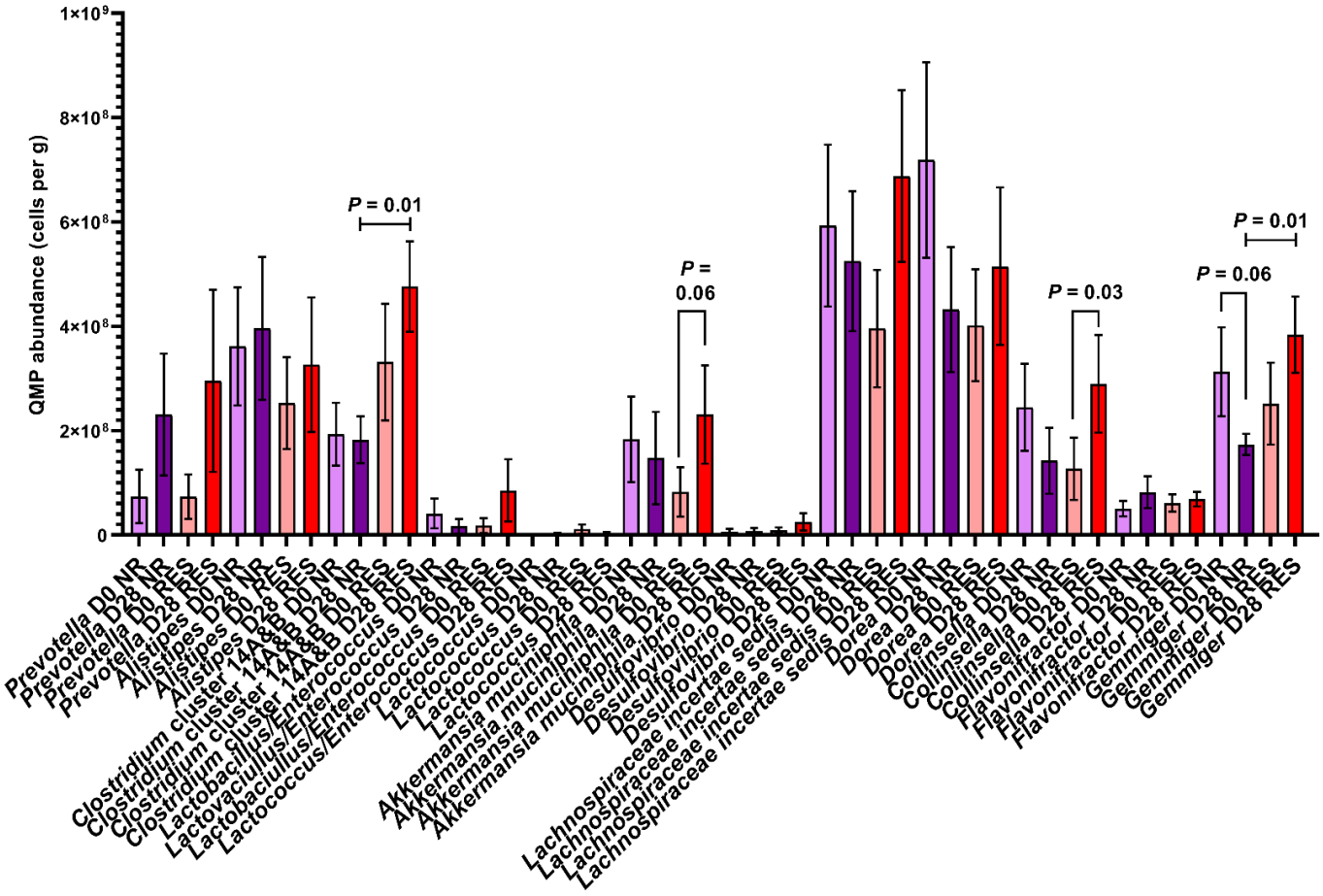

Figure 5.3.4. Quantitative microbiome profiling (QMP) of 16S rRNA sequencing data segregated by genus and RES (*n* = 12) and NR (*n* = 11) status at D0 and D28. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responder, RES = responder

5.3.4.2 Quantitative Microbiome Profiling – a deeper dive into species level differences – why stopping at genus level is not enough.

In order to try and gain a deeper insight into what drives differences between NR/RES status to 2'FL supplementation we conducted species level analysis (where possible) using QMP ASV data. Firstly, looking at bifidobacterial species there were clear significant differences seen between NR vs RES at completion of the intervention (Figure 5.4.1). Largest changes in *Bifidobacterium* species were documented in *B. longum* subsp. *infantis* recording significant day x responder status [F (1,22) = 4.626, *P* = 0.039] and responder status [F (1,22) = 1.852, *P* = 0.043] interactions. Follow up analysis revealed significant increases in *B. longum* subsp. *infantis* counts in the RES group only at Day 28 going from $2.36 \times 10^8 \pm 1.02 \times 10^8$ (SE) to $9.02 \times 10^8 \pm 4.07 \times 10^8$ (6.42×10^8 mean difference) cells per gram (*P* = 0.001). Number of *B. longum* subsp. *infantis* recorded at D28 in Res being statistically significant from changes seen in NR (*P* = 0.03). The only other significant difference detected between RES and NR in *Bifidobacterium* species was documented in *B. animalis* subsp. *lactis* with increases detected in the NR group only (*P* ≤ 0.001). The numbers of in *B. animalis* subsp. *lactis* documented in NR at D28 being statically significant from RES (*P* = 0.009) (Figure 5.4.1 and Appendix 3). Subsequent, analysis also revealed several within group changes including increases in *B. pseudocatenulatum* (*P* = 0.023) and *Bifidobacterium* (others) (*P* = 0.023) in the RES group only (Figure 5.4.1). The only other increases in *Bifidobacterium* seen in the NR group was documented in *B. bifidum*. however, this was not statistically significant compared to with baseline values (*P* = 0.095) (Figure 5.4.1). There was also a trend towards significant decreases in *Bifidobacterium* (others) identified in the NR group (*P* = 0.057).

Large increases were also seen in *Roseburia faecis* in the RES group at D28 going from $3.28 \times 10^8 \pm 7.79 \times 10^7$ (SE) to $1.50 \times 10^9 \pm 4.98 \times 10^8$ (SE) (1.17×10^9 mean difference) cells per gram

(*P* = 0.006). Numbers in the RES group at D28 also being statistically different from the NR group (*P* = 0.03) (Figure 5.4.2). Increases in *Blautia wexlerae* were also identified in the RES group going from $7.81 \times 10^8 \pm 3.76 \times 10^8$ (SE) to $1.19 \times 10^9 \pm 4.19 \times 10^8$ (SE) (4.09×10^8 mean difference). The numbers recorded at D28 in the RES group being significantly different from NR (*P* = 0.02). There were also significant increases in *Eubacterium hadrum* in the RES group only going from 4.09×10^8 (1.69×10^8) to 5.90×10^8 (1.66×10^8) (1.81×10^8 mean difference). D28 values in the RES group being statically different from NR (*P* = 0.04). The only other significant difference detected at species level between NR and RES was identified in *Coprococcus eutactus* (*P* = 0.03). Lastly, there was a trend towards significant differences between NR and RES at completion in *Bacteroides plebeius* (*P* = 0.08) with numbers only significantly increasing in the NR group only (*P* = 0.01) (Figure 5.4.3 Appendix 3).

As per genus level, there were also several other significant within group changes including increases in *Roseburia hominis* (*P* = 0.04), *Roseburia intestinalis* (*P* = 0.03), *Blautia hansenii* (*P* = 0.03), *Blautia luti* (*P* = 0.04) *Eubacterium hadrum* (*Anaerostipes*) (*P* = 0.003), *Lactobacillus ruminis* (*P* = 0.005), *Ruminococcus bromi* (*P* = 0.003), *Prevotella copri* (*P* = 0.04), *Ruminococcus faecis* (*P* = 0.02), *Ruminococcus bromii* (*P* = 0.02), *Ruminococcus obeum* (*P* = 0.04), *Coprococcus* others (*P* = 0.001) in the RES group, (Figures 5.4.2 and 5.4.3 and Appendix 3).

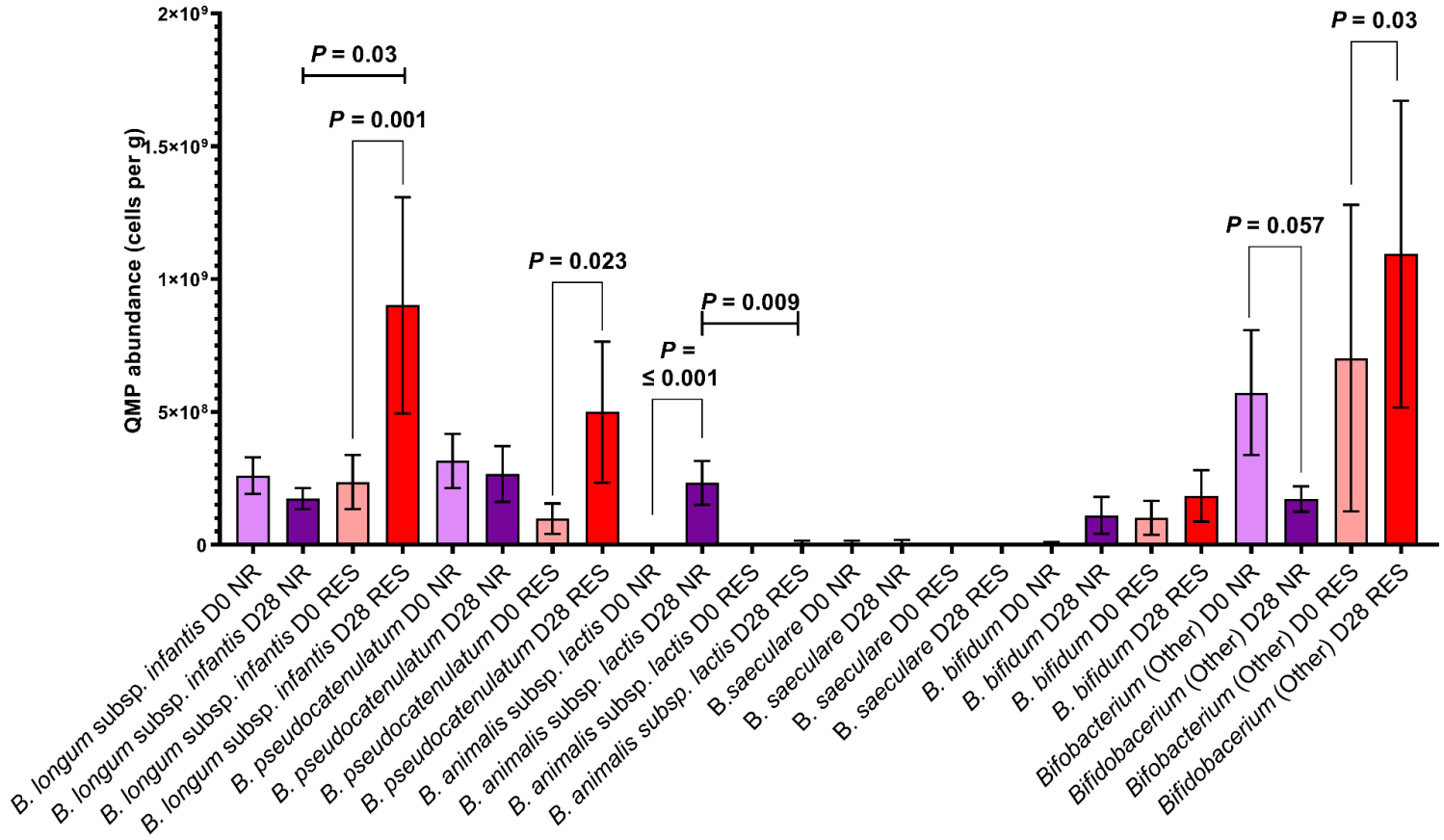

Figure 5.4.1. Quantitative microbiome profiling (QMP) of 16S rRNA *Bifidobacterium* taxa segregated by species and RES (*n* = 12) and NR (*n* = 11) status at D0 and D28. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responder, RES = responder

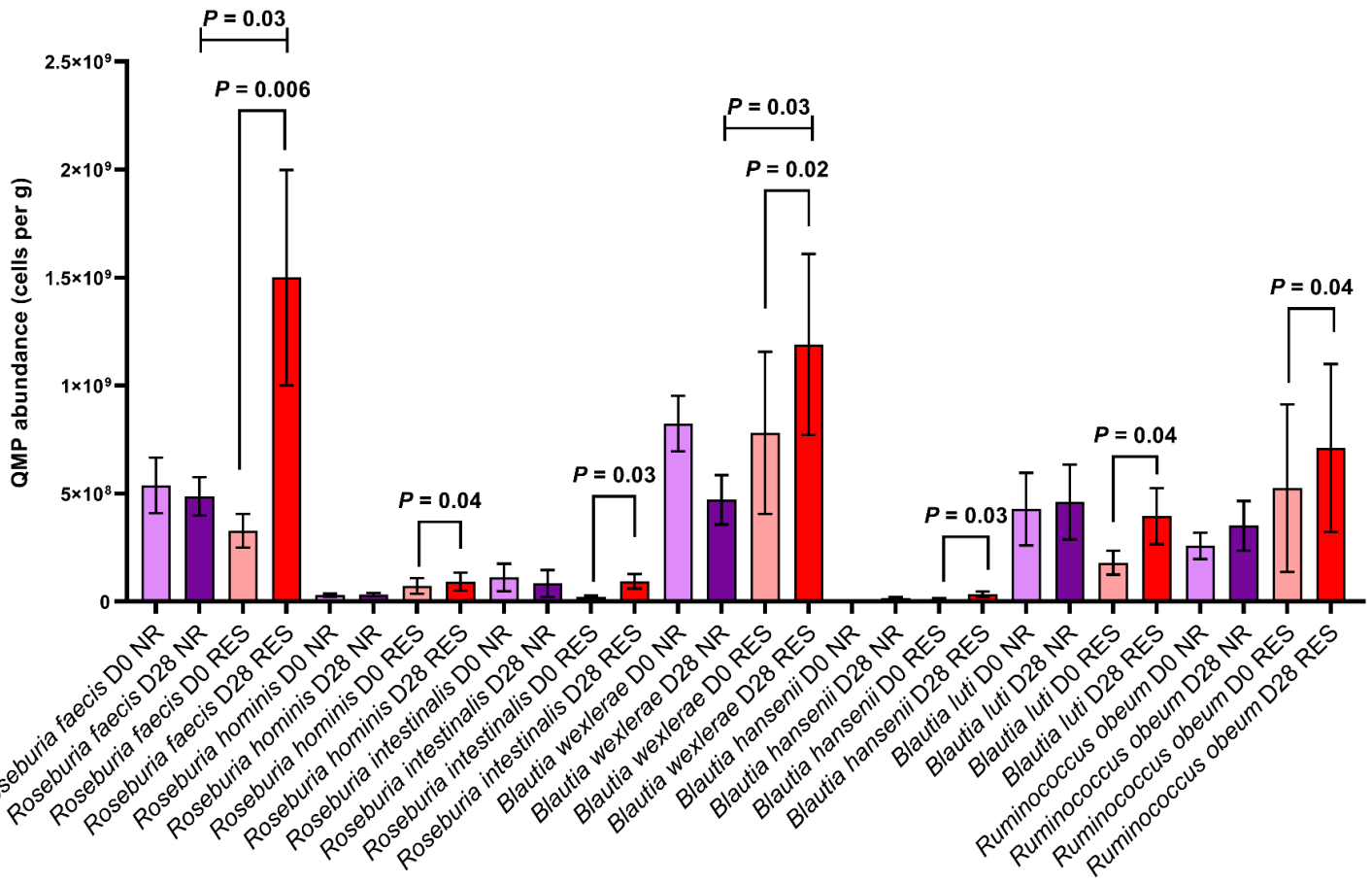

Figure 5.4.2. Quantitative microbiome profiling (QMP) of 16S rRNA taxon data at species level segregated by RES (*n* = 12) and NR (*n* = 11) status at D0 and D28. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responder, RES = responder

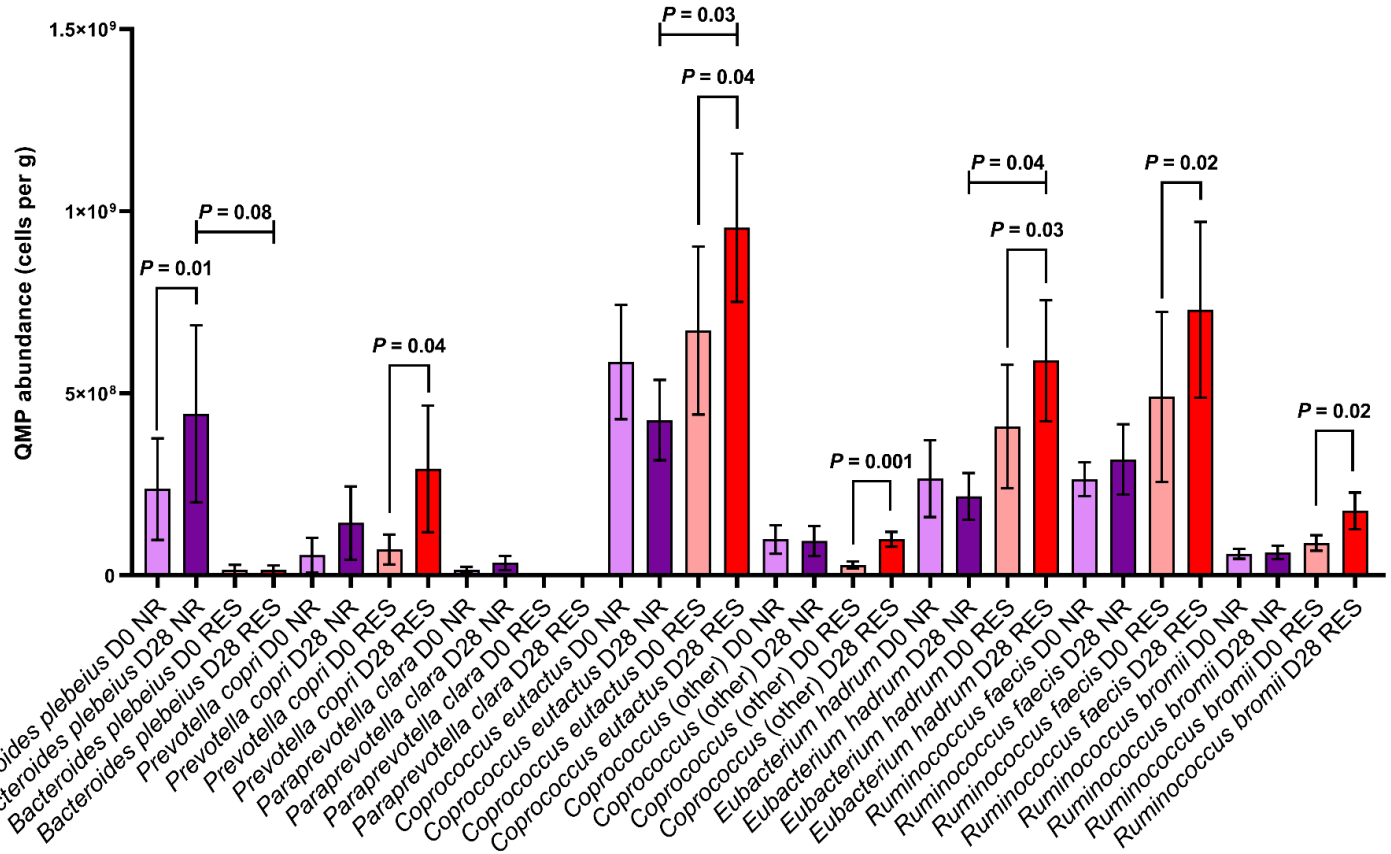

Figure 5.4.3. Quantitative microbiome profiling (QMP) of 16S rRNA taxon data at species level segregated by RES (*n* = 12) and NR (*n* = 11) status at D0 and D28. Mean and standard error (SE). Numbers are expressed as cells per gram of faeces. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responder, RES = responder

5.3.5 Bowel habit and function

Changes in gastrointestinal sensations (flatulence, intestinal bloating, abdominal pressure, abdominal pain and feeling of fullness), stool consistency (as per Bristol Stool Form Scale) (Lewis and Heaton, 1997) and stool frequency were self-recorded daily throughout the one-week run-in and 28-day intervention period. Scores of 0, 1, 2, and 3 corresponded to none, mild, moderate, and severe (Ramnani *et al.*, 2010; Costabile *et al.*, 2008; Walton *et al.*, 2012). Data are presented as an average of the one-week run in period and the last week of the intervention phase (D22-28) and are reported in Figure 5.5.

No differences in stool frequency, stool consistency (trends towards softer stools), flatulence, intestinal bloating, abdominal pressure, abdominal pain or feeling of fullness were detected across the entire 2'FL cohort (Appendix 4). Similarly, upon separation into NR/RES status no differences in gastrointestinal sensation (flatulence, intestinal bloating, abdominal pressure, abdominal pain and feeling of fullness) were detected (Appendix 4). Significant reductions in stool frequency were detected in the RES group at completion ($P = 0.033$), There was a significant reduction in stool consistency detected in the RES group only ($P = 0.003$) (Figure 5.5D). Finally, no differences in stool frequency were either within (pre vs post) or between responder status at completion (Figure 5.5 A and B and Appendix 4).

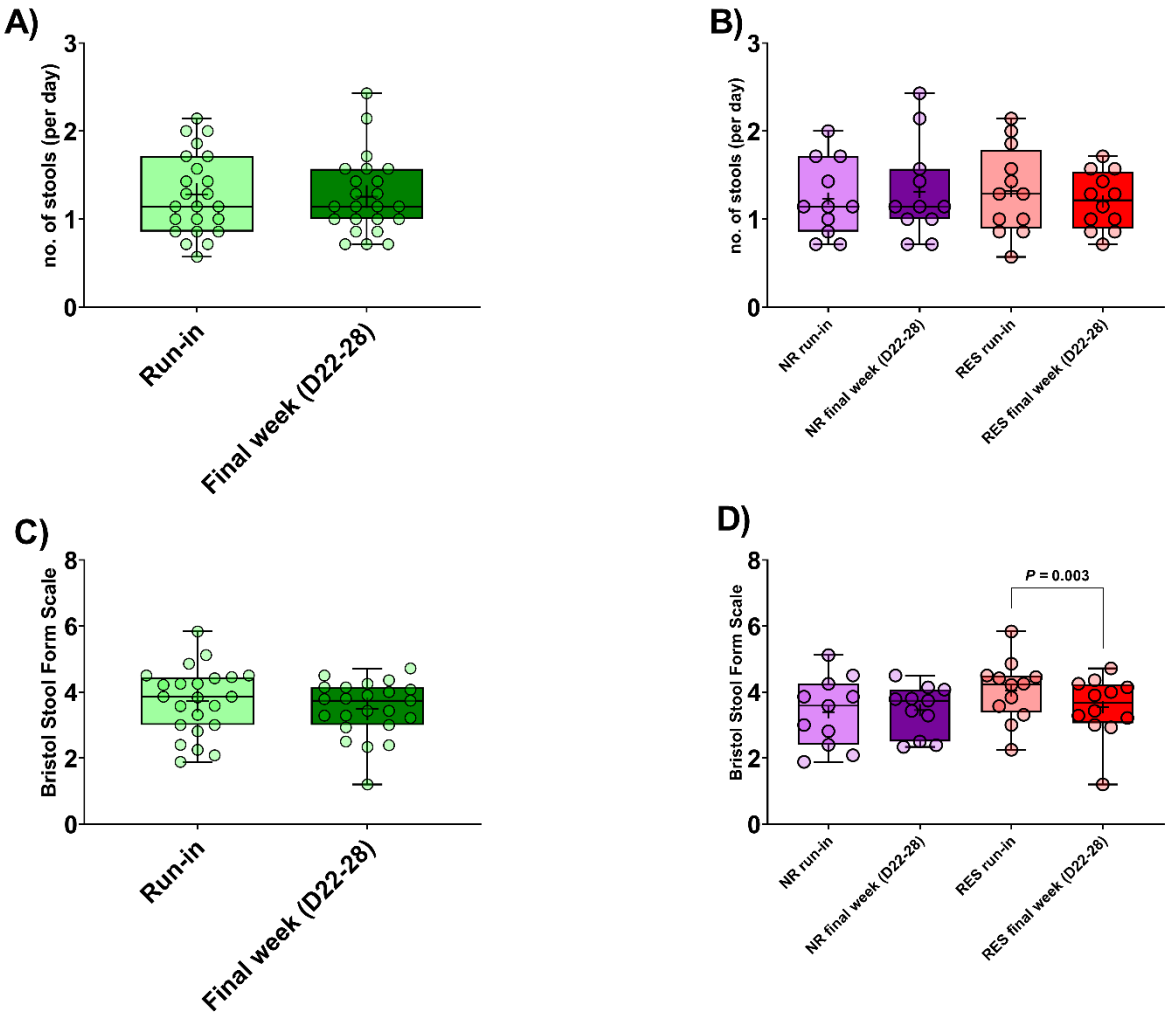

Figure 5.5. Gastrointestinal scores for stool frequency and stool consistency at baseline and again at last week of intervention (D22-28) in the entire 2'FL cohort (*n* = 23) **(A and C)** and segregated via RES (*n* = 12) and NR (*n* = 11) status **(B and D)**. Box and Whisker plot (min and max) all points, mean and medium. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responders, RES = responders

5.3.6 Mood state

5.3.6.1 Beck Depression Inventory

Figure 5.6.1 presents the baseline and post-intervention BDI scores broken down across both the entire cohort and segregated via RES/NR status. At completion of the intervention significant decreases (improvements) in BDI scores were detected across the entire cohort (*P* = 0.001) (Figure 5.6.1A). Upon segregation, analysis revealed significant decreases in BDI scores in the RES group only (*P* = 0.005) (Figure 5.6.1B), however, this was not statistically different from NR (*P* = 0.37).

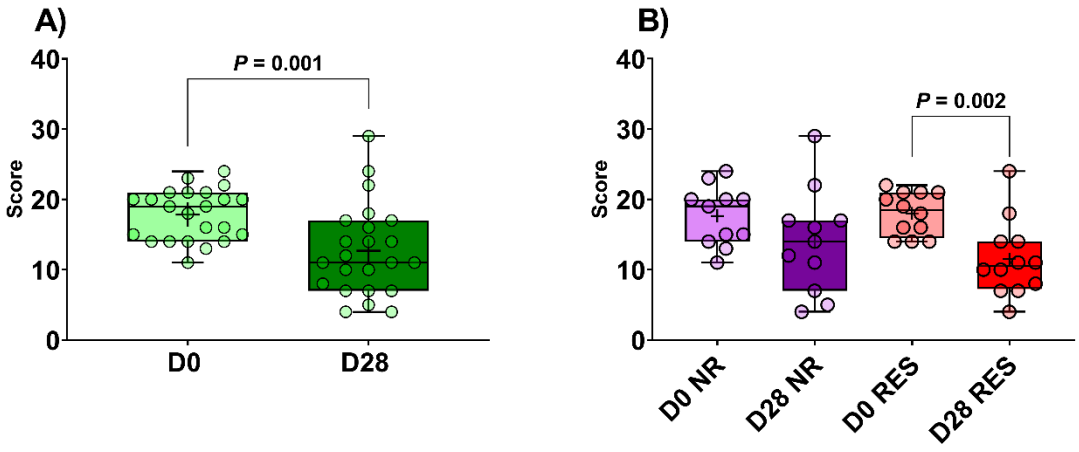

Figure 5.6.1 Beck Depression Inventory (BDI) scores across both the entire cohort (*n* = 23) **(A)** and segregated by RES (*n* = 12) and NR (*n* = 11) status **(B)** at baseline and completion. Box and Whisker plot (min and max) all points, mean and medium. Results that are statistically significant within group (pre and post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responders, RES = responders

5.3.6.2 STAI Y1 and STAI Y2

Figure 5.6.2 and 5.6.3 reports the changes in STAI Y1 and STAI Y2 scores. There were significant reductions (indicative of improvements in anxiety) in STAI Y1 and STAI Y2 scores detected across the entire cohort (*P* = 0.006) and (*P* = 0.002) respectively (Figures 56.2A and 56.3A). Segregation into NR and RES revealed significant reductions (improvements) in STAI Y1 (*P* = 0.009) and STAI Y2 (*P* ≤ 0.001) in the RES group only (Figure 5.6.2B and 5.6.3B). No significant differences were observed between NR and RES in either STAI Y1 or Y2 scores at completion (Appendix 5), although a trend towards significance was documented in STAI Y2 scores (*P* = 0.066).

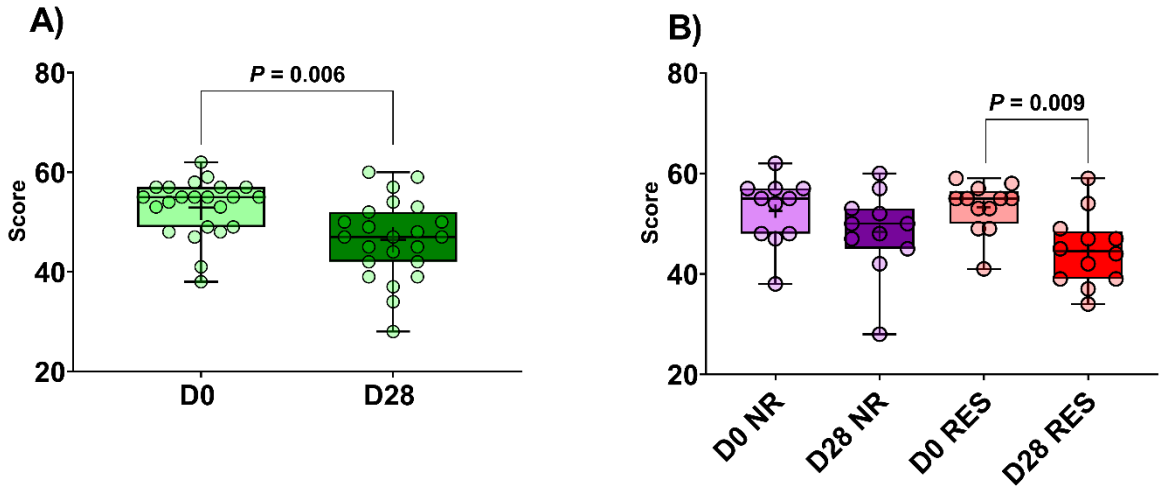

Figure 5.6.2. State Trait Anxiety Inventory (STAI) Y1 scores across both the entire cohort (*n* = 23) **(A)** and segregated by RES (*n* = 12) and NR (*n* = 11) status **(B)** at baseline and completion. Box and Whisker plot (min and max) all points, mean and medium. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responders, RES = responders

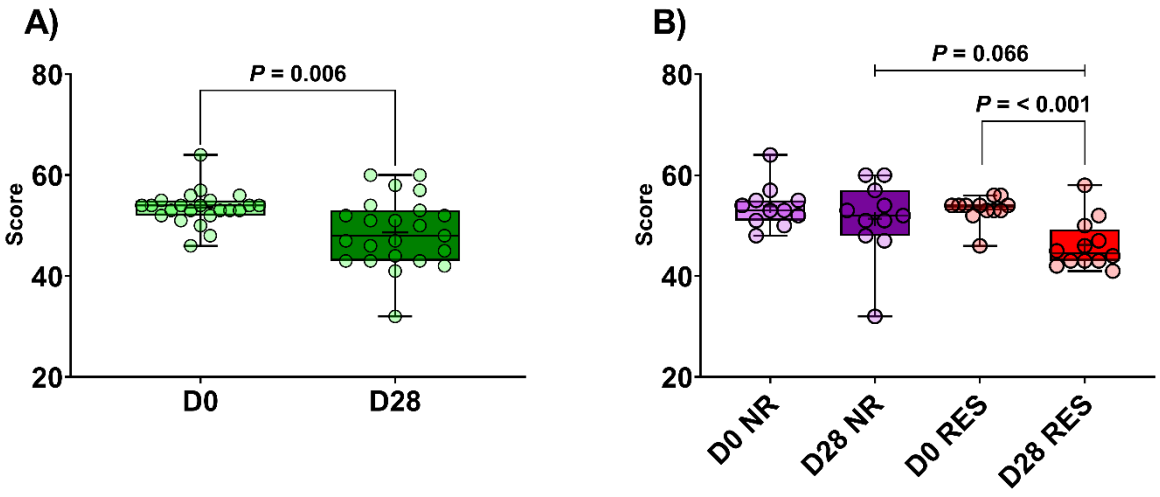

Figure 5.6.3. State Trait Anxiety Inventory (STAI) Y2 scores across both the entire cohort (*n* = 23) **(A)** and segregated by RES (*n* = 12) and NR (*n* = 11) status **(B)** at baseline and completion. Box and Whisker plot (min and max) all points, mean and medium. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responders, RES = responders

5.3.6.3 Positive and negative affect score – short form (PANAS-SF)

Significant positive affect (PA) and negative affect (NA) scores are reported in Figure 5.6.4 and 5.6.5. Analysis of the entire cohort revealed significant increases and in PA (*P* = 0.005) and decreases in NA (*P* = 0.002) scores respectively. These trends reflected those seen upon separation into NR and RES with significant improvement seen in PA (*P* ≤ 0.001) and NA (*P* = 0.003) scores in the RES group only (Figure 5.6.4 and 5.6.5). There was also a trend towards significant improvements detected in PA scores in the NR group at completion (*P* = 0.066). No differences were observed between NR and RES at completion in either PA (*P* = 0.49) or NA (*P* = 0.13) scores.

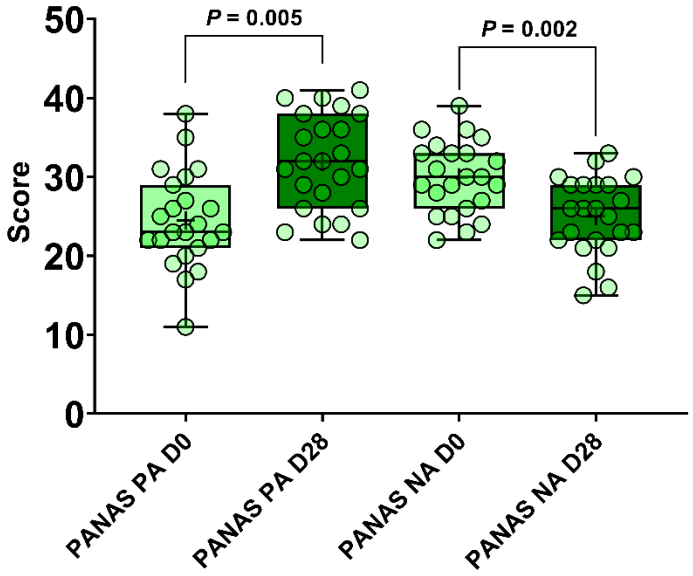

Figure 5.6.4. Positive and Negative Affect Schedule – Short Form (PANAS-SF) scores across the entire cohort (*n* = 23) at baseline and completion. Box and Whisker plot (min and max) all points, mean and medium. Results that are statically significant are displayed by specified *P* values. **Abbreviations:** NR = non-responders, RES = responders; PA = positive affect; NA = Negative affect

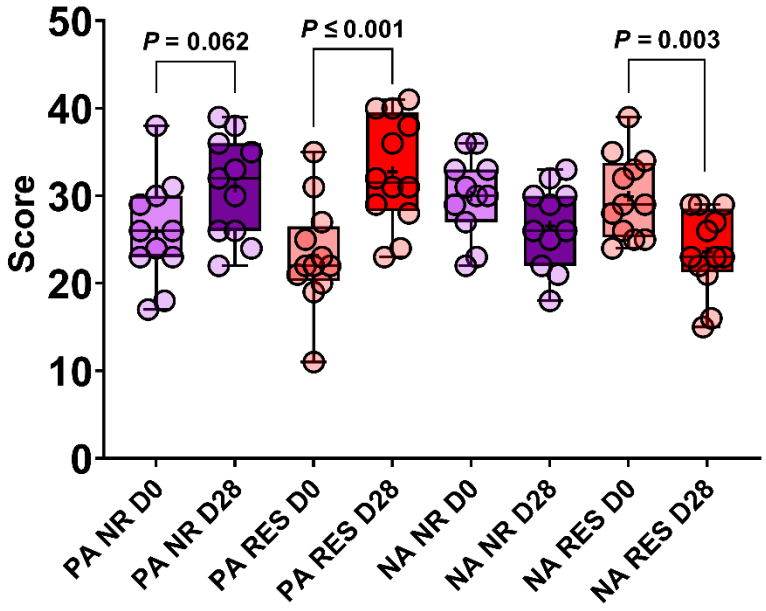

Figure 5.6.5. Positive and Negative Affect Schedule – Short Form (PANAS-SF) scores segregated by RES (*n* = 12) and NR (*n* = 11) status at baseline and completion. Box and Whisker plot (min and max) all points, mean and medium. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responders, RES = responders; PA = positive affect; NA = Negative affect

5.3.6.3 Pittsburgh Sleep Quality Index (PSQI)

Figure 5.6.6 A and B reports PSQI scores for both the entire 2'FL cohort and segregated by RES/NR status. Analysis revealed significant decreases (representing improved sleep quality) in PSQI scores across the entire 2'FL cohort (*P* = 0.005) (Figure 5.6.6A). Subsequent analysis revealed that PSQI scores were only significantly lower in the NR group (*P* = 0.001) (Figure 5.6.6B), however, this was not statically significant from RES (*P* = 0.27).

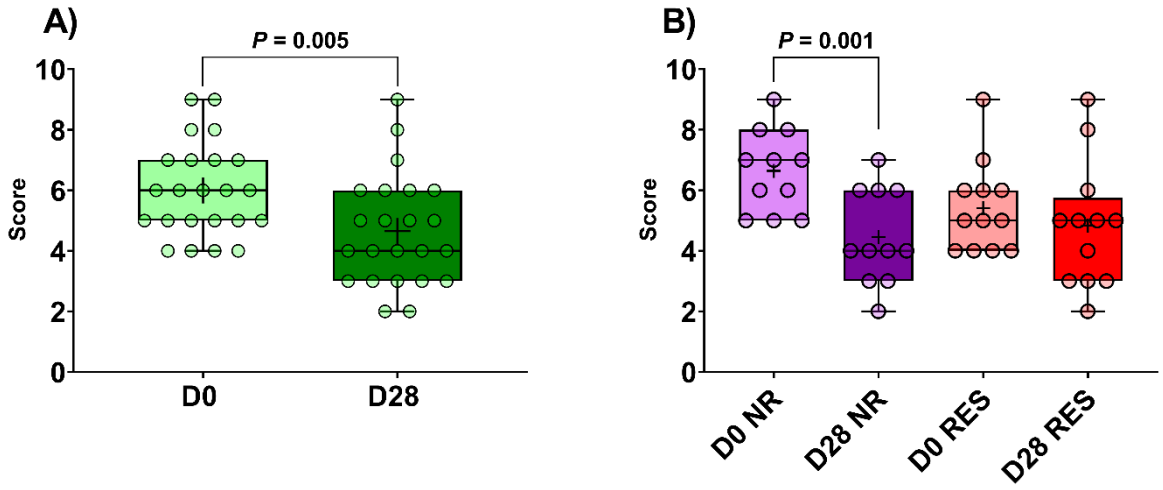

Figure 5.6.6. Pittsburgh Sleep Quality Index (PSQI) scores for both the entire cohort (*n* = 23) **(A)** and segregated via RES (*n* = 12) and NR (*n* = 11) status **(B)**. Box and Whisker plot (min and max) all points, mean and medium. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responders, RES = responders

5.3.6.4 Cortisol Awakening Response

Cortisol Awakening Response (CAR) scores across the entire cohort and segregated by RES/NR status are reported in Figure 5.6.7 A and B.

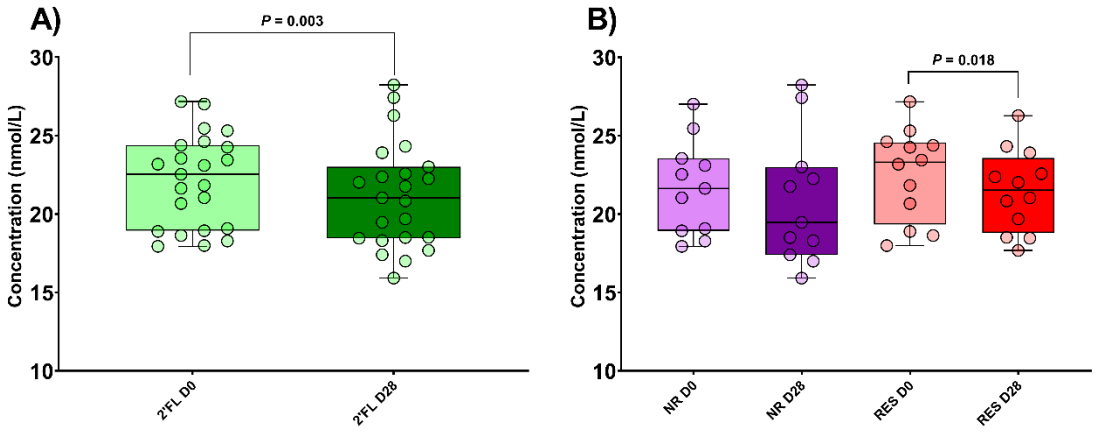

Figure 5.6.7. Cortisol awakening response (CAR) for both the entire cohort **(A)** (*n* = 23) and segregated via RES (*n* = 12) and NR (*n* = 11) status **(B)**. Box and Whisker plot (min and max) all points, mean and medium. Results that are statistically significant within group (pre vs post) and between responder status are displayed by specified *P* values. **Abbreviations:** NR = non-responders, RES = responders

At completion CAR values were significantly lower across the entire 2'FL cohort (*P* = 0.003) (Figure 5.6.7A). Upon separation into RES and NR status significant improvements in CAR values were recorded in the RES group only (*P* = 0.018) (Figure 5.6.7B). While there was a trend towards reductions in CAR detected in the NR group (*P* = 0.064). No significant differences were detected between NR and RES in CAR values at D28 (*P* = 0.66).

5.3.7 ^{1}H-NMR spectroscopic profiles

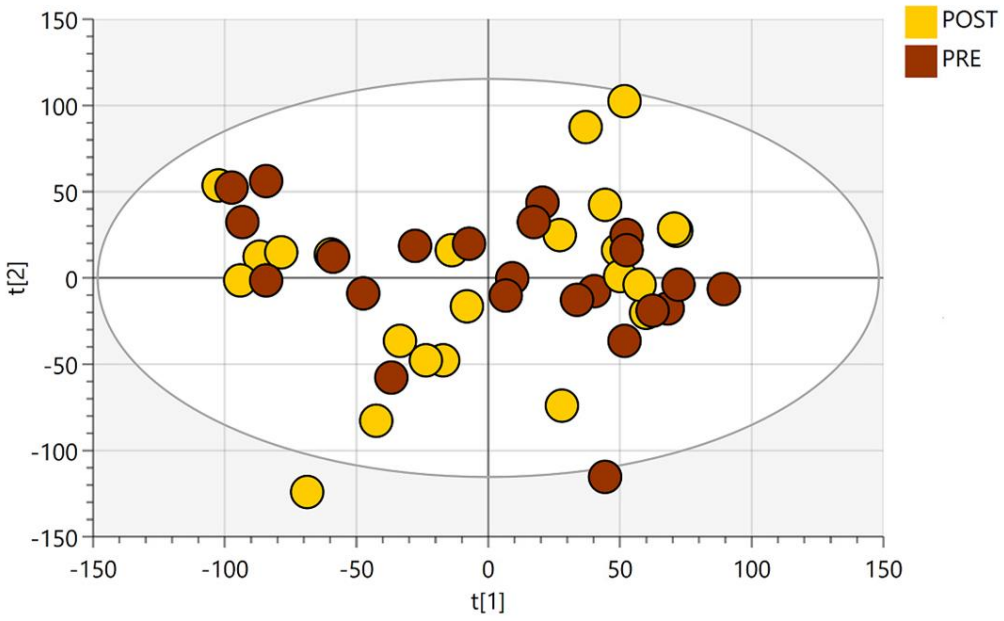

Figure 5.7.1. Urinary ^{1}H magnetic resonance (^{1}H-NMR) profiles for entire 2'FL cohort. Unsupervised principal components analysis (PCA) scores plot of pre and post intervention urine samples. R^2Cum = 0.626, Q^2Cum = 0.321.

Metabolic profiles of urine samples across the entire 2'FL cohort were analysed using unsupervised (PCA) methods (first seven components) showing the separation between pre and post intervention (R^2Cum = 0.626, Q^2Cum = 0.321) (Figure 5.7.1). Analysis revealed no clustering between pre and post intervention, Similarly, upon reclassification of the data into RES/NR status analysis revealed no clustering between NR and RES (Figure 5.7.2).

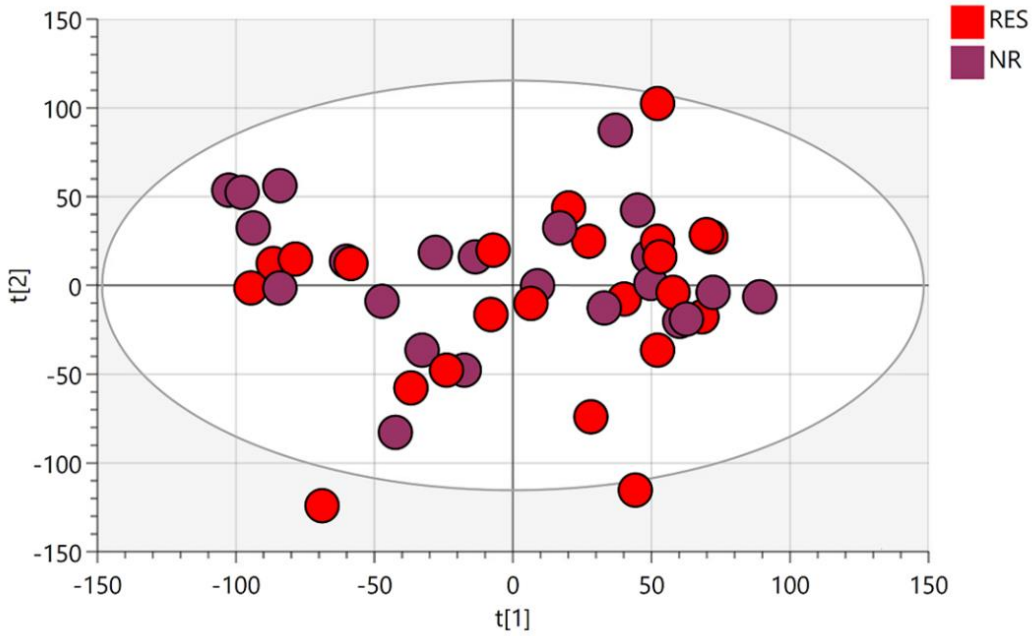

Figure 5.7.2. Urinary ^{1}H magnetic resonance (^{1}H-NMR) profiles for entire 2'FL cohort segregated into RES and NR. Unsupervised principal components analysis (PCA) scores plot of pre and post intervention urine samples. R^2Cum = 0.626, Q^2Cum = 0.321. **Abbreviations:** NR = Non-responders; RES = Responders.

OPLS-DA confirming these results with analysis indicating that the model had no predictive power to differentiate between individuals based upon RES/NR classification - R^2Y = 0.211, Q^2Cum = -0.266 (Figure 5.7.3). As a result, no subsequent further analyse was carried out.

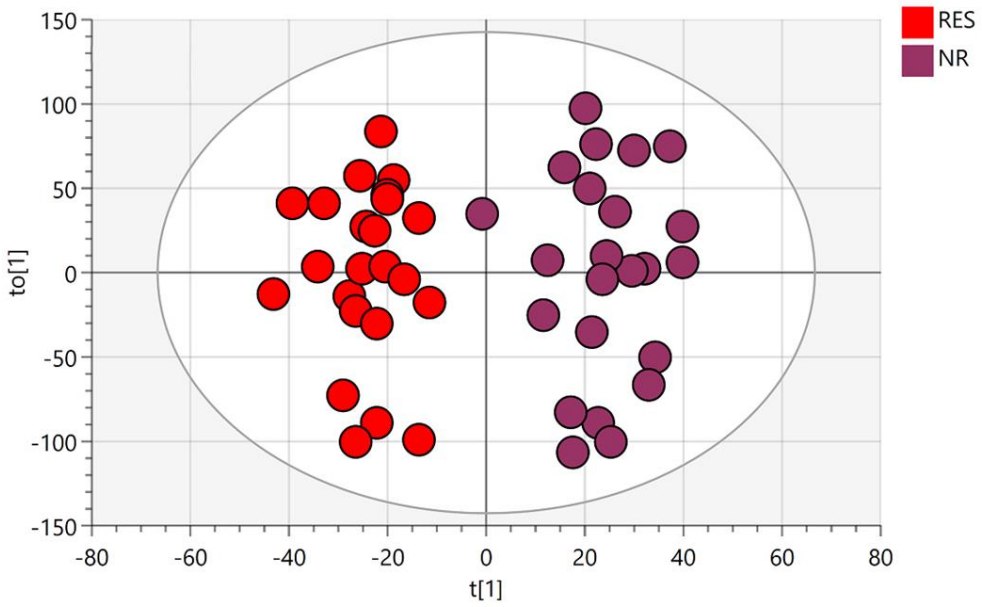

Figure 5.7.3. OPLS-DA scores plot of pre and post intervention urine samples showing RES and NR to 2'FL supplementation. R^2Y = 0.211, Q^2Cum = -0.266. **Abbreviations:** NR = Non-responders; RES = Responders.

5.4 Discussion

In this follow up paper to the EFFICAD trial we aimed to identify differences in microbial composition between NR and RES to 2'FL supplementation and the key factors driving this response. Initial analysis at genus level revealed significant increases in a number of bacterial taxa in the RES group including *Bifidobacterium*, *Blautia*, *Roseburia*, *Faecalibacterium prausnitzii*, *Ruminococcus* and *Eubacterium* (Figures 5.3.2 and 5.3.3 and 5.3.4 and Appendix 4). However, very few differences in bacterial genera were seen between NR and RES on completion. These differences included *Bifidobacterium* (*P* = 0.008), *Roseburia* (*P* = 0.03), *Blautia* (*P* = 0.02), *Anaerostipes* (*P* = 0.03), *Clostridium* cluster 14A&B (*P* = 0.01) and *Gemmiger* (*P* = 0.01). The largest changes in bacterial genus were in *Bifidobacterium* counts. This adds to the evidence that bifidobacteria are one of the most dominant HMOs utilisers within the gut (Thomson, Medina and Garrido, 2018; Jackson, Wijeyesekera and Rastall, 2022). However, not

all bifidobacteria are readily able to utilise HMOs (Sela *et al.*, 2008; Locascio *et al.*, 2007). This suggests that identifying differences at genus level may simply not be enough to distinguish NR from RES. So, in order to further identify differences between NR and RES, full ASV analysis was conducted at species level (where possible).

Upon separation of microbial taxa into species, significant differences between NR and RES were detected in *B. longum* subsp. *infantis* ($P = 0.03$), *B. animalis* subsp. *lactis* ($P = 0.009$), *Roseburia faecis* ($P = 0.03$), *Eubacterium hadrum* ($P = 0.03$), *Coprococcus eutactus* ($P = 0.04$) and *Blautia wexlerae* ($P = 0.03$). The increases in *B. longum* subsp. *infantis* are unsurprising as *B. longum* subsp. *infantis* is considered a cornerstone of HMOs degradation as it possesses all the necessary loci to degrade and assimilate a wide variety of HMOs (Zhang *et al.*, 2022). Research on the ability of *Roseburia faecis* to utilise HMOs is limited to pure culture experiments that suggest that it is not readily able to utilise HMOs. However, the same authors documented that in the presence of *Akkermansia muciniphila* significant increases in *Roseburia* spp. were detected (Pichler *et al.*, 2020). In our study cohort, while increases in *Akkermansia* were documented in the RES group, these were not statistically significant compared to baseline or to NR ($P = 0.11$) (Appendix 2). It has been shown that increases in numbers of *Roseburia* spp. often coincide with increases in bifidobacteria (Kim *et al.*, 2020; Riviere *et al.*, 2016). However, while large changes in *B. longum* subsp. *infantis* were detected in the RES group, the degradation of HMOs by *B. longum* subsp. *infantis* occurs intracellularly and cannot contribute to cross-feeding of HMOs (Garrido *et al.*, 2013). It does, however, stimulate changes in the wider microbiota via the production of acetate and lactate (Chia *et al.*, 2021).

Although not significantly different to NR, our RES cohort had significant increases in *B. pseudocatenulatum* were detected on completion ($P = 0.023$). *B. pseudocatenulatum* is a

common constituent of the adult microbiota possessing a plethora of loci able to utilise a wide variety of HMOs, specifically fucosylated HMOs via extracellular degradation (Shani *et al.*, 2022; Lawson *et al.*, 2020). Increases seen in *Roseburia faecis* likely occurred as a result of scavenging both fucose and lactose motifs as well as acetate and lactate from the degradation and utilisation of 2'FL by *B. longum* subsp. *infantis* and *B. pseudocatenulatum* (Louis and Flint, 2009; Riviere *et al.*, 2016). These mechanisms also may accounting for the changes seen in *Roseburia intestinalis, Blautia wexlerae*, *Blautia luti*, *Coprococcus eutactus*, *Eubacterium hallii*, *Eubacterium hadrum* and *Ruminococcus faecis*, *Ruminococcus bromii* and *Prevotella copri* with numbers increasing in the RES group, while numbers remained virtually unchanged or decreased in NR (Figure 5.4.2 and 5.4.3 and Appendix 3).

As stated, while we detected significant increases in a number of several species od bacteria at completion in RES group (Figures 5.4.1, 5.4.2 and 5.4.3), with the exception of *B*. *longum* subsp. *infantis*, *Blautia wexelerae,* Roseburia *faecis, Eubacterium hadrum* and *Coprococcus eutactus* no other significant differences compared with NR were detected. This may be related to the small sample size and a need for a greater number of participants to achieve statistical significance. Additionally, we were only able to analysis species level changes which might have limited scope in separating RES from NR, as there are vast differences in microbial responses to HMOs, even from the same genus and species. Differences between NR and RES might reside only at strain level (Gotoh *et al.*, 2018; Garrido *et al.*, 2015; Bunesova, Lacroix and Schwab, 2016; Ward *et al.*, 2007; Marcobal *et al.*, 2010). Nevertheless, these results add to the speculative evidence that the composition of the gut microbiota is a key predictor of whether an individual is likely to be a RES/NR to HMOs supplementation (Jackson, Wijeyesekera and Rastall, 2022).

Upon segregation by RES/NR status we detected several improvements in mood state scores (BDI, STAI Y1, STAI Y2, PANAS-SF PA and NA) only in the RES group, although none of these were significantly different to NR (Appendix 5). While there is an increasing body of evidence supporting the relationship between the gut and brain, many of the mechanisms remain poorly understood. Taking this into consideration several microorganisms found within the gut including *B. longum* subsp. *infantis*, *Blautia wexelerae*, *Eubacterium hadrum* and *Eubacterium halli* have been identified as γ-aminobutyric acid (GABA) producers and were significantly increased in RES upon completion (Strandwitz *et al.*, 2019; Otaru *et al.*, 2021; Duranti *et al.*, 2020).

GABA plays several essential roles, including being responsible for the synthesis and production of several other neurotransmitters including serotonin, tryptophan and dopamine (Al-Khafaji *et al.*, 2020). GABA concentrations are also often correlated with an individual's levels of depression and a variety of mood disorders (Petty, 1995; Brady *et al.*, 2013). Given that we saw significant increases in the number of GABA producing bacteria in the RES group it could be speculated that increases in GABA concentrations also would have occurred. However, as we were unable to collect blood samples, we cannot confirm this and this should be the focus of future work.

Several GABA producing bacteria, including *Eubacterium halli*, which was significantly enhanced in the RES group, have been associated with improvements in cognitive function (Bajaj *et al.*, 2016). Lower abundances of both *Eubacterium* and *Blautia* have been associated with increased levels of depression (Yang *et al.*, 2020). Furthermore, *B. longum* subsp. *infantis*, which was again enhanced in the RES group possesses the highest anti-inflammatory properties of all bifidobacteria, improving intestinal barrier function and decreasing pro-inflammatory cytokine production (Soliman *et al.*, 2012; Underwood *et al.*, 2015). *B. longum*

has also been shown to improve sleep quality scores during periods of high exam stress (Moloney *et al.*, 2021).

While we did not analyse changes in organic acids, it is highly likely that significant increases in acetate, lactate, propionate and butyrate concentrations occurred in the RES group as a result of a number of significant shifts in microbial response. Finding a means of increasing organic acid production could potentially have beneficial effects on mood state. Organic acids play vital roles in neurotransmitter production via regulation of the expression of tryptophan 5-hydroxylase 1 and tyrosine hydroxylase, enzymes involved in the synthesis of serotonin, dopamine, noradrenaline and adrenaline respectively (Dalile *et al.*, 2019; Reigstad *et al.*, 2015).

Acetate and lactate can function as endocrine signaling molecules (Silva, Bernardi and Frozza, 2020) with acetate also regulating GABA production in the hypothalamus (Frost *et al.*, 2014). While propionate can reduce blood brain barrier (BBB) permeability and protect against lipopolysaccharide-mediated blood brain barrier disruption (Hoyles *et al.*, 2018). Furthermore, butyrate can aid in GABA expression as well as increasing serotonin production via regulation of enterochromaffin cells (Reigstad *et al.*, 2015). GABA has been shown in animal models to exert anti-inflammatory effects including increasing global histone acetylation (Rada-Iglesias *et al.*, 2007) and regulation of brain-derived neurotrophic factor and glial-derived neurotrophic factor, proteins involved in the growth and differentiation of neurons in both the central and peripheral nervous system (Moris and Vega, 2003; Intlekofer *et al.*, 2013; Savignac *et al.*, 2013).

Our study is not without limitations. Firstly, the sample size was small at 23 individuals and a larger sample size would likely be required to achieve greater levels of statistical significance.

Secondly, we were unable to collect blood samples due to restrictions placed on us by the Covid-19 pandemic. Despite this, the results of this study add to the increasing body of evidence that identifying key differences in individual microbiotas can help us to distinguish whether an individual is likely to be wither a RES/NR to a HMO supplementation and potential effects on mood state.

3.8 Conclusion

In summary, we can conclude that differences in a number of key genera and species namely *B. longum* subsp. *infantis* (*Bifidobacterium*), *Roseburia faecis*, *Blautia wexlerae, Coprococcus eutactus*, and *Eubacterium hadrum* were detected between RES and NR at completion of the intervention. Additionally, while not significantly different, RES documented significant increases in a number of bacterial species including *B. pseudocatenulatum, Blautia luti*, *Blautia hansenii*, *Roseburis hominis*, *Roseburia intestinalis*, *Eubacterium hallii*, *Eubacterium hadrum*, *Prevotella copri*, *Coprococcus* (others), *Ruminococcus obeum* and *Ruminococcus faecis* (all $P \leq 0.05$). Similarly, while not significantly different from NR significant improvements in mood state parameters were only identified in the RES group. Nevertheless, the results of this follow-up paper indicate that shifting the compositional nature of the gut microbiota could have a profound impact on mood state. It is imperative that future studies focus on larger sample sizes and conducting sequencing down to strain level in order to identify key microbial differences between RES and NR to 2'FL supplementation.

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General discussion

To date, the majority of prebiotics are low molecular weight carbohydrates which, due to their structure, display a high degree of selectivity towards bifidobacteria as they possess the necessary glycosidases and transport systems to degrade and assimilate a wide variety of low molecular weight carbohydrates. Yet, while much of the research to date on prebiotics has focused on increasing bifidobacterial counts (Kolida and Gibson, 2007; Vandeputte *et al.*, 2017; Wang and Gibson, 1993) bifidobacteria do not produce either propionate or butyrate. Acetate and lactate can, however, be utilised by several bacterial taxa including *Bacteroides*, *Roseburia* and *Faecalibacterium* amongst others to generate propionate and butyrate (Louis and Flint, 2017). This reliance on cross-feeding, however, can lead to a high degree of unpredictability with diet-related changes in the gut microbiota and metabolites (Carlson *et al.*, 2017). As a result, there is increasing interest in combining prebiotics with other oligo/polysaccharides to gain more controllable changes in the microbiota and therefore butyrate and propionate production (Collins *et al.*, 2021).

Subsequently, there is an increasing body of evidence supporting the relationship between the gut and the brain, a term coined the gut-brain axis, and its influence on cognitive performance, mood state, and mental health disorders (Cryan *et al.*, 2020; Morais, Schreiber and Mazmanian, 2021). Of all mental health disorders, anxiety and depression are the most prevalent, costing the health services in excess of 1 trillion US$ per year (Chisholm *et al.*, 2016; Dieleman *et al.*, 2016). Thus it has become apparent that finding novel approaches to tackle the burden of health while also relieving increasing pressure on the healthcare system. As discussed in previous chapters, due to the relationship between the gut and brain one way to improve anxiety and depression may be through targeted manipulation of the gut microbiota.

One method of targeted manipulation could be use of the prebiotic ITF and prebiotic candidates - 2'FL and β-glucan.

In Chapter 2, changes in microbial composition and SCFA production were monitored using ITF and 2'FL and β-glucan alone and combination. *Bifidobacterium* was significantly higher across all substrates tested. *Faecalibacterium*, *Roseburia*, and *Clostridium* cluster IX were also significantly increased in treatments possessing combinations of ITF and β-glucan and OF and 2'FL. This coincided with considerable increases seen in both propionate and butyrate throughout the course of fermentation, implying that combining prebiotics with other fermentable carbohydrates can potentially result in a more targeted manipulation of the gut microbiota and metabolite production. In Chapter 3, we monitored changes in microbial load and organic acid and neurotransmitter production using *in vitro* batch culture fermentation at pH 5.4-5.6 to mimic the proximal region of the colon over 48 h using OF and 2'FL individually and combination as a secondary prescreening tool to the human intervention trial. Physiologically relevant increases in GABA production were detected across all substrates but were significantly greater in OF and combinations of OF/2'FL. This coincided with substantial increases in both bifidobacteria and *Bacteroides*. These results suggest that prebiotics may be able to stimulate beneficial increases not only microbial populations but more importantly in neurotransmitter production. To date, the majority of work focusing on changes in neurotransmitter production via targeted manipulation of the gut microbiota revolves around probiotics, with little known of the ability of prebiotics to stimulate neurotransmitter production. At face value this seemingly makes sense given the large heterogeneity seen in an individual gut microbiota, not only whether an individual possesses the necessary microorganisms to utilise the prebiotic, but whether the microorganisms are able to stimulate meaningful changes in neurotransmitter production.

Taking this into consideration both OF and 2'FL induce specific changes in the gut microbiota, mainly in bifidobacteria, *Bacteroides* and to a lesser extent *Lactobacillus* (Jackson *et al.*, 2022). The wider affects associated with prebiotic consumption include increases in several potentially beneficial propionate and butyrate producing bacteria, namely *Roseburia*, *Blautia* and *Faecalibacterium* as result of cross-feeding (Riviere *et al.*, 2016). These could possibly have advantageous effects on improvements in mood states via increasing not only GABA producing bacteria but also SCFA production, the benefits of which have been discussed in Chapter 3, Chapter 4 and Chapter 5. However, it is important to acknowledge that despite increases in neurotransmitter production throughout our batch cultures, much remains unknown about the ability of bacterial derived neurotransmitters to cross the blood brain barrier (Strandwitz, 2018).

In Chapter 4, the main objective was to take a combination of prebiotic and prebiotic candidate, screened during Chapter 2 and 3, and determine if differences in microbial composition occurred compared to sole supplementation using a 5-week, double-blind, randomized, controlled trial. A secondary outcome was to assess if prebiotics and candidates could improve anxiety and depression scores and cortisol awakening response. OF, 2'FL and combinations of OF/2'FL resulted in substantial differences in microbial response. Bifidogenic effects were largest in OF and OF/2'FL treatment groups, while the bifidogenic effect was highly heterogenous amongst 2'FL volunteers. This indicates that a strong responder/non-responder status exists in response to 2'FL supplementation. Increases in several propionate and butyrate bacteria including *Roseburia*, *Prevotella* and *Faecalibacterium* also demonstrated increases in OF, OF/2'FL and 2'FL treatments but were greatest in OF and OF/2'FL combination. This implies that OF and, more importantly, combining OF with 2'FL is likely to be a means of offsetting the discrepancies associated with non-responders to 2'FL supplementation.

In the human trial OF, 2'FL and combinations of OF/2'FL resulted in substantial improvements in BDI, STAI Y1 and Y2, PANAS-SF and CAR and this is the first prebiotic study to identify these effects. While it is hard to identify the mechanisms by which alterations in the gut microbiota drives changes in mood state, we were able to identify several significant correlations between bacterial taxa and mood state parameters. The strongest of correlations between bacterial taxa and mood state revolved around bifidobacteria. Several less significant correlations were identified with mood state including *Faecalibacterium*, *Blautia*, *Lactobacillus/Enterococcus*, *Lachnospiraceae incertae sedis* amongst others. In Chapters 2 and 3 we saw substantial increases in SCFA, namely acetate, propionate, and butyrate, in response to ITF and 2'FL supplementation. SCFA are hard to measure *in vivo* as faecal samples are not a good proxy for colonic SCFA production (Ramnani *et al.*, 2010), and we were unable to collect blood samples. The large increases in bacterial taxa suggest that circulatory and colonic SCFA production was also likely to be elevated as a result. The evidence for the supporting role of SCFA in regulation of the gut-brain axis is via modulation of neurological, immunological, or endocrine pathways is increasing (Silva, Bernardi and Frozza, 2020; Dalile *et al.*, 2019). Consequently, it is likely that supplementation with OF and/or 2'FL beneficially shaped the gut microbiota, along with altering metabolic activity, and subsequent improvements in mood state.

In response to the large heterogeneity seen between individuals to 2'FL supplementation, additional analysis was carried out to identify potential microbial differences segregating RES from NR. Segregation at genus level revealed several differences in bacterial taxa between responders and non-responders – namely in bifidobacteria, *Roseburia*, *Gemmiger* and *Anaerostipes* and *Blautia*. Notably recent evidence suggests that the ability of the gut microbiota to dissect and assimilate HMOs may lie at species and even strain level (Gotoh *et al.*, 2018; Sakanaka *et al.*, 2020; Yu, Chen and Newburg, 2013). At species level, 2'FL

modulated changes in a number of bacterial taxa in RES only. *B. longum* subsp. *infantis* and *B. pseudocatenulatum* were significantly increased while numbers declined in NR. Numbers of *Roseburia faecis*, *Blautia Wexlerae*, *Eubacterium halli* and *Bacteroides fragilis* also increased dramatically upon 2'FL supplementation. In contrast, with the exception of *Bacteroides fragilis*, numbers of these bacterial taxa either remained unchanged or declined in number in NR. Consequently, the changes seen in bacterial taxa correlate with those seen in mood state parameters and cortisol awakening response in RES and NR. However, these were not significantly different from NR ($P \geq 0.05$). Nevertheless, these results add to the increasing body of evidence supporting that the initial gut microbiota composition is a key characteristic in determining if an individual is likely to be either a RES/NR to HMOs supplementation and subsequently with mood state (Jackson, Wijeyesekera and Rastall, 2022; Al-Khafaji *et al.*, 2020; Appleton, 2018).

Future directions and limitations

In chapter two, a flour fraction, rich in β-glucan, was used in the *in vitro* fermentation to explore changes in microbial composition and metabolite production. While starch and glucose were removed prior to fermentation, the fraction did not undergo any simulated upper GI tract pre-digestive process to remove any residual proteins, and as a result the findings of this study should be interpreted cautiously. However, while upper tract pre-digestive processes are becoming increasingly common prior to *in vitro* fermentation it is still not without limitation. For example, it has been documented that lactulose was able to diffuse through the dialysis tubing and was lost during dialysis. Furthermore, it has been demonstrated that roughly 10 % of all dietary protein reaches the colon intact (Yao, Muir and Gibson, 2016) and the small amount of protein found in the β-glucan likely has little impact on

microbial response. Nevertheless, it would be beneficial to replicate a higher protein ITF/2'FL control just for comparative purposes.

While we did see substantial increase in GABA production, it should be noted that concentrations of several neurotransmitters including dopamine and serotonin remained virtually unchanged. This is likely due to the lack of the necessary enteroendocrine cells, required precursors and co-enzymes such as tyrosine and pyridoxine-5-phosphate needed to generate neurotransmitter biosynthesis (Reigstad *et al.*, 2015; Stover and Field, 2015). Consequently *in vitro* batch cultures in this instance are not the best proxy for assessing changes in neuroactive metabolite production. However, they do provide means of pre-screening a wide variety of substrates for potential changes prior to *in vivo* supplementation.

It must be acknowledged that while we were able to document increases in several bacterial taxa along with identifying several key taxa-taxa and taxa-mood state correlations in the human study, differentiating correlation from causation is difficult. Furthermore, due to restrictions placed on us as a result of the Covid-19 pandemic we were unable to collect blood samples which could have potentially given us greater insight into associations between microbial interactions and the relationship between microbial metabolites including GABA and acetate, and the gut-brain axis.

The PSQI questionnaire was used to capture changes in sleep quality throughout the course of the intervention. However, despite being a validated questionnaire it is not without limitation with it relying heavily on accurate recall of, and documentation of, changes in sleep quality over a large recall period. It is also insensitive to either the over or underestimation of changes in several critical sleep parameters including depth and efficiency (Faulkner and Sidey-Gibbons, 2019), making it hard to distinguish good from poor sleepers. Yet, out of all sleep

questionnaires, and specifically the PSQI used in this study, it is still the most recognised in assessing changes in subjective sleep quality demonstrating the best validity and reliability, specifically in the ability to identity differences between individuals of known-group traits such as anxiety, depression and schizophrenia (Fabbri *et al.*, 2021).

Finally, even though we were able to detect significant differences in several bacterial taxa namely bifidobacteria, *Roseburia*, *Anaerostipes*, *Gemmiger* and *Blautia* at genus and *B. longum* subsp. *infantis* and *Roseburia faecis* at species level between RES and NR (all $P \leq 0.05$) very few other significant differences were identified. Firstly, this is likely to be due to the limited sample size and therefore overall power. Secondly, the use of QMP to assess changes in microbial load meant it is currently not possible to log resulting microbial data prior to comparisons. This is due to the impacts that both library preparation and rarefaction processes have on excluding bacteria from a given individual's microbiota that would otherwise be captured via targeted analysis. Further, we were only able to sequence to species level, whereas the ability of the gut microbiota to utilise HMOs appears to lie at strain level. (Jackson, Wijeyesekera and Rastall, 2022). Consequently, it would be highly beneficial for future studies to focus on larger sample sizes, and sequencing down to strain level to identify changes in microbial responses if differences between RES and NR to 2'FL are ever going to be fully understood.

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Appendices

Appendix 2.1 Mean SCFA values using pH-controlled *in vitro* batch culture fermentation at 0, 4, 8, 24, 36 and 48 h.

SCFA	Acetate														
Time (h)	T 0	T 4			T 8			T 24			T 36			T 48		
Substrate	Mean (SE)	Mean (SE)	*P*	*Q*	Mean (SE)	*P*	*Q*	Mean (SE)	*P*	*Q*	Mean (SE)	*P*	*Q*	Mean (SE)	*P*	*Q*
OF	7.18 (0.95)	32.28 (8.21)	0.11	0.11	80.95 (7.72	**0.009**	**0.011**	99.86 (6.78)	**0.005**	**0.011**	103.72 (8.54)	**0.008**	**0.011***	113.94 (8.48)	**0.006**	**0.011*a**
OFI	3.55 (0.69)	9.04 (1.27)	**0.016**	**0.016***	53.28 (4.95)	**0.01**	**0.016***	73.51 (5.47)	**0.006**	**0.016***	88.71 (7.36)	**0.008**	**0.016***	95.68 (11.46)	**0.016**	**0.016*a**
ITF-mix	7.17 (0.94)	32.61 (8.05)	0.105	0.105	75.21 (13.12)	**0.031**	**0.039***	100.58 (10.38)	**0.011**	**0.019***	104.77 (8.59)	**0.007**	**0.018***	113.46 (8.03)	**0.005**	**0.018*a**
2'FL	3.51 (0.71)	8.34 (1.18)	**0.031**	**0.044***	58.94 (12.44)	**0.046**	**0.046***	81.26 (6.85)	**0.007**	**0.034***	96.28 (13.47)	**0.021**	**0.044***	104.68 (19.35)	**0.035**	**0.044*a**
β-glucan	7.18 (0.92)	28.79 (6.36)	0.097	0.097	59.36 (1.83)	**0.003**	**0.014***	75.73 (7.13)	**0.013**	**0.017****	84.99 (6.56)	**0.009**	**0.017**	94.49 (8.18)	**0.01**	**0.017*a**
OFI/2'FL 50/50	3.47 (0.72)	8.52 (1.44)	**0.03**	**0.037***	53.61 (9.56)	**0.037**	**0.037***	71.24 (4.62)	**0.005**	**0.023***	85.78 (10.50)	**0.018**	**0.037***	97.14 (14.64)	**0.025**	**0.037*a**
OFI/2'FL 85/15	3.54 (0.70)	8.63 (1.50)	**0.034**	**0.043***	57.08 (11.59)	**0.044**	**0.044***	75.26 (4.84)	**0.005**	**0.023***	82.39 (10.98)	**0.019**	**0.043***	93.94 (10.43)	**0.032**	**0.043*a**

GC-FID analysis of acetate concentration in the supernatant of effluents collected from vessel 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). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with respective 0 h sampling (grey columns). Red text indicates trending towards significance. Significant differences between substrates at 48h are indicated by differing letters.
Abbreviations: OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

SCFA	Acetate (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OFI/2'L 95/5	3.54 (0.68)	8.81 (1.61)	**0.046**	**0.046***	59.29 (10.70)	**0.02**	**0.026***	73.48 (3.93)	**0.003**	**0.014***	87.45 (8.52)	**0.011**	**0.026***	97.84 (13.49)	**0.021**	**0.026*a**
OF/2'FL	7.20 (0.94)	40.29 (9.48)	0.086	0.086	90.67 (11.28)	**0.016**	**0.020***	112.67 (8.06)	**0.005**	**0.009****	115.94 (6.65)	**0.003**	**0.009****	121.84 (8.59)	**0.005**	**0.009**a**
ITF-mix/2'FL	7.20 (0.93)	39.15 (10.36)	**0.014**	**0.016***	91.14 (11.69)	**0.016**	**0.016***	111.037 (7.72)	**0.005**	**0.010****	116.30 (8.21)	**0.005**	**0.010****	121.03 (9.41)	**0.006**	**0.010**a**
OF/β-glucan	7.14 (0.93)	34.34 (7.64)	0.087	0.087	60.77 (6.23)	**0.017**	**0.029***	91.11 (12.69)	**0.025**	**0.031***	95.12 (10.84)	**0.017**	**0.029***	101.81 (10.55)	**0.014**	**0.029*a**
OFI/β-glucan 50/50	7.21 (0.94)	31.94 (6.74)	0.084	0.084	65.83 (5.34)	**0.009**	**0.020***	79.36 (7.37)	**0.012**	**0.020***	88.38 (7.79)	**0.011**	**0.020***	94.74 (11.87)	**0.02**	**0.025*a**
ITF-mix/β-glucan	7.18 (0.94)	35.81 (7.27)	0.073	0.073	66.30 (2.98)	**0.004**	**0.018***	88.83 (10.86)	**0.02**	**0.024***	96.53 (10.43)	**0.015**	**0.024***	98.47 (11.23)	**0.017**	**0.024*a**
Negative	7.13 (0.94)	9.64 (0.60)	0.059	0.059	11.85 (0.039)	**0.006**	**0.009****	20.40 (0.28)	**0.007**	**0.009****	21.29 (0.08)	**0.001**	**0.007****	22.18 (0.47)	**0.004**	**0.009**b**

GC-FID analysis of acetate concentration in the supernatant of effluents collected from vessel 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). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with respective 0 h sampling (grey columns). Red text indicates trending towards significance. Significant differences between substrates at 48h are indicated by differing letters.
Abbreviations: OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

SCFA	Propionate														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	3.80 (0.77)	12.99 (1.63)	**0.048**	**0.048***	20.53 (2.12)	**0.023**	**0.029***	31.26 (3.53)	**0.014**	**0.026***	32.18 (3.41)	**0.013**	**0.026***	35.15 (4.18)	**0.015**	**0.026*cd**
OFI	3.25 (0.10)	6.84 (2.95)	0.36	0.36	18.39 (1.92)	**0.016**	**0.021***	25.76 (2.29)	**0.011**	**0.021***	28.82 (2.86)	**0.013**	**0.021***	35.41 (1.92)	**0.004**	**0.019*cd**
ITF-mix	3.77 (0.76)	11.54 (1.11)	0.05	0.05	20.19 (1.57)	**0.012**	**0.028***	36.88 (4.13)	**0.017**	**0.028***	37.75 (4.14)	**0.017**	**0.028***	40.67 (6.08)	**0.027**	**0.034*bc**
2'FL	3.29 (0.13)	5.51(1.65)	0.336	0.336	10.45 (1.09)	**0.026**	**0.032***	18.89 (1.52)	**0.011**	**0.018***	23.56 (1.97)	**0.011**	**0.018***	25.54 (1.84)	**0.008**	**0.018*d**
β-glucan	3.79 (0.76)	11.52 (0.99)	**0.047**	**0.047***	22.56 (1.40)	**0.027**	**0.033***	54.14 (0.85)	**≤ 0.001**	**≤ 0.001*****	58.09 (0.83)	**≤ 0.001**	**≤ 0.001*****	59.5 (1.14)	**≤ 0.001**	**≤ 0.001***a**
OFI/2'FL 50/50	3.20 (0.09)	6.03 (1.92)	0.291	0.291	12.94 (1.49)	**0.024**	**0.031***	24.49 (2.04)	**0.009**	**0.016***	29.57 (0.97)	**0.001**	**0.005****	31.17 (1.12)	**0.002**	**0.005**cd**
OFI/2'FL 85/15	3.24 (0.10)	6.02 (2.04)	0.322	0.322	15.23 (2.27)	**0.035**	**0.044***	25.35 (3.20)	**0.021**	**0.036***	26.61 (3.24)	**0.02**	**0.036***	31.89 (1.62)	**0.004**	**0.018*cd**
OFI/2'L 95/5	3.29 (0.12)	7.08 (2.77)	0.319	0.319	19.31 (1.95)	**0.015**	**0.018***	26.1 (2.26)	**0.011**	**0.018***	30.58 (1.71)	**0.004**	**0.011***	35.56 (1.13)	**0.001**	**0.007**cd**
OF/2'FL	3.76 (0.76)	13.01 (1.55)	**0.036**	**0.036***	24.04 (2.76)	**0.026**	**0.036***	31.89 (4.64)	**0.008**	**0.021***	36.33 (5.84)	**0.006**	**0.021***	38.8 (6.32)	**0.031**	**0.036*cd**

GC-FID analysis of propionate concentration in the supernatant of effluents collected from vessel 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). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with respective 0 h sampling (grey columns). Red text indicates trending towards significance. Significant differences between substrates at 48h are indicated by differing letters.
Abbreviations: OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

SCFA	Propionate (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	3.81 (0.77)	13.11 (1.84)	**0.044**	**0.044***	24.42 (2.20)	**0.014**	**0.017***	28.73 (1.33)	**≤ 0.001**	**≤ 0.001*****	32.51 (0.48)	**≤ 0.001**	**≤ 0.001*****	35.78 (1.44)	**≤ 0.001**	**≤ 0.001***cd
OF/β-glucan	3.79 (0.76)	12.06 (1.08)	**0.041**	**0.041***	26.39 (1.61)	**0.001**	**0.004****	46.02 (2.06)	**0.002**	**0.004****	47.21 (2.21)	**0.002**	**0.004****	56.35 (4.36)	**0.005**	**0.006**a**
OFI/β-glucan 50/50	3.83 (0.76)	10.7 (0.66)	**0.039**	**0.039***	24.03 (1.38)	**0.001**	**0.006****	44.67 (1.59)	**0.003**	**0.007****	50.22 (2.75)	**0.005**	**0.007****	52.79 (2.94)	**0.005**	**0.007**a**
ITF-mix/β-glucan	3.81 (0.76)	11.29 (0.70)	**0.032**	**0.032***	25.29 (1.55)	**0.002**	**0.004****	44.13 (2.29)	**0.002**	**0.004****	45.88 (1.78)	**0.002**	**0.004****	50.86 (3.48)	**0.005**	**0.006**ab**
Negative	3.78 (0.76)	4.25 (0.61)	**0.043**	0.058	4.65 (0.83)	**0.048**	0.058	5.86 (0.33)	0.058	0.058	6.05 (0.34)	0.057	0.058	6.37 (0.42)	0.038	0.058**e**

GC-FID analysis of propionate concentration in the supernatant of effluents collected from vessel 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). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with respective 0 h sampling (grey columns). Red text indicates trending towards significance. Significant differences between substrates at 48h are indicated by differing letters.
Abbreviations: OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

SCFA	Butyrate															
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	1.45 (0.21)	2.4 (0.30)	**0.076**	**0.094**	7.01 (0.95)	**0.035**	0.094	13.50 (3.77)	0.094	0.094	14.84 (3.60)	**0.072**	**0.094**	16.18 (3.54)	**0.059**	**0.094c**
OFI	1.01 (0.42)	1.57 (0.62)	0.1	0.102	5.95 (3.80)	0.102	0.102	13.08 (2.34)	**0.025**	**0.041***	15.71 (1.61)	**0.007**	**0.019***	17.11 (1.24)	**0.003**	**0.013*c**
ITF-mix	1.45 (0.21)	2.68 (0.63)	0.156	0.156	6.44 (1.10)	0.062	0.103	12.89 (3.71)	0.1	0.125	15.98 (3.47)	0.057	0.103	17.07 (3.49)	0.051	0.103c
2'FL	1.00 (0.42)	1.17 (0.41)	0.052	0.065	2.51 (1.07)	0.148	0.148	7.17 (0.95)	**0.028**	**0.047***	9.56 (0.54)	**0.012**	**0.047***	11.90 (1.44)	**0.027**	**0.047*c**
β-glucan	1.43 (0.20)	4.1 (0.88)	0.059	0.059	8.88 (1.44)	**0.044**	0.055	23.25 (1.02)	**0.003**	**0.013***	27.76 (2.65)	**0.01**	**0.017***	28.76 (2.60)	**0.009**	**0.017*a**
OFI/2'FL 50/50	1.00 (0.42)	1.28 (0.44)	0.093	0.093	1.96 (0.41)	0.06	0.074	8.68 (0.19)	**≤ 0.001**	**0.004***	10.36 (0.65)	**0.007**	**0.012***	11.72 (0.48)	**0.006**	**0.012*c**
OFI/2'FL 85/15	1.01 (0.41)	1.4 (0.44)	**0.009**	**0.021***	2.21 (0.45)	**0.007**	**0.021***	12.41 (1.91)	**0.017**	**0.021***	13.80 (2.39)	**0.023**	**0.023***	15.95 (2.30)	**0.016**	**0.021*bc**
OFI/2'L 95/5	1.01 (0.41)	1.51 (0.57)	0.086	0.086	3.37 (0.10)	**0.039**	**0.049***	14.97 (2.87)	**0.03**	**0.049***	15.93 (2.52)	**0.019**	**0.049***	18.31 (1.82)	**0.007**	**0.034*bc**
OF/2'FL	1.44 (0.21)	3.03 (0.10)	**0.027**	0.093	5.37 (1.08)	0.093	0.093	13.14 (3.62)	0.092	0.093	14.67 (3.40)	0.066	0.093	15.36 (3.30)	0.057	0.093c

GC-FID analysis of butyrate concentration in the supernatant of effluents collected from vessel 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). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with respective 0 h sampling (grey columns). Red text indicates trending towards significance. Significant differences between substrates at 48h are indicated by differing letters.
Abbreviations: OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

SCFA	Butyrate (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	1.46 (0.21)	2.87 (0.10)	**0.041**	0.088	6.49 (1.40)	0.088	0.088	13.98 (3.65)	0.083	0.088	14.87 (3.72)	0.076	0.088	15.57 (3.93)	0.076	0.088c
OF/β-glucan	1.44 (0.20)	3.78 (0.79)	0.061	0.077	8.28 (1.93)	0.082	0.082	22.38 (0.89)	**0.002**	**0.005****	23.82 (0.63)	**≤ 0.001**	**0.005****	26.53 (1.55)	**0.004**	**0.007**a
OFI/β-glucan 50/50	1.47 (0.21)	3.22 (0.46)	**0.022**	**0.022***	9.55 (1.08)	**0.021**	**0.022***	20.43 (1.48)	**0.006**	**0.013***	23.15 (1.85)	**0.008**	**0.013***	25.50 (1.59)	**0.005**	**0.013*ab**
ITF-mix/β-glucan	1.47 (0.21)	3.43 (0.61)	**0.042**	0.053	7.74 (1.87)	0.091	0.091	22.18 (0.78)	**0.001**	**0.005****	24.04 (0.96)	**0.002**	**0.005****	26.30 (1.65)	**0.005**	**0.008**a
Negative	1.44 (0.21)	1.87 (0.08)	0.148	0.148	2.32 (0.24)	0.054	0.067	2.74 (0.41)	0.051	0.067	2.95 (0.41)	**0.048**	0.067	3.21 (0.50)	**0.031**	0.067b

GC-FID analysis of butyrate concentration in the supernatant of effluents collected from vessel 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). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with respective 0 h sampling (grey columns). Red text indicates trending towards significance. Significant differences between substrates at 48h are indicated by differing letters.
Abbreviations: OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

SCFA	Total SCFA															
Time (h)	T 0	T 4			T 8			T 24			T 36			T 48		
Substrate	Mean (SE)	Mean (SE)	*P*	*Q*	Mean (SE)	*P*	*Q*	Mean (SE)	*P*	*Q*	Mean (SE)	*P*	*Q*	Mean (SE)	*P*	*Q*
OF	12.43 (1.79)	47.66 (9.44)	**0.088**	**0.088**	108.5 (10.13)	**0.01**	**0.012***	144.61 (6.49)	**0.003**	**0.008****	150.75 (8.66)	**0.005**	**0.008****	165.28 (7.39)	**0.003**	**0.008**abc**
OFI	7.81 (1.00)	17.45 (4.81)	0.128	0.128	77.62 (6.55)	**0.007**	**0.009****	112.36 (4.72)	**0.002**	**0.005****	133.24 (5.74)	**0.002**	**0.005****	148.19 (9.27)	**0.005**	**0.008**abc**
ITF-mix	12.42 (1.42)	46.83 (9.64)	0.09	0.09	101.85 (15.60)	**0.026**	**0.033***	150.37 (9.30)	**0.004**	**0.007****	158.50 (8.21)	**0.003**	**0.007****	171.20 (5.35)	**0.001**	**0.005**abc**
2'FL	7.80 (1.01)	15.02 (3.08)	0.081	0.081	71.90 (13.53)	**0.04**	0.05	107.32 (6.59)	**0.003**	**0.017***	129.40 (12.58)	**0.011**	**0.027***	142.12 (19.04)	**0.02**	**0.033*bc**
β-glucan	12.40 (1.40)	44.42 (8.05)	0.075	0.075	90.81 (3.02)	**≤ 0.001**	**0.004****	153.12 (6.27)	**0.003**	**0.006****	170.84 (8.34)	**0.004**	**0.006****	182.74 (10.10)	**0.004**	**0.006**c**
OFI/2'FL 50/50	7.67 (0.76)	15.82 (2.14)	**0.031**	**0.039***	68.51 (5.33)	**0.004**	**0.024***	104.41 (2.91)	**0.011**	**0.021***	125.72 (7.90)	**0.011**	**0.024***	140.03 (8.07)	**0.014**	**0.024*bc**
OFI/2'FL 85/15	7.79	16.05 (3.90)	0.106	0.106	74.53 (13.31)	**0.037**	**0.047***	113.03 (6.85)	**0.003**	**0.017***	122.80 (11.53)	**0.009**	**0.023***	141.78 (15.84)	**0.014**	**0.023*abc**
OFI/2'L 95/5	7.84 (0.98)	17.40 (4.74)	0.128	0.128	81.97 (8.95)	**0.014**	**0.018***	114.54 (6.97)	**0.003**	**0.008****	133.96 (5.45)	**0.002**	**0.008****	151.71 (12.20)	**0.007**	**0.012**abc**
OF/2'FL	12.40 (1.41)	56.34 (10.70)	0.066	0.066	120.08 (14.17)	**0.016**	**0.02***	157.70 (6.74)	**0.002**	**0.003****	166.93 (4.02)	**≤ 0.001**	**0.002****	176.00 (5.43)	**≤ 0.001**	**0.002**ab**

GC-FID analysis of Total SCFA concentration in the supernatant of effluents collected from vessel 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). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with respective 0 h sampling (grey columns). Red text indicates trending towards significance. Significant differences between substrates at 48h are indicated by differing letters.
Abbreviations: OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

SCFA	Total SCFA (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	12.47 (1.41)	55.13 (11.67)	0.08	0.08	122.04 (14.64)	**0.016**	**0.019***	153.75 (10.93)	**0.005**	**0.01****	163.67 (11.71)	**0.005**	**0.01****	172.38 (12.90)	**0.006**	**0.01**abc**
OF/β-glucan	12.37 (1.41)	50.18 (9.43)	0.072	0.072	95.43 (4.11)	**0.004**	**0.009****	159.52 (12.75)	**0.009**	**0.01***	166.15 (10.61)	**0.006**	**0.01****	184.68 (7.25)	**0.002**	**0.009**a**
OFI/β-glucan 50/50	12.50 (1.42)	45.85 (7.83)	0.068	0.068	99.41 (4.88)	**0.004**	**0.012***	144.45 (9.94)	**0.007**	**0.012***	161.75 (10.45)	**0.006**	**0.012***	173.03 (14.35)	**0.009**	**0.012*abc**
ITF-mix/β-glucan	12.46 (1.42)	50.53 (8.55)	0.06	0.06	99.32 (0.96)	**≤ 0.001**	**≤ 0.001****	155.14 (10.01)	**0.006**	**0.008****	166.45 (10.18)	**0.006**	**0.008****	175.63 (10.90)	**0.006**	**0.008**abc**
Negative	12.36 (1.41)	15.76 (0.55)	0.091	0.091	18.82 (1.38)	**0.003**	**0.006****	29.00 (0.34)	**0.005**	**0.006****	30.29 (0.31)	**0.004**	**0.006****	31.76 (0.36)	**0.004**	**0.006**d**

GC-FID analysis of Total SCFA concentration in the supernatant of effluents collected from vessel 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). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with respective 0 h sampling (grey columns). Red text indicates trending towards significance. Significant differences between substrates at 48h are indicated by differing letters.
Abbreviations: OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Appendix 2.2 Individual SCFA *in vitro* fermentation concentrations

Acetate							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	Donor 1	9.07	17.47	93.95	104.22	106.81	119.03
	Donor 2	6.29	33.52	67.24	86.56	87.64	97.39
	Donor 3	6.18	45.84	81.67	108.80	116.72	125.41
OFI	Donor 1	3.25	7.48	63.08	84.43	103.42	118.46
	Donor 2	2.54	8.09	46.83	67.45	81.76	86.48
	Donor 3	4.87	11.56	49.94	68.64	80.95	82.09
ITF-mix	Donor 1	9.08	17.12	98.72	111.07	111.76	123.38
	Donor 2	6.29	36.56	53.35	79.84	87.78	97.58
	Donor 3	6.22	44.14	73.58	110.88	114.77	119.43
2'FL	Donor 1	3.12	6.22	80.14	89.93	122.78	142.91
	Donor 2	2.53	8.48	37.05	67.75	78.81	80.39
	Donor 3	4.88	10.31	59.65	86.11	87.25	90.74
β-glucan	Donor 1	9.02	16.34	55.91	61.76	72.28	81.57
	Donor 2	6.29	37.26	60.01	85.18	88.53	92.26
	Donor 3	6.23	32.78	62.18	80.26	94.17	109.63
OFI/2'FL 50/50	Donor 1	3.02	6.36	72.31	79.73	102.94	124.41
	Donor 2	2.51	7.94	47.75	67.61	87.67	92.76
	Donor 3	4.87	11.25	40.77	66.37	66.72	74.27
OFI/2'FL 85/15	Donor 1	3.19	6.50	79.66	84.52	104.23	126.77
	Donor 2	2.54	7.87	41.22	68.18	69.49	76.56
	Donor 3	4.88	11.52	50.37	73.09	73.46	78.48
OFI/2'FL 95/5	Donor 1	3.13	6.11	69.02	79.77	103.66	124.80
	Donor 2	2.62	8.64	42.24	66.25	83.86	85.37
	Donor 3	4.87	11.67	56.62	74.41	74.82	83.36
OF/2'FL	Donor 1	9.08	21.40	104.79	120.83	120.17	130.98
	Donor 2	6.28	51.11	68.37	96.55	102.90	104.66
	Donor 3	6.24	48.37	98.84	120.64	124.73	129.87
ITF-mix/2fl	Donor 1	9.05	38.50	111.69	122.95	127.94	131.26
	Donor 2	6.35	50.86	71.21	96.58	100.46	102.24
	Donor 3	6.20	48.09	90.51	113.58	120.49	129.59
OF/β-glucan	Donor 1	9.02	19.05	48.31	70.03	78.54	86.89
	Donor 2	6.24	42.21	66.95	89.42	91.30	96.34
	Donor 3	6.17	41.75	67.05	113.90	115.51	122.18
OFI/β-glucan	Donor 1	9.09	18.52	62.37	70.03	75.19	75.69
	Donor 2	6.35	37.47	58.82	74.13	87.78	91.99
	Donor 3	6.20	39.82	76.31	93.91	102.17	116.53
ITF-mix/β-glucan	Donor 1	9.05	21.49	63.51	72.90	80.78	80.83
	Donor 2	6.30	45.12	63.13	84.03	92.57	95.25
	Donor 3	6.20	40.82	72.25	109.57	116.24	119.33
Negative	Donor 1	6.09	7.72	10.92	16.09	18.31	18.40
	Donor 2	4.38	8.40	10.72	15.30	16.53	18.34
	Donor 3	5.52	9.80	11.06	18.76	19.17	20.69

Propionate							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	Donor 1	4.97	9.98	21.09	29.14	29.72	32.75
	Donor 2	4.07	15.58	16.62	38.14	38.93	43.29
	Donor 3	2.36	13.41	23.89	26.49	27.91	29.40
OFI	Donor 1	3.33	4.42	20.81	23.32	26.53	32.02
	Donor 2	3.37	3.39	14.60	23.63	25.43	35.56
	Donor 3	3.04	12.70	19.76	30.34	34.51	38.65
ITF-mix	Donor 1	4.96	9.33	21.99	30.66	30.74	31.97
	Donor 2	4.01	12.37	17.06	44.70	45.08	52.39
	Donor 3	2.36	12.91	21.52	35.30	37.42	37.64
2'FL	Donor 1	3.46	3.99	10.85	17.10	20.84	24.33
	Donor 2	3.36	3.74	8.40	17.65	22.46	23.14
	Donor 3	3.04	8.80	12.11	21.91	27.39	29.15
β-glucan	Donor 1	4.95	9.73	28.84	55.11	56.60	58.09
	Donor 2	4.06	11.68	17.15	54.86	59.48	61.76
	Donor 3	2.36	13.15	21.70	52.44	58.19	58.64
OFI/2'FL 50/50	Donor 1	3.20	4.48	15.05	28.03	31.51	32.34
	Donor 2	3.34	3.77	10.06	20.95	28.56	28.93
	Donor 3	3.04	9.85	13.71	24.49	28.65	32.22
OFI/2'FL 85/15	Donor 1	3.38	4.60	17.50	24.56	26.00	30.91
	Donor 2	3.30	3.42	10.69	20.25	21.34	29.72
	Donor 3	3.05	10.04	17.51	31.26	32.50	35.06
OFI/2'FL 95/5	Donor 1	3.46	5.03	16.38	24.07	27.50	33.56
	Donor 2	3.36	3.66	23.01	23.61	30.83	35.65
	Donor 3	3.05	12.56	18.53	30.61	33.40	37.46
OF/2'FL	Donor 1	4.93	10.79	23.86	25.19	30.52	31.78
	Donor 2	4.02	16.00	19.35	30.80	37.28	51.42
	Donor 3	2.33	12.25	28.91	29.68	31.18	33.21
ITF-mix/2fl	Donor 1	4.98	10.67	26.75	29.57	33.27	38.10
	Donor 2	4.09	16.72	20.02	30.49	32.64	36.11
	Donor 3	2.37	11.93	26.48	28.12	31.61	33.14
OF/β-glucan	Donor 1	4.95	9.91	28.59	44.69	45.69	58.22
	Donor 2	4.04	13.11	27.34	50.06	51.57	62.79
	Donor 3	2.37	13.17	23.24	43.32	44.38	48.03
OFI/β-glucan	Donor 1	5.00	9.42	25.42	41.95	44.75	46.92
	Donor 2	4.08	11.03	25.41	44.60	52.42	55.76
	Donor 3	2.39	11.65	21.27	47.47	53.48	55.70
ITF-mix/β-glucan	Donor 1	5.01	9.88	26.82	44.08	44.74	47.88
	Donor 2	4.02	12.04	26.87	48.13	49.37	57.79
	Donor 3	2.39	11.94	22.19	40.19	43.54	46.90
Negative	Donor 1	4.95	5.23	6.13	6.37	6.41	7.01
	Donor 2	4.02	4.38	4.54	5.23	5.37	5.58
	Donor 3	2.70	3.27	3.51	5.32	5.50	5.79

Butyrate							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	Donor 1	1.03	2.10	8.02	21.00	21.93	23.18
	Donor 2	1.68	2.10	7.90	10.30	12.46	13.64
	Donor 3	1.63	2.99	5.11	9.18	10.14	11.73
OFI	Donor 1	0.60	0.96	1.24	9.79	12.88	16.18
	Donor 2	0.57	0.95	3.14	11.86	15.79	15.57
	Donor 3	1.85	2.80	3.46	17.61	18.45	19.56
ITF-mix	Donor 1	1.04	1.97	8.55	19.96	21.38	22.76
	Donor 2	1.67	2.13	4.85	7.40	9.51	10.71
	Donor 3	1.64	3.95	5.92	11.31	17.05	17.74
2'FL	Donor 1	0.62	0.87	1.81	5.53	10.03	12.39
	Donor 2	0.55	0.67	1.11	8.83	10.17	14.11
	Donor 3	1.84	1.98	4.60	7.15	8.47	9.20
β-glucan	Donor 1	1.03	2.39	11.48	23.92	27.21	27.93
	Donor 2	1.64	5.28	8.66	21.25	23.47	24.72
	Donor 3	1.62	4.64	6.50	24.59	32.59	33.62
OFI/2'FL 50/50	Donor 1	0.61	0.72	2.05	8.52	11.52	12.57
	Donor 2	0.56	0.98	1.21	8.46	9.29	11.67
	Donor 3	1.85	2.14	2.63	9.07	10.28	10.91
OFI/2'FL 85/15	Donor 1	0.63	0.94	1.99	10.11	11.63	14.60
	Donor 2	0.56	0.97	1.57	10.92	11.19	12.81
	Donor 3	1.84	2.27	3.07	16.20	18.57	20.44
OFI/2'FL 95/5	Donor 1	0.62	1.05	3.55	12.33	13.67	17.51
	Donor 2	0.58	0.85	3.30	11.87	13.16	15.63
	Donor 3	1.84	2.64	3.24	20.70	20.97	21.79
OF/2'FL	Donor 1	1.02	3.12	7.52	19.49	20.49	20.77
	Donor 2	1.67	3.15	4.10	6.94	8.72	9.38
	Donor 3	1.64	2.83	4.50	12.98	14.80	15.93
ITF-mix/2fl	Donor 1	1.04	3.04	9.20	21.01	22.10	23.16
	Donor 2	1.70	2.86	5.71	8.75	9.70	10.00
	Donor 3	1.64	2.71	4.56	12.19	12.82	13.55
OF/β-glucan	Donor 1	1.03	2.39	11.55	22.31	23.82	28.20
	Donor 2	1.66	5.12	8.40	20.89	22.72	23.44
	Donor 3	1.62	3.84	4.88	23.95	24.91	27.95
OFI/β-glucan	Donor 1	1.05	2.33	10.43	20.71	24.83	27.17
	Donor 2	1.69	3.89	7.39	17.73	19.47	22.33
	Donor 3	1.66	3.44	10.82	22.83	25.16	27.01
ITF-mix/β-glucan	Donor 1	1.04	2.35	10.85	21.93	24.59	27.83
	Donor 2	1.71	4.45	7.97	20.97	22.17	23.00
	Donor 3	1.65	3.50	4.40	23.63	25.35	28.07
Negative	Donor 1	0.83	1.40	1.72	2.02	2.32	2.88
	Donor 2	1.12	1.18	2.33	2.98	3.20	3.40
	Donor 3	1.75	2.16	2.24	2.57	2.66	2.94

Total SCFA							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	Donor 1	9.07	17.47	93.95	104.22	106.81	119.03
	Donor 2	6.29	33.52	67.24	86.56	87.64	97.39
	Donor 3	6.18	45.84	81.67	108.80	116.72	125.41
OFI	Donor 1	3.25	7.48	63.08	84.43	103.42	118.46
	Donor 2	2.54	8.09	46.83	67.45	81.76	86.48
	Donor 3	4.87	11.56	49.94	68.64	80.95	82.09
ITF-mix	Donor 1	9.08	17.12	98.72	111.07	111.76	123.38
	Donor 2	6.29	36.56	53.35	79.84	87.78	97.58
	Donor 3	6.22	44.14	73.58	110.88	114.77	119.43
2'FL	Donor 1	3.12	6.22	80.14	89.93	122.78	142.91
	Donor 2	2.53	8.48	37.05	67.75	78.81	80.39
	Donor 3	4.88	10.31	59.65	86.11	87.25	90.74
β-glucan	Donor 1	9.02	16.34	55.91	61.76	72.28	81.57
	Donor 2	6.29	37.26	60.01	85.18	88.53	92.26
	Donor 3	6.23	32.78	62.18	80.26	94.17	109.63
OFI/2'FL 50/50	Donor 1	3.02	6.36	72.31	79.73	102.94	124.41
	Donor 2	2.51	7.94	47.75	67.61	87.67	92.76
	Donor 3	4.87	11.25	40.77	66.37	66.72	74.27
OFI/2'FL 85/15	Donor 1	3.19	6.50	79.66	84.52	104.23	126.77
	Donor 2	2.54	7.87	41.22	68.18	69.49	76.56
	Donor 3	4.88	11.52	50.37	73.09	73.46	78.48
OFI/2'FL 95/5	Donor 1	3.13	6.11	69.02	79.77	103.66	124.80
	Donor 2	2.62	8.64	42.24	66.25	83.86	85.37
	Donor 3	4.87	11.67	56.62	74.41	74.82	83.36
OF/2'FL	Donor 1	9.08	21.40	104.79	120.83	120.17	130.98
	Donor 2	6.28	51.11	68.37	96.55	102.90	104.66
	Donor 3	6.24	48.37	98.84	120.64	124.73	129.87
ITF-mix/2fl	Donor 1	9.05	38.50	111.69	122.95	127.94	131.26
	Donor 2	6.35	50.86	71.21	96.58	100.46	102.24
	Donor 3	6.20	48.09	90.51	113.58	120.49	129.59
OF/β-glucan	Donor 1	9.02	19.05	48.31	70.03	78.54	86.89
	Donor 2	6.24	42.21	66.95	89.42	91.30	96.34
	Donor 3	6.17	41.75	67.05	113.90	115.51	122.18
OFI/β-glucan	Donor 1	9.09	18.52	62.37	70.03	75.19	75.69
	Donor 2	6.35	37.47	58.82	74.13	87.78	91.99
	Donor 3	6.20	39.82	76.31	93.91	102.17	116.53
ITF-mix/β-glucan	Donor 1	9.05	21.49	63.51	72.90	80.78	80.83
	Donor 2	6.30	45.12	63.13	84.03	92.57	95.25
	Donor 3	6.20	40.82	72.25	109.57	116.24	119.33
Negative	Donor 1	6.09	7.72	10.92	16.09	18.31	18.40
	Donor 2	4.38	8.40	10.72	15.30	16.53	18.34
	Donor 3	5.52	9.80	11.06	18.76	19.17	20.69

Individual donor SCFA data concentrations – acetate, propionate, butyrate and total SCFA across 0, 4, 8, 24, 36 and 48 h fermentation. Concentration in (mmol l-1).

Appendix 2.3 Mean bacterial populations using pH-controlled *in vitro* batch culture fermentation at 0, 4, 8, 24, 36 and 48 h

Probe	Total Bacteria (Eub I-II-III)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	**OF**	8.36 (0.15)	8.90 (0.13)	0.068	**0.014***	9.29 (0.04)	**0.015**	**0.004****	9.02 (0.11)	**0.005**	**0.003****	8.85 (0.12)	**0.006**	**0.003****	8.74 (0.13)	**0.01**
OFI	**OFI**	8.26 (0.13)	8.62 (0.16)	0.159	0.278	8.98 (0.10)_	**0.012**	0.054	8.75 (0.08)	**0.021**	0.054	8.53 (0.05)	0.25	0.328	8.43 (0.08)	0.508
ITF-mix	**ITF-mix**	8.36 (0.15)	8.82 (0.16)	**0.046**	**0.048***	9.29 (0.03)	**0.018**	**0.032***	9.12 (0.13)	**0.001**	**0.005***	8.94 (0.14)	**0.009**	**0.023***	8.81 (0.17)	**0.036**
2'FL	**2'FL**	8.27 (0.12)	8.48 (0.10)	0.145	0.247	8.90 (0.20)	0.145	0.247	8.78 (0.10)	0.075	0.247	8.58 (0.06)	0.215	0.247	8.53 (0.04)	0.236
β-glucan	**β-glucan**	8.36 (0.14)	8.80 (0.09)	0.087	0.073	9.14 (0.06)	**0.03**	**0.042***	9.06 (0.11)	**0.007**	**0.028***	8.93 (0.08)	**0.02**	**0.042***	8.84 (0.03)	0.063
OFI/2'FL 50/50	**OFI/2'FL 50/50**	8.28 (0.12)	8.486	0.171	0.224	8.89 (0.17)	0.141	0.224	8.70 (0.08)	**0.05**	0.224	8.61 (0.04)	0.144	0.224	8.43 (0.06)	0.479
OFI/2'FL 85/15	**OFI/2'FL 85/15**	8.28 (0.13)	8.65 (0.12)	0.134	0.23	8.86 (0.16)	0.158	0.23	8.69 (0.10)	0.175	0.23	8.56 (0.05)	0.091	0.23	8.48 (0.04)	0.373
OFI/2'L 95/5	**OFI/2'L 95/5**	8.29 (0.13)	8.74 (0.12)	0.148	0.169	8.90 (0.19)	0.161	0.169	8.86 (0.70)	**0.023**	0.119	8.70 (0.04)	0.068	0.169	8.55 (0.07)	0.127
OF/2'FL	**OF/2'FL**	8.36 (0.15)	8.87 (0.14)	0.057	**0.012***	9.36 (0.03)	**0.014**	**0.005****	9.11 (0.09)	**0.013**	**0.005****	8.99 (0.14)	**0.013**	**0.005****	8.85 (0.18)	**0.029**

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: total bacteria (Eub338 I-II-III). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Total Bacteria (Eub I-II-II) (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	8.36 (0.14)	8.87 (0.14)	0.06	**0.013***	9.34 (0.01)	**0.019**	**0.006***	9.09 (0.10)	**0.004**	**0.004****	8.92 (0.11)	**0.01**	**0.005****	8.86 (0.10)	**0.021**	**0.006****
OF/β-glucan	8.36 (0.15)	8.87 (0.15)	0.125	**0.026***	9.33 (0.05)	**0.01**	**0.005***	9.09 (0.12)	**0.004**	**0.004****	8.98 (0.10)	**0.025**	**0.007****	8.90 (0.12)	**0.014**	**0.005****
OFI/β-glucan 50/50	8.36 (0.15)	8.84 (0.16)	0.138	0.058	9.29 (0.04)	**0.014**	**0.010***	9.10 (0.11)	**0.006**	**0.010****	8.93 (0.11)	**0.011**	**0.010****	8.82 (0.10)	**0.042**	**0.022***
ITF-mix/β-glucan	8.36 (0.15)	8.85 (0.16)	0.145	**0.031***	9.29 (0.05)	**0.019**	**0.005***	9.12 (0.11)	**0.003**	**0.003****	8.99 (0.09)	**0.02**	**0.005****	8.90 (0.09)	**0.019**	**0.005****
Negative	8.36 (0.15)	8.44 (0.07)	0.286	0.645	8.32 (0.06)	0.369	0.645	8.06 (0.03)	0.92	0.966	7.84 (0.03)	0.712	0.935	7.74 (0.05)	0.054	0.281

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: total bacteria (Eub338 I-II-III). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Bif 164															
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	7.15 (0.21)	8.39 (0.26)	**0.004**	**0.020***	8.99 (0.04)	**0.011**	**0.027***	8.72 (0.20)	**0.029**	**0.033***	8.64 (0.16)	**0.03**	**0.033***	8.55 (0.17)	**0.033**	**0.033***
OFI	7.14 (0.13)	7.91 (0.23)	**0.023**	**0.023***	8.61 (0.01)	**0.009**	**0.023***	8.44 (0.04)	**0.016**	**0.023***	8.26 (0.11)	**0.023**	**0.023***	8.19 (0.14)	**0.023**	**0.023***
ITF-mix	7.15 (0.20)	8.22 (0.29)	**0.019**	**0.019***	8.82 (0.16)	**0.002**	**0.012***	8.70 (0.23)	**0.015**	**0.019***	8.65 (0.21)	**0.013**	**0.019***	8.60 (0.23)	**0.018**	**0.019***
2'FL	7.14 (0.12)	7.78 (0.23)	**0.036**	**0.045***	8.37 (0.14)	0.07	0.07	8.48 (0.10)	**0.01**	**0.020***	8.40 (0.10)	**0.011**	**0.020***	8.37 (0.09)	**0.012**	**0.020***
β-glucan	7.16 (0.20)	8.19 (0.08)	**0.041**	**0.041***	8.53 (0.08)	**0.034**	**0.041***	8.50 (0.04)	**0.028**	**0.041***	8.43 (0.05)	**0.031**	**0.041***	8.41 (0.06)	**0.033**	**0.041***
OFI/2'FL 50/50	7.14 (0.12)	7.90 (0.19)	**0.011**	**0.014***	8.47 (0.27)	**0.022**	**0.022***	8.47 (0.02)	**0.009**	**0.014***	8.34 (0.15)	**0.006**	**0.014***	8.27 (0.12)	**0.005**	**0.014***
OFI/2'FL 85/15	7.13 (0.12)	7.89 (0.35)	0.107	0.107	8.38 (0.30)	**0.04**	0.05	8.41 (0.09)	**0.019**	**0.034***	8.28 (0.05)	**0.017**	**0.034***	8.26 (0.11)	**0.02**	**0.034***
OFI/2'L 95/5	7.15 (0.12)	8.01 (0.41)	0.105	0.105	8.46 (0.25)	**0.022**	**0.027***	8.51 (0.04)	**0.011**	**0.018***	8.48 (0.02)	**0.01**	**0.018***	8.37 (0.03)	**0.006**	**0.018***
OF/2'FL	7.15 (0.21)	8.44 (0.24)	**0.008**	**0.024***	9.01 (0.10)	**0.009**	**0.024***	8.83 (0.23)	**0.021**	**0.029***	8.78 (0.24)	**0.023**	**0.029***	8.71 (0.26)	**0.03**	**0.030***

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Bifidobacterium* spp. (Bif164). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Bif164 (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	7.16 (0.21)	8.42 (0.25)	**0.012**	**0.028 ***	9.15 (0.02)	**0.01**	**0.028 ***	8.79 (0.21)	**0.028**	**0.028 ***	8.68 (0.16)	**0.023**	**0.028 ***	8.67 (0.16)	**0.023**	**0.028 ***
OF/β-glucan	7.16 (0.21)	8.49 (0.26)	**0.002**	**0.008 ***	8.99 (0.14)	**0.01**	**0.019 ***	8.83 (0.20)	**0.022**	**0.022 ***	8.71 (0.18)	**0.015**	**0.019 ***	8.69 (0.18)	**0.014**	**0.019 ***
OFI/β-glucan 50/50	7.16 (0.21)	8.42 (0.26)	**0.002**	**0.012 ***	9.01 (0.06)	**0.013**	**0.019 ***	8.84 (0.15)	**0.019**	**0.019 ***	8.62 (0.14)	**0.018**	**0.019 ***	8.61 (0.14)	**0.017**	**0.019 ***
ITF-mix/β-glucan	7.16 (0.21)	8.41 (0.26)	**0.002**	**0.010 ***	8.96 (0.14)	**0.01**	**0.017 ***	8.89 (0.17)	**0.018**	**0.018 ***	8.74 (0.14)	**0.013**	**0.017 ***	8.69 (0.13)	**0.014**	**0.017 ***
Negative	7.16 (0.21)	7.30 (0.22)	0.051	0.257	7.39 (0.27)	0.354	0.469	7.13 (0.22)	0.56	0.56	7.03 (0.30)	0.375	0.469	6.98 (0.31)	0.309	0.469

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Bifidobacterium* spp. (Bif164). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Lab158															
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	6.36 (0.09)	6.48 (0.10)	0.263	0.659	7.13 (0.25)	0.141	0.659	6.60 (0.28)	0.583	0.751	6.41 (0.35)	0.921	0.921	6.03 (0.48)	0.601	0.751
OFI	6.25 (0.18)	6.56 (0.02)	0.272	0.272	6.73 (0.14)	0.152	0.253	6.27 (0.18)	0.27	0.272	5.95 (0.17)	**0.02**	0.1	5.98 (0.08)	0.148	0.253
ITF-mix	6.37 (0.09)	6.39 (0.09)	0.423	0.528	6.84 (0.14)	0.081	0.403	6.48 (0.02)	0.245	0.478	6.34 (0.02)	0.727	0.727	6.10 (0.10)	0.287	0.478
2'FL	6.26 (0.19)	6.54 (0.12)	0.146	0.365	6.63 (0.14)	0.259	0.432	6.10 (0.27)	0.417	0.521	6.25 (0.26)	0.873	0.873	5.79 (0.18)	**0.004**	**0.018***
β-glucan	6.38 (0.09)	6.44 (0.11)	0.464	0.464	6.60 (0.26)	0.389	0.464	6.20 (0.03)	0.107	0.179	6.11 (0.04)	0.104	0.179	6.03 (0.04)	0.098	0.179
OFI/2'FL 50/50	6.27 (0.18)	6.58 (0.07)	0.213	0.266	6.76 (0.20)	0.211	0.266	6.19 (0.18)	**0.02**	0.052	6.32 (0.13)	0.809	0.809	5.68 (0.27)	**0.021**	0.052
OFI/2'FL 85/15	6.26 (0.19)	6.54 (0.02)	0.259	0.604	6.51 (0.03)	0.362	0.604	6.32 (0.26)	0.713	0.713	6.19 (0.24)	0.692	0.713	5.85 (0.23)	0.05	0.251
OFI/2'L 95/5	6.25 (0.21)	6.67 (0.07)	0.26	0.325	6.57 (0.16)	0.235	0.325	6.17 (0.10)	0.549	0.549	5.76 (0.32)	0.056	0.14	5.78 (0.25)	**0.007**	**0.033***
OF/2'FL	6.36 (0.08)	6.53 (0.08)	**0.004**	**0.019***	7.02 (0.20)	0.141	0.352	6.56 (0.25)	0.625	0.781	6.32 (0.16)	0.868	0.868	5.97 (0.20)	0.242	0.403

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Lactobacillus/Enterococcus* spp. (Lab158). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Lab158 (continued)															
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	6.36 (0.09)	6.59 (0.05)	0.109	0.273	7.08 (0.17)	0.063	0.273	6.60 (0.20)	0.49	0.649	6.30 (0.12)	0.787	0.787	6.21 (0.12)	0.519	0.649
OF/β-glucan	6.36 (0.09)	6.57 (0.01)	0.123	0.308	7.05 (0.24)	0.121	0.308	6.82 (0.20)	0.445	0.564	6.49 (0.11)	0.564	0.564	6.20 (0.14)	0.503	0.564
OFI/β-glucan 50/50	6.37 (0.09)	6.58 (0.05)	0.236	0.589	6.94 (0.19)	0.132	0.589	6.73 (0.45)	0.574	0.717	6.33 (0.10)	0.848	0.848	6.12 (0.19)	0.401	0.669
ITF-mix/β-glucan	6.36 (0.09)	6.60 (0.04)	0.168	0.451	6.72 (0.11)	0.18	0.451	6.79 (0.46)	0.509	0.637	6.29 (0.16)	0.762	0.762	6.16 (0.16)	0.494	0.637
Negative	6.38 (0.09)	6.37 (0.11)	0.8	0.8	6.11 (0.32)	0.469	0.587	5.46 (0.20)	**0.029**	0.14	5.32 (0.34)	0.084	0.14	5.22 (0.32)	0.069	0.14

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Lactobacillus/Enterococcus* spp. (Lab158). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Bac303															
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	6.77 (0.07)	7.23 (0.31)	0.168	0.421	7.71 (0.48)	0.15	0.421	7.28 (0.40)	0.353	0.588	7.08 (0.35)	0.587	0.733	6.96 (0.36)	0.813	0.813
OFI	6.66 (0.23)	7.13 (0.24)	0.161	0.201	7.72 (0.19)	0.086	0.143	7.54 (0.02)	0.059	0.143	7.26 (0.15)	0.069	0.143	7.06 (0.23)	0.233	0.233
ITF-mix	6.77 (0.07)	7.17 (0.29)	0.24	0.535	7.59 (0.38)	0.166	0.535	7.23 (0.36)	0.321	0.535	6.93 (0.28)	0.679	0.849	6.74 (0.26)	0.92	0.92
2'FL	6.67 (0.21)	6.97 (0.10)	0.211	0.211	7.48 (0.36)	0.137	0.211	7.46 (0.06)	**0.039**	0.197	7.24 (0.09)	0.201	0.211	7.04 (0.05)	0.195	0.211
β-glucan	6.77 (0.07)	7.17 (0.25)	0.208	0.216	8.08 (0.46)	0.108	0.179	7.52 (0.20)	0.092	0.179	7.49 (0.22)	0.107	0.179	7.28 (0.23)	0.216	0.216
OFI/2'FL 50/50	6.67 (0.22)	6.89 (0.08)	0.414	0.414	7.40 (0.20)	0.19	0.237	7.47 (0.08)	0.074	0.232	7.32 (0.02)	0.109	0.232	7.03 (0.15)	0.139	0.232
OFI/2'FL 85/15	6.66 (0.23)	7.13 (0.24)	0.189	0.189	7.58 (0.20)	0.13	0.163	7.40 (0.07)	0.13	0.163	7.43 (0.03)	0.063	0.153	7.20 (0.06)	0.092	0.153
OFI/2'L 95/5	6.66 (0.24)	7.01 (0.15)	0.34	0.34	7.65 (0.18)	0.089	0.112	7.51 (0.11)	**0.033**	0.082	7.36 (0.04)	0.076	0.112	7.03 (0.21)	**0.006**	**0.028***
OF/2'FL	6.77 (0.07)	7.18 (0.34)	0.285	0.641	7.82 (0.34)	0.092	0.458	7.11 (0.28)	0.384	0.641	6.84 (0.27)	0.836	0.836	6.64 (0.27)	0.728	0.836

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: most *Bacteroidacae* and *Prevotellaceae* (Bac303). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Bac303 (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	6.78 (0.07)	7.06 (0.32)	0.375	0.469	7.80 (0.52)	0.184	0.469	7.36 (0.40)	0.261	0.469	7.18 (0.32)	0.338	0.469	6.71 (0.36)	0.886	0.886
OF/β-glucan	6.78 (0.07)	7.18 (0.27)	0.21	0.21	8.05 (0.40)	0.089	0.148	7.69 (0.31)	0.077	0.148	7.48 (0.18)	0.052	0.148	7.24 (0.20)	0.173	0.21
OFI/β-glucan 50/50	6.78 (0.06)	7.07 (0.)22	0.215	0.215	8.26 (0.43)	0.082	0.166	7.79 (0.38)	0.1	0.166	7.450 (0.24)	0.085	0.166	7.33 (0.30)	0.188	0.215
ITF-mix/β-glucan	6.78 (0.06)	7.09 (0.25)	0.268	0.335	8.10 (0.43)	0.097	0.182	7.61 (0.23)	0.067	0.182	7.46 (0.26)	0.109	0.182	7.09 (0.29)	0.426	0.426
Negative	6.74 (0.05)	6.89 (0.21)	0.392	0.49	6.99 (0.19)	0.382	0.49	6.78 (0.14)	0.704	0.704	6.44 (0.18)	0.175	0.438	5.93 (0.27)	**0.034**	0.168

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: most *Bacteroidacae* and *Prevotellaceae* (Bac303). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Erec482														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	8.062 (0.11)	8.37 (0.10)	0.074	0.124	8.68 (0.11)	**0.013**	0.063	8.50 (0.07)	**0.027**	0.068	8.22 (0.11)	0.14	0.175	7.98 (0.14)	0.813	0.813
OFI	7.79 (0.07)	8.15 (0.18)	0.242	0.559	8.14 (0.38)	0.45	0.563	7.92 (0.32)	0.728	0.728	7.49 (0.20)	0.336	0.559	7.25 (0.30)	0.233	0.233
ITF-mix	8.06 (0.10)	8.35 (0.16)	**0.037**	0.092	8.91 (0.07)	**0.03**	0.092	8.68 (0.16)	0.059	0.099	8.39 (0.17)	0.145	0.177	8.16 (0.11)	0.92	0.92
2'FL	7.79 (0.08)	8.01 (0.10)	0.235	0.392	8.03 (0.43)	0.636	0.772	7.92 (0.38)	0.772	0.772	7.47 (0.08)	0.16	0.392	7.03 (0.26)	0.195	0.211
β-glucan	8.06 (0.10)	8.25 (0.19)	0.272	0.272	8.71 (0.05)	**0.023**	0.065	8.62 (0.26)	0.081	0.127	8.47 (0.23)	0.102	0.127	8.31 (0.14)	0.216	0.216
OFI/2'FL 50/50	7.80 (0.08)	7.92 (0.15)	0.452	0.728	8.05 (0.36)	0.582	0.728	7.84 (0.33)	0.9	0.9	7.61 (0.20)	0.527	0.728	7.22 (0.28)	0.139	0.232
OFI/2'FL 85/15	7.81 (0.08)	8.12 (0.20)	0.292	0.467	8.15 (0.27)	0.374	0.467	7.89 (0.33)	0.819	0.819	7.54 (0.19)	0.345	0.467	7.34 (0.27)	0.092	0.153
OFI/2'L 95/5	7.80 (0.08)	8.18 (0.20)	0.238	0.546	8.26 (0.31)	0.327	0.546	8.18 (0.26)	0.298	0.546	7.82 (0.26)	0.946	0.946	7.34 (0.44)	**0.006**	**0.028 ***
OF/2'FL	8.05 (0.10)	8.33 (0.10)	**0.044**	0.055	8.88 (0.09)	**0.03**	**0.050 ***	8.56 (0.07)	**0.024**	**0.050 ***	8.30 (0.04)	**0.01**	**0.049 ***	7.95 (0.10)	0.728	0.836

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Clostridium coccoides-Eubacterium rectale* group (Erec482). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Erec482 (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	8.07 (0.01)	8.35 (0.11)	0.107	0.134	8.64 (0.09)	0.083	0.134	8.53 (0.06)	0.091	0.134	8.31 (0.11)	0.067	0.134	8.16 (0.11)	0.886	0.886
OF/β-glucan	8.05 (0.10)	8.22 (0.11)	0.167	0.167	8.70 (0.01)	**0.021**	0.051	8.49 (0.07)	**0.02**	0.051	8.37 (0.05)	0.103	0.148	8.24 (0.34)	0.173	0.21
OFI/β-glucan 50/50	8.06 (0.11)	8.24 (0.14)	0.198	0.248	8.76 (0.06)	**0.049**	0.121	8.47 (0.18)	0.091	0.152	8.38 (0.14)	**0.037**	0.121	8.12 (0.13)	0.188	0.215
ITF-mix/β-glucan	8.06 (0.11)	8.27 (0.16)	0.19	0.19	8.74 (0.06)	**0.01**	**0.024 ***	8.43 (0.07)	**0.013**	**0.024 ***	8.37 (0.08)	**0.014**	**0.024 ***	8.22 (0.07)	0.426	0.426
Negative	8.04 (0.10)	8.06 (0.04)	0.86	0.86	7.86 (0.08)	0.399	0.499	7.34 (0.14)	0.06	0.124	6.93 (0.23)	0.074	0.124	6.59 (0.22)	**0.034**	0.168

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Clostridium coccoides-Eubacterium rectale* group (Erec482). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Rrec584															
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	6.77 (0.17)	7.45 (0.28)	0.137	0.342	7.63 (0.21)	0.131	0.342	7.49 (0.14)	0.278	0.463	6.89 (0.29)	0.721	0.721	6.69 (0.30)	0.813	0.813
OFI	6.73 (0.06)	6.99 (0.20)	0.358	0.665	6.98 (0.48)	0.665	0.665	7.10 (0.31)	0.414	0.665	6.50 (0.39)	0.651	0.665	6.10 (0.42)	0.233	0.233
ITF-mix	6.78 (0.16)	7.54 (0.41)	0.11	0.184	7.84 (0.22)	**0.017**	0.087	7.59 (0.25)	0.087	0.184	7.18 (0.24)	0.232	0.29	6.75 (0.19)	0.92	0.92
2'FL	6.75 (0.06)	6.92 (0.05)	0.235	0.392	6.97 (0.33)	0.571	0.714	6.54 (0.56)	0.74	0.74	5.96 (0.34)	0.136	0.341	5.41 (0.28)	0.195	0.211
β-glucan	6.79 (0.23)	7.48 (0.48)	0.159	0.159	8.15 (0.08)	**0.004**	**0.019 ***	8.14 (0.31)	**0.029**	0.061	7.77 (0.25)	**0.037**	0.061	7.25 (0.31)	0.216	0.216
OFI/2'FL 50/50	6.75 (0.05)	6.87 (0.08)	0.342	0.57	7.25 (0.35)	0.328	0.57	6.66 (0.40)	0.854	0.854	6.58 (0.20)	0.497	0.622	6.01 (0.29)	0.139	0.232
OFI/2'FL 85/15	6.73 (0.06)	7.04 (0.23)	0.373	0.513	7.16 (0.36)	0.379	0.513	7.13 (0.33)	0.411	0.513	6.57 (0.36)	0.727	0.727	6.28 (0.29)	0.092	0.153
OFI/2'L 95/5	6.74 (0.05)	7.19 (0.03)	0.339	0.565	7.36 (0.39)	0.282	0.565	6.99 (0.25)	0.475	0.594	6.54 (0.33)	0.626	0.626	6.24 (0.36)	**0.006**	**0.028 ***
OF/2'FL	6.77 (0.16)	7.29 (0.34)	0.113	0.189	7.74 (0.15)	**0.002**	**0.012 ***	7.44 (0.03)	0.051	0.127	7.02 (0.25)	0.514	0.566	6.56 (0.21)	0.728	0.836

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Roseburia* (Rrec584). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Rrec584 (continued)															
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	6.77 (0.16)	7.43 (0.32)	0.052	0.065	7.80 (0.24)	**0.006**	**0.014***	7.57 (0.20)	**0.003**	**0.013***	7.28 (0.10)	**0.046**	0.065	6.74 (0.22)	0.886	0.886
OF/β-glucan	6.78 (0.16)	7.46 (0.37)	0.087	0.087	8.05 (0.09)	**0.003**	**0.013***	7.41 (0.27)	**0.046**	0.073	7.29 (0.27)	0.051	0.073	6.51 (0.17)	0.173	0.21
OFI/β-glucan 50/50	6.77 (0.17)	7.49 (0.35)	0.063	0.104	7.66 (0.27)	**0.023**	0.089	7.21 (0.09)	**0.036**	0.089	6.99 (0.23)	0.24	0.3	6.53 (0.32)	0.188	0.215
ITF-mix/β-glucan	6.77 (0.16)	7.49 (0.40)	0.103	0.171	8.09 (0.20)	**0.001**	**0.004***	7.47 (0.24)	0.086	0.171	7.01 (0.24)	0.221	0.276	6.67 (0.21)	0.426	0.426
Negative	6.76 (0.15)	6.84 (0.09)	0.468	0.468	6.57 (0.15)	0.38	0.468	5.65 (0.52)	0.169	0.281	5.30 (0.52)	0.144	0.281	5.04 (0.44)	**0.034**	0.168

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Roseburia* (Rrec584). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Ato291														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	6.57 (0.05)	7.54 (0.17)	**0.036**	**0.036***	7.96 (0.13)	**0.015**	**0.018***	7.65 (0.06)	**≤ 0.001**	**0.002****	7.52 (0.09)	**0.002****	**0.003**	7.38 (0.05)	0.054	**≤ 0.001**
OFI	6.54 (0.21)	7.56 (0.15)	0.105	0.131	7.84 (0.06)	**0.029**	0.073	7.57 (0.17)	**0.002**	**0.008****	7.36 (0.19)	**0.044**	0.073	7.04 (0.19)	0.186	0.188
ITF-mix	6.57 (0.05)	7.46 (0.18)	0.05	0.05	7.91 (0.16)	**0.024**	**0.043***	7.63 (0.11)	**0.02**	**0.043***	7.38 (0.09)	**0.026**	**0.043***	7.26 (0.08)	0.084	**0.037**
2'FL	6.54 (0.22)	7.16 (0.11)	0.178	0.178	7.55 (0.27)	0.167	0.178	7.61 (0.06)	**0.03**	0.152	7.28 (0.09)	0.11	0.178	7.11 (0.11)	0.112	0.177
β-glucan	6.57 (0.05)	7.55 (0.07)	**0.014**	**0.044***	7.60 (0.09)	**0.017**	**0.044***	7.28 (0.11)	**0.049**	0.068	7.21 (0.15)	0.08	0.08	7.14 (0.09)	0.093	0.054
OFI/2'FL 50/50	6.54 (0.20)	7.27 (0.16)	0.166	0.415	7.63 (0.30)	0.159	0.415	7.09 (0.20)	0.304	0.443	6.82 (0.33)	0.658	0.658	6.73 (0.02)	0.115	0.354
OFI/2'FL 85/15	6.54 (0.20)	7.39 (0.37)	0.175	0.438	7.57 (0.29)	0.156	0.438	7.07 (0.27)	0.374	0.624	6.77 (0.26)	0.658	0.714	6.75 (0.36)	0.358	0.714
OFI/2'L 95/5	6.54 (0.20)	7.46 (0.29)	0.2	0.2	7.65 (0.18)	0.091	0.152	7.65 (0.09)	**0.012**	0.062	7.33 (0.17)	0.056	0.14	7.02 (0.27)	0.266	0.188
OF/2'FL	6.56 (0.05)	7.58 (0.13)	**0.018**	**0.018***	7.89 (0.11)	**0.015**	**0.018***	7.61 (0.02)	**0.005**	**0.017***	7.54 (0.03)	**0.007**	**0.017***	7.37 (0.06)	0.057	**0.01**

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Atopobium* cluster (ato291). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Ato291 (continued)															
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	6.57 (0.06)	7.53 (0.11)	**0.016**	**0.016***	7.76 (0.07)	**0.003**	**0.005****	7.65 (0.12)	**0.007**	**0.009****	7.47 (0.05)	**≤ 0.001**	**0.003****	7.41 (0.05)	0.053	**0.002**
OF/β-glucan	6.58 (0.06)	7.50 (0.23)	0.074	0.074	7.93 (0.03)	**0.005**	**0.013***	7.75 (0.14)	**0.026**	**0.032***	7.65 (0.11)	**0.017**	**0.028***	7.45 (0.07)	0.074	**0.005**
OFI/β-glucan 50/50	6.57 (0.05)	7.50 (0.22)	0.061	0.091	7.77 (0.05)	**0.008**	**0.039***	7.61 (0.12)	**0.024**	0.059	7.40 (0.19)	0.073	0.091	7.27 (0.23)	0.228	0.125
ITF-mix/β-glucan	6.57 (0.05)	7.48 (0.23)	0.073	0.073	7.87 (0.04)	**0.004**	**0.020***	7.73 (0.09)	**0.013**	**0.020***	7.64 (0.09)	**0.016**	**0.020***	7.52 (0.07)	0.074	**0.016**
Negative	6.578 (0.05)	6.76 (0.16)	0.228	0.354	6.62 (0.13)	0.647	0.647	6.50 (0.11)	0.284	0.354	6.40 (0.13)	0.212	0.354	6.26 (0.18)	0.178	0.129

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Atopobium* cluster (ato291). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Prop853														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	6.49 (0.13)	7.18 (0.19)	0.072	0.181	7.31 (0.22)	0.064	0.181	6.98 (0.16)	0.361	0.601	6.74 (0.22)	0.87	0.87	6.65 (0.17)	0.575	6.49 (0.13)
OFI	6.40 (0.47)	6.69 (0.50)	0.125	0.626	7.02 (0.27)	0.293	0.733	6.68 (0.29)	0.647	0.809	6.36 (0.41)	0.938	0.938	6.12 (0.37)	0.595	6.40 (0.47)
ITF-mix	6.52 (0.14)	6.89 (0.12)	0.08	0.127	7.31 (0.23)	**0.04**	0.127	7.11 (0.22)	0.091	0.127	7.02 (0.23)	0.108	0.127	6.89 (0.22)	0.127	6.52 (0.14)
2'FL	6.39 (0.49)	6.73 (0.25)	0.312	0.873	6.66 (0.36)	0.349	0.873	6.76 (0.22)	0.582	0.898	6.58 (0.23)	0.782	0.898	6.32 (0.15)	0.898	6.39 (0.49)
β-glucan	6.50 (0.13)	7.10 (0.07)	0.068	0.085	7.52 (0.05)	**0.009**	**0.046 ***	7.23 (0.07)	**0.029**	0.068	7.18 (0.06)	**0.041**	0.068	7.09 (0.08)	0.09	6.50 (0.13)
OFI/2'FL 50/50	6.39 (0.51)	6.48 (0.48)	0.06	0.302	7.14 (0.10)	0.262	0.562	6.81 (0.17)	0.411	0.562	6.78 (0.15)	0.449	0.562	6.37 (0.06)	0.975	6.39 (0.51)
OFI/2'FL 85/15	6.40 (0.49)	6.85 (0.30)	0.207	0.518	7.18 (0.11)	0.18	0.518	6.97 (0.20)	0.46	0.714	6.86 (0.29)	0.571	0.714	6.41 (0.20)	0.985	6.40 (0.49)
OFI/2'L 95/5	6.40 (0.48)	6.88 (0.20)	0.255	0.488	6.89 (0.29)	0.379	0.488	7.02 (0.32)	0.058	0.288	6.71 (0.18)	0.404	0.488	6.08 (0.24)	0.488	6.40 (0.48)

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: Clostridial cluster IX (Prop853). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations**: OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Prop853 (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF/2'FL	6.49 (0.13)	7.07 (0.05)	**0.034**	**0.035***	7.30 (0.08)	**0.007**	**0.035***	7.04 (0.16)	**0.035**	**0.035***	6.88 (0.16)	**0.017**	**0.035***	6.81 (0.12)	**0.021**	6.49 (0.13)
ITF-mix/2'FL	6.51 (0.12)	7.17 (0.15)	**0.005**	**0.023***	7.29 (0.17)	**0.014**	**0.024***	7.16 (0.13)	**0.013**	**0.024***	7.02 (0.15)	**0.045**	0.057	6.93 (0.21)	0.126	6.51 (0.12)
OF/β-glucan	6.50 (0.12)	7.18 (0.04)	**0.032**	0.08	7.47 (0.05)	**0.01**	0.05	7.15 (0.09)	0.055	0.092	7.04 (0.09)	0.078	0.097	6.91 (0.12)	0.174	6.50 (0.12)
OFI/β-glucan 50/50	6.50 (0.12)	7.25 (0.15)	**0.007**	**0.011***	7.48 (0.12)	**0.003**	**0.008****	7.30 (0.09)	**0.003**	**0.008****	7.19 (0.02)	**0.023**	**0.029***	6.99 (0.10)	0.156	6.50 (0.12)
ITF-mix/β-glucan	6.51 (0.14)	7.15 (0.08)	**0.007**	**0.033***	7.42 (0.09)	**0.015**	**0.037***	7.09 (0.10)	0.051	0.085	7.02 (0.09)	0.072	0.091	6.93 (0.14)	0.144	6.51 (0.14)
Negative	6.50 (0.12)	7.03 (0.16)	0.073	0.367	7.02 (0.15)	0.273	0.517	6.63 (0.10)	0.529	0.529	6.40 (0.32)	0.447	0.529	6.27 (0.30)	0.31	6.50 (0.12)

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: Clostridial cluster IX (Prop853). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations**: OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Fprau655														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	7.34 (0.13)	8.01 (0.04)	**0.024**	**0.017***	8.18 (0.06)	0.062	**0.033***	7.36 (0.02)	0.142	0.06	7.02 (0.03)	**0.013**	**0.014***	6.71 (0.04)	**0.006**	**0.012***
OFI	7.29 (0.26)	7.34 (0.23)	0.082	0.219	7.43 (0.05)	0.608	0.639	6.95 (0.03)	0.282	0.37	5.94 (0.33)	0.1	0.219	5.89 (0.31)	0.125	0.219
ITF-mix	7.34 (0.13)	7.96 (0.08)	**0.004**	**0.016***	8.21 (0.14)	**0.021**	**0.044***	7.49 (0.22)	0.536	0.45	7.10 (0.26)	0.454	0.45	6.82 (0.23)	0.159	0.223
2'FL	7.22 (0.27)	7.31 (0.21)	0.254	0.445	7.13 (0.31)	0.74	0.777	7.05 (0.36)	0.653	0.777	6.33 (0.18)	0.18	0.445	6.23 (0.22)	0.178	0.445
β-glucan	7.34 (0.13)	8.10 (0.05)	**0.024**	**0.017***	8.34 (0.14)	**0.004**	**0.008****	7.93 (0.13)	**0.009**	**0.009****	7.56 (0.16)	0.185	0.097	7.32 (0.15)	0.891	0.374
OFI/2'FL 50/50	7.20 (0.26)	7.30 (0.20)	0.518	0.68	7.21 (0.06)	0.956	>.999	6.93 (0.21)	0.297	0.564	6.63 (0.19)	0.322	0.564	6.28 (0.29)	0.227	0.564
OFI/2'FL 85/15	7.22 (0.26)	7.41 (0.15)	0.235	0.34	7.37 (0.109)	0.259	0.34	6.99 (0.25)	0.664	0.697	6.54 (0.12)	0.162	0.34	6.21 (0.25)	0.188	0.34
OFI/2'L 95/5	7.22 (0.26)	7.43 (0.12)	0.357	0.469	7.19 (0.15)	0.916	0.962	6.91 (0.07)	0.308	0.469	6.47 (0.24)	0.272	0.469	5.94 (0.31)	0.155	0.469
OF/2'FL	7.34 (0.12)	7.89 (0.13)	**0.043**	0.112	8.10 (0.13)	**0.031**	0.112	7.42 (0.20)	0.719	0.943	7.32 (0.22)	0.913	0.958	7.03 (0.19)	0.177	0.31

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Faecalibacterium prausnitzii* (Fprau655). Mean (*n* = 3) and standard error (SE). * (*Q* ≤ 0.05), ** (*Q* ≤ 0.01), and *** (*Q* ≤ 0.001) indicates significance compared with 0 h sampling (grey columns). **Abbreviations**: OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Fprau655 (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	7.34 (0.13)	8.01 (0.10)	**0.029**	0.075	7.99 (0.12)	**0.017**	0.075	7.53 (0.09)	0.051	0.09	7.19 (0.22)	0.533	0.559	6.98 (0.23)	7.34 (0.13)	8.01 (0.10)
OF/β-glucan	7.34 (0.13)	7.98 (0.01)	**0.041**	0.109	8.27 (0.06)	**0.011**	0.06	7.56 (0.25)	0.406	0.533	7.42 (0.19)	0.634	0.666	7.11 (0.15)	7.34 (0.13)	7.98 (0.01)
OFI/β-glucan 50/50	7.34 (0.12)	7.96 (0.063)	0.057	0.085	8.10 (0.06)	**0.049**	0.085	7.32 (0.18)	0.924	0.971	7.03 (0.05)	0.065	0.085	6.44 (0.16)	7.34 (0.12)	7.96 (0.063)
ITF-mix/β-glucan	7.35 (0.12)	8.01 (0.02)	**0.023**	0.061	8.167 (0.03)	**0.018**	0.061	7.68 (0.17)	0.124	0.163	7.33 (0.22)	0.929	0.975	7.03 (0.18)	7.35 (0.12)	8.01 (0.02)
Negative	7.34 (0.12)	7.64 (0.01)	0.76	0.798	7.46 (0.12)	0.505	0.663	6.80 (0.08)	0.026	0.061	6.18 (0.18)	**0.035**	0.061	5.99 (0.10)	7.34 (0.12)	7.64 (0.01)

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Faecalibacterium prausnitzii* (Fprau655). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations**: OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	DSV587															
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	5.82 (0.23)	6.35 (0.44)	0.138	0.339	6.56 (0.55)	0.203	0.339	6.39 (0.39)	0.089	0.339	5.93 (0.32)	0.41	0.513	5.71 (0.43)	0.675	0.675
OFI	5.73 (0.32)	5.46 (0.53)	0.549	0.801	5.89 (0.54)	0.762	0.801	5.64 (0.38)	0.801	0.801	5.12 (0.28)	0.271	0.711	5.11 (0.30)	0.284	0.711
ITF-mix	5.82 (0.22)	6.03 (0.31)	0.145	0.387	6.64 (0.55)	0.155	0.387	6.18 (0.44)	0.252	0.42	5.79 (0.35)	0.896	0.896	5.85 (0.38)	0.882	0.896
2'FL	5.73 (0.31)	5.92 (0.32)	0.44	0.653	5.41 (0.45)	0.546	0.653	6.08 (0.10)	0.278	0.653	5.55 (0.20)	0.653	0.653	5.51 (0.11)	0.642	0.653
β-glucan	5.83 (0.23)	6.22 (0.41)	0.221	0.25	6.41 (0.42)	0.143	0.25	6.15 (0.36)	0.135	0.25	6.07 (0.36)	0.25	0.25	6.03 (0.33)	0.218	0.25
OFI/2'FL 50/50	5.74 (0.31)	5.82 (0.39)	0.848	0.889	6.70 (0.08)	0.13	0.364	5.85 (0.35)	0.832	0.889	5.80 (0.21)	0.889	0.889	5.14 (0.36)	0.146	0.364
OFI/2'FL 85/15	5.74 (0.31)	5.75 (0.17)	0.965	0.965	6.21 (0.43)	0.343	0.571	5.81 (0.30)	0.674	0.843	5.23 (0.52)	0.14	0.351	4.63 (0.23)	**0.01**	**0.048 ***
OFI/2'L 95/5	5.73 (0.32)	5.98 (0.03)	0.484	0.484	5.90 (0.29)	0.236	0.295	5.37 (0.32)	0.099	0.164	4.98 (0.25)	**0.024**	0.06	4.62 (0.26)	**0.015**	0.06
OF/2'FL	5.82 (0.22)	6.24 (0.43)	0.214	0.357	6.59 (0.54)	0.172	0.357	6.34 (0.50)	0.294	0.367	6.04 (0.29)	0.07	0.349	5.91 (0.26)	0.471	0.471

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Desulfovibrio* spp. (DSV867). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	DSV587 (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	5.82 (0.23)	6.29 (0.42)	0.132	0.331	6.19 (0.39)	0.237	0.394	6.15 (0.49)	0.348	0.436	5.78 (0.31)	0.714	0.714	5.36 (0.20)	0.092	0.331
OF/β-glucan	5.82 (0.23)	6.42 (0.48)	0.131	0.242	6.58 (0.47)	0.09	0.242	6.39 (0.48)	0.145	0.242	6.05 (0.41)	0.316	0.395	5.84 (0.38)	0.886	0.886
OFI/β-glucan 50/50	5.82 (0.22)	6.51 (0.53)	0.162	0.269	6.36 (0.41)	0.117	0.269	6.08 (0.50)	0.506	0.506	5.72 (0.33)	0.504	0.506	5.58 (0.31)	0.145	0.269
ITF-mix/β-glucan	5.82 (0.23)	6.40 (0.46)	0.133	0.166	6.33 (0.36)	0.07	0.146	6.08 (0.46)	0.429	0.429	5.40 (0.16)	0.088	0.146	5.40 (0.16)	**0.045**	0.146
Negative	5.82 (0.22)	5.95 (0.29)	0.511	0.815	5.86 (0.31)	0.815	0.815	5.84 (0.20)	0.686	0.815	5.46 (0.30)	0.288	0.72	4.82 (0.17)	**0.015**	0.076

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Desulfovibrio* spp. (DSV867). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Chis150														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
OF	5.95 (0.40)	6.36 (0.38)	0.167	0.371	6.27 (0.38)	**0.009**	**0.043 ***	6.12 (0.33)	0.297	0.371	5.83 (0.31)	0.441	0.441	5.86 (0.34)	0.289	0.371
OFI	5.74 (0.19)	5.98 (0.11)	0.162	0.27	6.69 (0.01)	**0.042**	0.21	5.99 (0.20)	0.336	0.42	5.65 (0.26)	0.805	0.805	5.25 (0.20)	0.091	0.228
ITF-mix	5.96 (0.40)	6.15 (0.23)	0.382	0.503	6.29 (0.45)	0.311	0.503	5.76 (0.40)	0.648	0.648	5.78 (0.35)	0.147	0.503	5.56 (0.33)	0.403	0.503
2'FL	5.74 (0.19)	5.97 (0.36)	0.412	0.631	5.99 (0.34)	0.505	0.631	6.08 (0.31)	0.406	0.631	5.64 (0.25)	0.742	0.742	5.44 (0.291)	0.421	0.631
β-glucan	5.96 (0.41)	6.20 (0.31)	0.175	0.291	6.47 (0.48)	**0.038**	0.096	6.23 (0.38)	0.334	0.417	5.87 (0.39)	**0.016**	0.081	5.89 (0.52)	0.725	0.725
OFI/2'FL 50/50	5.73 (0.20)	5.97 (0.26)	0.47	0.73	6.49 (0.10)	0.055	0.277	5.99 (0.29)	0.584	0.73	5.81 (0.19)	0.748	0.748	5.34 (0.29)	0.27	0.675
OFI/2'FL 85/15	5.75 (0.20)	5.83 (0.34)	0.757	0.87	6.28 (0.18)	**0.003**	**0.016 ***	6.23 (0.03)	0.102	0.254	5.77 (0.34)	0.87	0.87	5.31 (0.10)	0.253	0.422
OFI/2'L 95/5	5.72 (0.20)	5.96 (0.13)	0.131	0.218	6.21 (0.19)	**0.022**	0.111	5.86 (0.18)	0.275	0.344	5.67 (0.17)	0.811	0.811	4.94 (0.27)	0.107	0.218
OF/2'FL	5.96 (0.40)	6.07 (0.42)	0.108	0.312	6.28 (0.36)	0.125	0.312	5.81 (0.34)	0.449	0.449	5.77 (0.31)	0.354	0.443	5.81 (0.34)	0.189	0.315

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Clostridium histolyticum* (Chis150). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Probe	Chis150 (continued)														
Time (h)	**T 0**	**T 4**			**T 8**			**T 24**			**T 36**			**T 48**		
Substrate	**Mean (SE)**	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***	**Mean (SE)**	***P***	***Q***
ITF-mix/2'FL	5.95 (0.41)	6.14 (0.34)	0.114	0.274	6.10 (0.43)	0.235	0.294	5.92 (0.39)	0.517	0.517	5.65 (0.34)	0.165	0.274	5.38 (0.19)	0.153	0.274
OF/β-glucan	5.96 (0.40)	6.31 (0.43)	0.186	0.232	6.44 (0.52)	0.087	0.145	5.73 (0.23)	0.337	0.337	5.85 (0.38)	**0.041**	0.145	5.77 (0.35)	0.066	0.145
OFI/β-glucan 50/50	5.96 (0.40)	6.21 (0.39)	0.037	0.091	6.43 (0.40)	**0.029**	0.091	6.09 (0.38)	0.339	0.565	5.77 (0.23)	0.579	0.723	5.89 (0.37)	0.775	0.775
ITF-mix/β-glucan	5.96 (0.41)	6.30 (0.43)	0.159	0.265	6.51 (0.33)	0.098	0.265	5.94 (0.28)	0.874	0.874	5.76 (0.30)	0.393	0.492	5.64 (0.27)	0.138	0.265
Negative	5.96 (0.40)	5.99 (0.45)	0.543	0.679	5.98 (0.53)	0.874	0.874	5.46 (0.36)	**0.019**	**0.032 ***	5.17 (0.40)	**0.007**	**0.031 ***	5.15 (0.40)	**0.012**	**0.031 ***

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Clostridium histolyticum* (Chis150). Mean (n = 3) and standard error (SE). * ($Q \leq 0.05$), ** ($Q \leq 0.01$), and *** ($Q \leq 0.001$) indicates significance compared with 0 h sampling (grey columns). **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Appendix 2.4 Individual donor FISH-FLOW results

Total Bacteria (EUB-I-II-III)							
		T 0	**T 4**	**T 8**	**T 24**	**T 36**	**T 48**
OF	**Donor 1**	8.47	9.16	9.35	9.15	8.98	8.89
	Donor 2	8.54	8.78	9.30	9.11	8.96	8.84
	Donor 3	8.07	8.74	9.22	8.80	8.62	8.48
OFI	**Donor 1**	8.01	8.47	8.81	8.60	8.60	8.55
	Donor 2	8.42	8.46	8.98	8.77	8.44	8.28
	Donor 3	8.36	8.94	9.16	8.87	8.56	8.44
ITF-mix	**Donor 1**	8.47	9.10	9.34	9.26	9.12	9.08
	Donor 2	8.54	8.82	9.28	9.26	9.01	8.85
	Donor 3	8.07	8.53	9.24	8.86	8.67	8.50
2'FL	**Donor 1**	8.04	8.33	9.03	8.75	8.70	8.61
	Donor 2	8.41	8.44	8.51	8.63	8.51	8.51
	Donor 3	8.36	8.67	9.16	8.96	8.54	8.47
β-glucan	**Donor 1**	8.47	8.98	9.05	9.07	8.92	8.90
	Donor 2	8.54	8.71	9.26	9.25	9.06	8.83
	Donor 3	8.07	8.70	9.12	8.87	8.79	8.79
OFI/2'FL 50/50	**Donor 1**	8.04	8.35	9.00	8.59	8.62	8.47
	Donor 2	8.44	8.45	8.55	8.67	8.54	8.31
	Donor 3	8.37	8.66	9.12	8.85	8.67	8.49
OFI/2'FL 85/15	**Donor 1**	8.01	8.56	9.04	8.73	8.47	8.57
	Donor 2	8.44	8.51	8.55	8.49	8.65	8.45
	Donor 3	8.38	8.89	9.00	8.81	8.55	8.42
OFI/2'FL 95/5	**Donor 1**	8.03	8.74	9.03	8.74	8.64	8.44
	Donor 2	8.45	8.52	8.52	8.86	8.68	8.52
	Donor 3	8.40	8.95	9.14	8.98	8.77	8.67
OF/2'FL	**Donor 1**	8.47	9.14	9.36	9.25	9.21	9.13
	Donor 2	8.54	8.80	9.42	9.13	9.03	8.92
	Donor 3	8.07	8.68	9.31	8.96	8.72	8.51
ITF-mix/2'FL	**Donor 1**	8.47	9.14	9.34	9.18	9.07	9.01
	Donor 2	8.54	8.79	9.35	9.20	8.99	8.89
	Donor 3	8.08	8.69	9.33	8.89	8.71	8.67
OF/β-glucan	**Donor 1**	8.47	9.17	9.41	9.23	9.15	9.08
	Donor 2	8.54	8.65	9.36	9.17	8.96	8.95
	Donor 3	8.08	8.79	9.24	8.86	8.82	8.68
OFI/β-glucan	**Donor 1**	8.47	9.14	9.34	9.24	9.08	8.99
	Donor 2	8.54	8.62	9.32	9.16	8.99	8.81
	Donor 3	8.07	8.75	9.22	8.88	8.72	8.66
ITF-mix/β-glucan	**Donor 1**	8.47	9.15	9.38	9.23	9.13	9.03
	Donor 2	8.55	8.62	9.26	9.23	9.01	8.95
	Donor 3	8.06	8.78	9.22	8.89	8.83	8.72
Negative	**Donor 1**	8.47	9.16	9.35	9.15	8.98	7.82
	Donor 2	8.54	8.52	8.39	8.01	7.81	7.67
	Donor 3	8.07	8.30	8.37	8.04	7.83	7.72

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: total bacteria (Eub338 I-II-III), **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Bif164							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	Donor 1	7.52	8.91	9.05	8.92	8.77	8.70
	Donor 2	6.81	8.04	9.00	8.92	8.83	8.73
	Donor 3	7.12	8.24	8.92	8.33	8.32	8.21
OFI	Donor 1	7.12	8.00	8.61	8.46	8.47	8.45
	Donor 2	6.94	7.47	8.63	8.51	8.15	7.96
	Donor 3	7.37	8.26	8.59	8.36	8.15	8.14
ITF-mix	Donor 1	7.52	8.80	9.13	9.12	9.03	9.00
	Donor 2	6.81	7.95	8.64	8.67	8.60	8.58
	Donor 3	7.12	7.90	8.69	8.32	8.32	8.21
2'FL	Donor 1	7.14	7.92	8.97	8.68	8.59	8.55
	Donor 2	6.94	7.33	7.58	8.33	8.27	8.26
	Donor 3	7.35	8.10	8.58	8.43	8.36	8.31
β-glucan	Donor 1	7.53	8.13	8.39	8.43	8.35	8.32
	Donor 2	6.82	8.10	8.52	8.50	8.40	8.38
	Donor 3	7.13	8.34	8.68	8.58	8.53	8.52
OFI/2'FL 50/50	Donor 1	7.14	8.03	8.84	8.51	8.53	8.41
	Donor 2	6.93	7.54	7.95	8.45	8.05	8.03
	Donor 3	7.36	8.12	8.61	8.45	8.45	8.37
OFI/2'FL 85/15	Donor 1	7.13	8.23	8.88	8.59	8.36	8.48
	Donor 2	6.92	7.15	7.85	8.38	8.26	8.14
	Donor 3	7.35	8.19	8.40	8.27	8.20	8.16
OFI/2'FL 95/5	Donor 1	7.14	8.38	8.83	8.59	8.52	8.37
	Donor 2	6.94	7.20	7.99	8.49	8.48	8.32
	Donor 3	7.36	8.44	8.54	8.44	8.44	8.41
OF/2'FL	Donor 1	7.52	8.92	9.17	9.15	9.12	9.07
	Donor 2	6.81	8.23	9.03	8.93	8.88	8.84
	Donor 3	7.12	8.18	8.82	8.39	8.32	8.20
ITF-mix/2'FL	Donor 1	7.53	8.91	9.17	8.99	8.86	8.86
	Donor 2	6.82	8.25	9.17	8.98	8.80	8.79
	Donor 3	7.13	8.11	9.11	8.37	8.36	8.35
OF/β-glucan	Donor 1	7.53	8.94	9.21	9.07	9.01	8.98
	Donor 2	6.82	8.05	9.02	8.98	8.73	8.71
	Donor 3	7.12	8.47	8.73	8.44	8.38	8.38
OFI/β-glucan	Donor 1	7.53	8.87	9.07	9.00	8.83	8.82
	Donor 2	6.82	7.96	9.08	8.97	8.68	8.66
	Donor 3	7.12	8.43	8.89	8.54	8.36	8.35
ITF-mix/β-glucan	Donor 1	7.54	8.84	9.19	9.11	8.98	8.90
	Donor 2	6.81	7.95	8.97	9.01	8.74	8.70
	Donor 3	7.12	8.43	8.71	8.55	8.48	8.45
Negative	Donor 1	7.53	7.65	7.55	7.51	7.50	7.46
	Donor 2	6.81	6.91	6.87	6.74	6.46	6.39
	Donor 3	7.12	7.33	7.76	7.15	7.12	7.10

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Bifidobacterium* spp. (Bif 164). **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Lab158							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	Donor 1	6.35	6.62	6.83	6.43	6.06	5.19
	Donor 2	6.21	6.30	7.64	7.15	7.10	6.86
	Donor 3	6.52	6.52	6.93	6.22	6.06	6.05
OFI	Donor 1	5.89	6.60	6.63	5.90	5.60	5.83
	Donor 2	6.51	6.53	6.57	6.51	6.12	6.04
	Donor 3	6.36	6.56	7.00	6.39	6.12	6.07
ITF-mix	Donor 1	6.36	6.42	6.57	6.46	6.33	6.08
	Donor 2	6.22	6.22	6.92	6.46	6.31	6.28
	Donor 3	6.52	6.52	7.02	6.52	6.38	5.93
2'FL	Donor 1	5.90	6.32	6.60	5.60	5.74	5.45
	Donor 2	6.51	6.55	6.42	6.15	6.56	6.07
	Donor 3	6.38	6.75	6.88	6.54	6.45	5.85
β-glucan	Donor 1	6.38	6.30	6.25	6.18	6.04	5.98
	Donor 2	6.22	6.38	6.44	6.16	6.14	6.10
	Donor 3	6.53	6.65	7.12	6.25	6.16	6.02
OFI/2'FL 50/50	Donor 1	5.91	6.49	6.66	5.85	6.31	5.17
	Donor 2	6.52	6.52	6.47	6.43	6.55	6.08
	Donor 3	6.38	6.71	7.15	6.28	6.11	5.79
OFI/2'FL 85/15	Donor 1	5.89	6.52	6.54	5.94	5.88	5.47
	Donor 2	6.51	6.58	6.45	6.82	6.67	6.27
	Donor 3	6.39	6.52	6.54	6.21	6.03	5.82
OFI/2'FL 95/5	Donor 1	5.84	6.80	6.37	5.97	5.12	5.29
	Donor 2	6.52	6.57	6.46	6.29	6.10	6.09
	Donor 3	6.39	6.66	6.88	6.26	6.07	5.95
OF/2'FL	Donor 1	6.37	6.52	6.78	6.55	6.35	5.61
	Donor 2	6.22	6.40	7.41	6.99	6.59	6.28
	Donor 3	6.51	6.66	6.85	6.13	6.02	6.02
ITF-mix/2'FL	Donor 1	6.35	6.49	6.74	6.54	6.19	6.09
	Donor 2	6.21	6.60	7.26	6.98	6.54	6.44
	Donor 3	6.52	6.67	7.24	6.29	6.16	6.09
OF/β-glucan	Donor 1	6.36	6.55	6.58	6.41	6.38	5.99
	Donor 2	6.21	6.57	7.34	7.64	6.71	6.45
	Donor 3	6.51	6.59	7.22	6.41	6.38	6.15
OFI/β-glucan	Donor 1	6.39	6.49	6.56	6.32	6.23	5.77
	Donor 2	6.21	6.67	7.17	7.63	6.53	6.40
	Donor 3	6.52	6.59	7.08	6.24	6.24	6.20
ITF-mix/β-glucan	Donor 1	6.34	6.54	6.56	6.41	6.04	5.98
	Donor 2	6.22	6.68	6.94	7.70	6.57	6.49
	Donor 3	6.52	6.59	6.67	6.25	6.25	6.02
Negative	Donor 1	6.38	6.42	5.53	5.16	4.67	4.60
	Donor 2	6.21	6.15	6.15	5.39	5.46	5.40
	Donor 3	6.53	6.53	6.65	5.83	5.83	5.68

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Lactobacillus*/*Enterococcus* (Lab158). **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

		Bac303					
		T 0	**T 4**	**T 8**	**T 24**	**T 36**	**T 48**
OF	**Donor 1**	6.76	6.81	6.88	6.51	6.39	6.30
	Donor 2	7.24	7.84	8.56	7.86	7.55	7.53
	Donor 3	6.67	7.03	7.70	7.46	7.29	7.05
OFI	**Donor 1**	6.24	7.03	7.94	7.54	7.15	7.01
	Donor 2	6.72	6.78	7.33	7.50	7.07	6.68
	Donor 3	7.02	7.58	7.87	7.57	7.55	7.49
ITF-mix	**Donor 1**	6.78	6.81	6.84	6.54	6.42	6.26
	Donor 2	6.89	7.73	7.99	7.76	6.97	6.82
	Donor 3	6.65	6.96	7.94	7.40	7.39	7.14
2'FL	**Donor 1**	6.27	6.90	7.45	7.36	7.43	6.95
	Donor 2	6.73	6.85	6.87	7.44	7.18	7.13
	Donor 3	7.01	7.16	8.12	7.56	7.13	7.03
β-glucan	**Donor 1**	6.78	6.84	7.17	7.18	7.10	6.93
	Donor 2	6.89	7.69	8.51	7.52	7.52	7.19
	Donor 3	6.65	6.98	8.57	7.87	7.84	7.71
OFI/2'FL 50/50	**Donor 1**	6.28	6.92	7.75	7.39	7.35	6.74
	Donor 2	6.72	6.74	7.06	7.63	7.29	7.25
	Donor 3	7.03	7.02	7.40	7.38	7.31	7.09
OFI/2'FL 85/15	**Donor 1**	6.24	7.02	7.89	7.42	7.36	7.09
	Donor 2	6.71	6.73	7.22	7.27	7.47	7.24
	Donor 3	7.04	7.55	7.63	7.52	7.46	7.28
OFI/2'FL 95/5	**Donor 1**	6.24	7.11	7.84	7.40	7.28	6.64
	Donor 2	6.71	6.70	7.29	7.42	7.41	7.09
	Donor 3	7.05	7.20	7.81	7.72	7.39	7.36
OF/2'FL	**Donor 1**	6.77	6.82	7.14	6.55	6.32	6.31
	Donor 2	6.89	7.86	8.21	7.29	6.97	6.44
	Donor 3	6.65	6.86	8.10	7.48	7.23	7.17
ITF-mix/2'FL	**Donor 1**	6.77	6.77	6.77	6.63	6.53	6.24
	Donor 2	6.89	7.70	8.46	8.02	7.57	6.47
	Donor 3	6.66	6.73	8.16	7.43	7.43	7.41
OF/β-glucan	**Donor 1**	6.77	6.81	7.26	7.23	7.14	6.84
	Donor 2	6.89	7.70	8.37	8.29	7.77	7.37
	Donor 3	6.66	7.03	8.52	7.56	7.52	7.50
OFI/β-glucan	**Donor 1**	6.77	6.86	7.42	7.16	7.04	6.76
	Donor 2	6.89	7.50	8.48	8.48	7.82	7.74
	Donor 3	6.67	6.84	8.87	7.74	7.62	7.50
ITF-mix/β-glucan	**Donor 1**	6.77	6.80	7.28	7.15	6.96	6.53
	Donor 2	6.89	7.59	8.31	7.92	7.79	7.22
	Donor 3	6.68	6.87	8.72	7.76	7.63	7.50
Negative	**Donor 1**	6.80	6.79	6.73	6.50	6.09	5.50
	Donor 2	7.23	7.28	7.36	6.96	6.66	6.43
	Donor 3	6.58	6.59	6.91	6.89	6.56	5.85

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: most *Bacteroidacae* and *Prevotellaceae* (Bac303). **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Erec482							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	Donor 1	8.22	8.56	8.90	8.64	8.42	8.25
	Donor 2	8.12	8.25	8.59	8.43	8.14	7.77
	Donor 3	7.85	8.29	8.53	8.42	8.08	7.91
OFI	Donor 1	7.70	8.02	7.53	7.41	7.34	6.94
	Donor 2	7.93	7.93	8.07	7.84	7.23	6.95
	Donor 3	7.75	8.50	8.82	8.52	7.89	7.85
ITF-mix	Donor 1	8.20	8.56	8.77	8.54	8.32	8.22
	Donor 2	8.11	8.46	9.01	9.00	8.72	8.31
	Donor 3	7.86	8.04	8.94	8.50	8.14	7.96
2'FL	Donor 1	7.67	7.89	7.41	7.41	7.50	7.04
	Donor 2	7.93	7.92	7.81	7.68	7.33	6.58
	Donor 3	7.76	8.19	8.85	8.65	7.59	7.49
β-glucan	Donor 1	8.20	8.63	8.66	8.77	8.67	8.50
	Donor 2	8.11	8.11	8.81	8.96	8.73	8.40
	Donor 3	7.86	8.00	8.66	8.12	8.00	8.03
OFI/2'FL 50/50	Donor 1	7.68	7.67	7.70	7.33	7.57	7.23
	Donor 2	7.94	7.93	7.67	7.73	7.27	6.73
	Donor 3	7.77	8.17	8.77	8.47	7.97	7.69
OFI/2'FL 85/15	Donor 1	7.69	7.92	7.96	7.35	7.35	7.21
	Donor 2	7.95	7.93	7.81	7.83	7.38	6.94
	Donor 3	7.78	8.52	8.67	8.49	7.92	7.85
OFI/2'FL 95/5	Donor 1	7.67	8.06	8.22	7.92	7.71	7.27
	Donor 2	7.94	7.92	7.74	7.93	7.43	6.62
	Donor 3	7.78	8.55	8.81	8.70	8.32	8.13
OF/2'FL	Donor 1	8.20	8.53	8.70	8.45	8.36	8.15
	Donor 2	8.11	8.29	9.02	8.53	8.23	7.91
	Donor 3	8.11	8.29	9.02	8.53	8.23	7.91
ITF-mix/2'FL	Donor 1	8.20	8.56	8.65	8.55	8.52	8.37
	Donor 2	8.13	8.21	8.48	8.41	8.24	7.98
	Donor 3	7.87	8.26	8.79	8.63	8.15	8.13
OF/β-glucan	Donor 1	8.20	8.45	8.71	8.64	8.45	8.28
	Donor 2	8.11	8.12	8.72	8.44	8.27	8.27
	Donor 3	7.85	8.09	8.68	8.40	8.37	8.17
OFI/β-glucan	Donor 1	8.20	8.51	8.74	8.83	8.63	8.37
	Donor 2	8.14	8.14	8.68	8.31	8.36	7.95
	Donor 3	7.85	8.06	8.87	8.28	8.14	8.03
ITF-mix/β-glucan	Donor 1	8.20	8.59	8.86	8.54	8.50	8.33
	Donor 2	8.12	8.13	8.70	8.43	8.38	8.22
	Donor 3	7.85	8.10	8.66	8.30	8.24	8.09
Negative	Donor 1	8.18	8.14	7.83	7.51	6.83	6.35
	Donor 2	8.11	8.03	7.74	7.07	6.60	6.39
	Donor 3	7.84	8.01	8.00	7.42	7.36	7.04

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Clostridium coccoides-Eubacterium rectale* group (Erec482). **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

		Ato291					
		T 0	**T 4**	**T 8**	**T 24**	**T 36**	**T 48**
OF	**Donor 1**	6.63	7.72	7.91	7.68	7.62	7.45
	Donor 2	6.60	7.20	7.77	7.74	7.60	7.42
	Donor 3	6.47	7.70	8.20	7.54	7.34	7.28
OFI	**Donor 1**	6.37	7.67	7.95	7.44	7.55	7.34
	Donor 2	6.95	7.26	7.80	7.90	7.55	7.06
	Donor 3	6.29	7.75	7.77	7.37	6.99	6.70
ITF-mix	**Donor 1**	6.65	7.59	7.72	7.60	7.35	7.12
	Donor 2	6.60	7.10	7.79	7.47	7.25	7.24
	Donor 3	6.48	7.68	8.23	7.84	7.55	7.41
2'FL	**Donor 1**	6.35	7.11	7.94	7.50	7.46	7.34
	Donor 2	6.97	7.01	7.04	7.68	7.19	7.01
	Donor 3	6.29	7.36	7.66	7.65	7.19	6.99
β-glucan	**Donor 1**	6.63	7.49	7.52	7.08	6.99	6.98
	Donor 2	6.60	7.47	7.50	7.27	7.16	7.14
	Donor 3	6.47	7.68	7.78	7.48	7.49	7.30
OFI/2'FL 50/50	**Donor 1**	6.38	7.55	8.03	7.12	7.02	6.82
	Donor 2	6.95	7.01	7.05	6.73	6.17	6.86
	Donor 3	6.30	7.24	7.80	7.41	7.27	6.50
OFI/2'FL 85/15	**Donor 1**	6.37	7.76	8.08	7.38	6.93	7.41
	Donor 2	6.94	6.98	7.08	6.53	6.27	6.17
	Donor 3	6.30	7.43	7.56	7.31	7.12	6.70
OFI/2'FL 95/5	**Donor 1**	6.36	7.85	7.97	7.51	7.53	7.33
	Donor 2	6.94	6.90	7.35	7.82	7.46	7.24
	Donor 3	6.31	7.53	7.62	7.62	6.99	6.49
OF/2'FL	**Donor 1**	6.64	7.75	7.77	7.58	7.51	7.44
	Donor 2	6.59	7.33	7.79	7.60	7.50	7.26
	Donor 3	6.47	7.65	8.12	7.65	7.60	7.42
ITF-mix/2'FL	**Donor 1**	6.66	7.68	7.74	7.68	7.52	7.43
	Donor 2	6.59	7.31	7.89	7.85	7.52	7.49
	Donor 3	6.47	7.60	7.64	7.43	7.36	7.31
OF/β-glucan	**Donor 1**	6.66	7.66	7.88	7.47	7.45	7.42
	Donor 2	6.62	7.05	7.92	7.87	7.81	7.59
	Donor 3	6.45	7.79	7.99	7.91	7.68	7.35
OFI/β-glucan	**Donor 1**	6.65	7.71	7.73	7.65	7.39	7.23
	Donor 2	6.6	7.06	7.7	7.38	7.06	6.9
	Donor 3	6.46	7.72	7.87	7.8	7.72	7.69
ITF-mix/β-glucan	**Donor 1**	6.65	7.57	7.8	7.54	7.49	7.37
	Donor 2	6.59	7.04	7.88	7.81	7.65	7.58
	Donor 3	6.48	7.83	7.92	7.82	7.79	7.61
Negative	**Donor 1**	6.67	6.97	6.86	6.7	6.64	6.60
	Donor 2	6.58	6.86	6.54	6.45	6.22	6.18
	Donor 3	6.48	6.45	6.45	6.34	6.34	6.24

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Atopobium* cluster (Ato291). **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Prop583							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	**Donor 1**	6.66	6.84	6.91	6.73	6.52	6.48
	Donor 2	6.95	7.51	7.66	6.94	6.52	6.48
	Donor 3	6.75	7.20	7.35	7.26	7.18	6.99
OFI	**Donor 1**	5.47	5.72	6.84	6.66	6.09	5.83
	Donor 2	6.82	6.93	6.67	6.19	5.82	5.68
	Donor 3	6.91	7.40	7.55	7.19	7.16	6.85
ITF-mix	**Donor 1**	6.35	6.65	6.86	6.69	6.55	6.46
	Donor 2	6.79	7.02	7.59	7.27	7.27	7.18
	Donor 3	6.40	7.00	7.48	7.38	7.22	7.02
2'FL	**Donor 1**	5.40	6.24	6.03	6.85	6.73	6.26
	Donor 2	6.85	6.87	6.70	6.34	6.13	6.08
	Donor 3	6.91	7.07	7.26	7.09	6.87	6.61
β-glucan	**Donor 1**	6.34	7.33	7.52	7.09	7.08	7.04
	Donor 2	6.75	7.09	7.59	7.25	7.16	6.97
	Donor 3	6.40	7.14	7.44	7.34	7.29	7.25
OFI/2'FL 50/50	**Donor 1**	5.38	5.52	7.09	6.60	6.58	6.47
	Donor 2	6.84	6.92	6.99	6.69	6.68	6.26
	Donor 3	6.95	7.01	7.34	7.15	7.07	6.38
OFI/2'FL 85/15	**Donor 1**	5.43	6.32	6.98	7.23	7.23	6.06
	Donor 2	6.84	6.87	7.22	6.58	6.30	6.42
	Donor 3	6.92	7.37	7.33	7.09	7.06	6.73
OFI/2'FL 95/5	**Donor 1**	5.45	6.52	6.66	6.38	6.35	5.81
	Donor 2	6.84	6.92	6.55	7.33	6.85	5.86
	Donor 3	6.92	7.20	7.46	7.36	6.94	6.56
OF/2'FL	**Donor 1**	6.34	6.97	7.15	6.73	6.62	6.61
	Donor 2	6.74	7.10	7.42	7.25	7.16	7.01
	Donor 3	6.40	7.12	7.32	7.15	6.84	6.81
ITF-mix/2'FL	**Donor 1**	6.37	6.94	6.97	6.92	6.73	6.52
	Donor 2	6.75	7.46	7.56	7.34	7.19	7.14
	Donor 3	6.42	7.12	7.34	7.21	7.15	7.14
OF/β-glucan	**Donor 1**	6.35	7.11	7.49	7.00	6.91	6.75
	Donor 2	6.75	7.18	7.55	7.12	7.00	6.82
	Donor 3	6.40	7.23	7.37	7.32	7.20	7.15
OFI/β-glucan	**Donor 1**	6.35	6.99	7.43	7.23	7.18	7.04
	Donor 2	6.75	7.50	7.70	7.48	7.23	6.80
	Donor 3	6.41	7.26	7.31	7.19	7.16	7.13
ITF-mix/β-glucan	**Donor 1**	6.34	7.05	7.48	6.89	6.86	6.69
	Donor 2	6.77	7.31	7.54	7.13	7.03	6.92
	Donor 3	6.41	7.09	7.25	7.24	7.18	7.17
Negative	**Donor 1**	6.64	6.76	6.79	6.70	6.67	6.41
	Donor 2	6.94	7.31	6.96	6.43	5.76	5.70
	Donor 3	6.74	7.01	7.30	6.77	6.76	6.70

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: Clostridial cluster IX (Prop853). **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Fprau655							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	Donor 1	7.59	7.96	8.12	7.32	7.00	6.76
	Donor 2	7.79	8.09	8.12	7.36	7.07	6.75
	Donor 3	7.46	7.97	8.30	7.40	6.98	6.62
OFI	Donor 1	7.16	7.27	6.62	6.56	5.30	5.45
	Donor 2	7.67	7.76	8.33	7.61	6.17	5.75
	Donor 3	6.77	7.00	7.35	6.67	6.35	6.48
ITF-mix	Donor 1	7.29	7.94	7.95	7.06	6.59	6.36
	Donor 2	7.57	8.12	8.42	7.79	7.40	7.05
	Donor 3	7.15	7.83	8.25	7.61	7.32	7.04
2'FL	Donor 1	7.20	7.26	6.64	6.41	6.20	6.14
	Donor 2	7.69	7.70	7.70	7.66	6.10	5.89
	Donor 3	6.77	6.97	7.04	7.08	6.69	6.65
β-glucan	Donor 1	7.28	8.19	8.16	7.76	7.29	7.07
	Donor 2	7.58	8.10	8.60	8.18	7.85	7.58
	Donor 3	7.16	8.00	8.25	7.83	7.55	7.32
OFI/2'FL 50/50	Donor 1	7.16	7.10	7.13	6.59	6.47	6.06
	Donor 2	7.68	7.69	7.35	7.32	6.40	5.92
	Donor 3	6.77	7.10	7.17	6.87	7.01	6.85
OFI/2'FL 85/15	Donor 1	7.18	7.44	7.24	7.45	6.31	6.17
	Donor 2	7.69	7.66	7.74	6.60	6.58	5.79
	Donor 3	6.78	7.14	7.13	6.94	6.71	6.66
OFI/2'FL 95/5	Donor 1	7.19	7.54	7.44	6.80	6.43	5.81
	Donor 2	7.69	7.55	7.22	7.04	6.08	5.47
	Donor 3	6.78	7.19	6.92	6.90	6.90	6.54
OF/2'FL	Donor 1	7.28	7.65	7.84	7.03	6.90	6.68
	Donor 2	7.58	8.09	8.28	7.67	7.63	7.34
	Donor 3	7.16	7.93	8.18	7.55	7.42	7.07
ITF-mix/2'FL	Donor 1	7.28	7.81	7.81	7.47	6.76	6.53
	Donor 2	7.59	8.17	8.20	7.71	7.49	7.16
	Donor 3	7.14	8.04	7.96	7.42	7.31	7.26
OF/β-glucan	Donor 1	7.27	7.98	8.16	7.08	7.08	6.88
	Donor 2	7.59	7.97	8.37	7.95	7.74	7.38
	Donor 3	7.16	7.99	8.28	7.63	7.43	7.08
OFI/β-glucan	Donor 1	7.28	8.08	8.20	7.03	6.97	6.13
	Donor 2	7.58	7.89	7.99	7.28	7.12	6.69
	Donor 3	7.17	7.89	8.11	7.64	7.00	6.50
ITF-mix/β-glucan	Donor 1	7.28	7.99	8.12	7.37	6.89	6.71
	Donor 2	7.59	8.05	8.21	7.96	7.63	7.33
	Donor 3	7.18	7.98	8.18	7.71	7.47	7.03
Negative	Donor 1	7.59	7.67	7.28	6.96	6.24	6.07
	Donor 2	7.78	7.63	7.39	6.71	5.84	5.79
	Donor 3	7.46	7.63	7.69	6.73	6.46	6.11

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Faecalibacterium prausnitzii* (Fprau655). **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

DSV587							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	Donor 1	5.37	5.46	5.65	5.62	5.28	4.89
	Donor 2	6.07	6.79	6.48	6.65	6.22	5.92
	Donor 3	6.02	6.79	7.55	6.90	6.28	6.32
OFI	Donor 1	5.45	4.47	4.81	4.90	4.60	4.55
	Donor 2	6.36	6.26	6.50	6.17	5.21	5.19
	Donor 3	5.37	5.65	6.36	5.85	5.55	5.59
ITF-mix	Donor 1	5.37	5.40	5.64	5.34	5.12	5.08
	Donor 2	6.06	6.35	6.73	6.82	5.96	6.26
	Donor 3	6.02	6.33	7.53	6.38	6.30	6.19
2'FL	Donor 1	5.47	5.37	4.51	6.08	5.17	5.45
	Donor 2	6.36	6.46	5.77	6.24	5.62	5.36
	Donor 3	5.37	5.93	5.93	5.91	5.85	5.73
β-glucan	Donor 1	5.37	5.46	5.65	5.43	5.39	5.38
	Donor 2	6.08	6.34	6.47	6.56	6.26	6.27
	Donor 3	6.03	6.86	7.10	6.46	6.57	6.43
OFI/2'FL 50/50	Donor 1	5.49	5.05	6.82	5.19	5.40	4.47
	Donor 2	6.36	6.28	6.55	5.97	5.88	5.71
	Donor 3	5.37	6.12	6.71	6.39	6.11	5.24
OFI/2'FL 85/15	Donor 1	5.49	5.60	6.73	5.34	4.77	4.57
	Donor 2	6.35	6.09	6.55	6.37	6.27	5.05
	Donor 3	5.37	5.54	5.36	5.71	4.67	4.26
OFI/2'FL 95/5	Donor 1	5.46	5.97	5.50	5.34	4.64	4.22
	Donor 2	6.37	6.03	6.47	5.94	5.46	5.12
	Donor 3	5.35	5.94	5.72	4.83	4.83	4.52
OF/2'FL	Donor 1	5.37	5.44	5.66	5.55	5.47	5.43
	Donor 2	6.07	6.40	6.60	6.19	6.37	6.35
	Donor 3	6.02	6.89	7.52	7.26	6.29	5.95
ITF-mix/2'FL	Donor 1	5.36	5.45	5.52	5.22	5.19	5.06
	Donor 2	6.06	6.74	6.88	6.88	5.94	5.74
	Donor 3	6.04	6.68	6.18	6.37	6.20	5.28
OF/β-glucan	Donor 1	5.35	5.47	5.64	5.44	5.23	5.08
	Donor 2	6.07	6.95	6.97	6.97	6.47	6.12
	Donor 3	6.02	6.84	7.14	6.77	6.45	6.32
OFI/β-glucan	Donor 1	5.38	5.44	5.59	5.24	5.08	4.99
	Donor 2	6.08	7.00	6.98	6.98	6.18	6.04
	Donor 3	6.01	7.07	6.50	6.03	5.93	5.70
ITF-mix/β-glucan	Donor 1	5.37	5.48	5.64	5.23	5.13	5.11
	Donor 2	6.07	6.92	6.83	6.83	5.38	5.64
	Donor 3	6.03	6.80	6.52	6.19	5.68	5.45
Negative	Donor 1	5.37	5.46	5.32	5.28	5.12	4.52
	Donor 2	6.06	5.93	5.88	5.83	4.97	4.81
	Donor 3	6.03	6.45	6.39	6.19	5.99	5.12

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Desulfovibrio* spp. (DSV867). **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Chis150							
		T 0	T 4	T 8	T 24	T 36	T 48
OF	**Donor 1**	5.17	5.64	5.53	5.45	5.28	5.19
	Donor 2	6.21	6.92	6.47	6.49	5.86	6.12
	Donor 3	6.47	6.52	6.80	6.40	6.33	6.28
OFI	**Donor 1**	5.48	5.77	6.71	5.60	5.20	4.85
	Donor 2	6.11	6.14	6.67	6.10	5.67	5.46
	Donor 3	5.61	6.02	6.68	6.26	6.09	5.44
ITF-mix	**Donor 1**	5.16	5.70	5.64	5.43	5.12	5.12
	Donor 2	6.21	6.25	6.06	5.30	5.91	6.21
	Donor 3	6.49	6.49	7.15	6.55	6.31	5.35
2'FL	**Donor 1**	5.49	5.28	5.33	5.53	5.17	4.91
	Donor 2	6.10	6.48	6.15	6.09	5.74	5.51
	Donor 3	5.62	6.14	6.49	6.61	6.02	5.91
β-glucan	**Donor 1**	5.17	5.59	5.53	5.51	5.10	4.85
	Donor 2	6.21	6.50	6.92	6.81	6.10	6.43
	Donor 3	6.50	6.52	6.97	6.37	6.40	6.41
OFI/2'FL 50/50	**Donor 1**	5.46	5.49	6.30	5.74	5.46	4.77
	Donor 2	6.12	6.03	6.53	5.67	5.86	5.54
	Donor 3	5.60	6.39	6.65	6.56	6.11	5.72
OFI/2'FL 85/15	**Donor 1**	5.49	5.16	6.08	6.21	5.42	5.27
	Donor 2	6.14	6.26	6.64	6.30	6.45	5.15
	Donor 3	5.61	6.06	6.11	6.19	5.45	5.50
OFI/2'FL 95/5	**Donor 1**	5.45	5.74	6.07	5.52	5.34	4.44
	Donor 2	6.12	6.17	6.58	6.14	5.76	5.00
	Donor 3	5.60	5.96	5.97	5.91	5.91	5.37
OF/2'FL	**Donor 1**	5.17	5.24	5.47	5.25	5.25	5.17
	Donor 2	6.20	6.40	6.32	5.73	5.73	5.96
	Donor 3	6.50	6.58	7.06	6.43	6.32	6.31
ITF-mix/2'FL	**Donor 1**	5.16	5.49	5.24	5.18	5.07	5.01
	Donor 2	6.21	6.32	6.53	6.08	5.64	5.66
	Donor 3	6.49	6.62	6.53	6.49	6.24	5.45
OF/β-glucan	**Donor 1**	5.17	5.47	5.41	5.31	5.10	5.08
	Donor 2	6.22	6.89	6.98	5.77	6.11	5.99
	Donor 3	6.49	6.56	6.93	6.09	6.34	6.23
OFI/β-glucan	**Donor 1**	5.17	5.44	5.64	5.32	5.23	5.19
	Donor 2	6.20	6.53	6.83	6.50	6.34	6.46
	Donor 3	6.50	6.66	6.84	6.44	5.72	6.00
ITF-mix/β-glucan	**Donor 1**	5.17	5.45	5.86	5.38	5.18	5.11
	Donor 2	6.20	6.84	6.97	6.16	6.16	5.79
	Donor 3	6.51	6.62	6.69	6.27	5.95	6.01
Negative	**Donor 1**	5.18	5.11	4.99	4.79	4.43	4.43
	Donor 2	6.19	6.28	6.15	5.56	5.28	5.20
	Donor 3	6.50	6.60	6.81	6.02	5.81	5.81

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: *Clostridium histolyticum* (Chis150). **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Appendix 2.5 Mean Quantitative Microbiome Profiling values of 16S rRNA genus using pH-controlled *in vitro* batch culture fermentation at 0, 4, 8, 24, 36 and 48

Substrates: OF (oligofructose)				
Genus	T0	T8	T0 vs T8	
			P	*Q*
Bifidobacterium	4.11 x10^7 (8.96 x 10^6)	1.19 x10^9 (9.95 x10^7)	0.074	0.115
Bacteroides	5.58 x 10^6 (1.74 x 10^6)	1.03 x 10^8 (7.76 x 10^8)	0.399	0.465
Prevotella	3.30 x 10^6 (1.68 x 10^6)	8.84 x 10^6 (4.42 x 10^6)	0.519	0.96
Alistipes	6.99 x 10^5 (4.23 x 10^5)	1.78 x 10^6 (1.67 x 10^6)	0.691	0.96
Roseburia	3.73 x 10^7 (1.98 x 10^7)	1.25 x 10^8 (1.07 x 10^8)	0.519	0.88
***Clostridium* cluster IVXA + IVXB**	4.14 x 10^6 (2.02 x 10^6)	3.17 x 10^6 (1.50 x 10^6)	0.843	0.96
Faecalibacterium prausnitzii and relatives	3.30 x 10^7 (8.35 x 10^6)	5.81 x 10^7 (5.81 x 10^6)	0.165	0.702
***Ruminococcaceae* (excluding Fprau)**	2.81 x 10^7 (9.01 x 10^6)	3.21 x 10^7 (1.52 x 10^7)	0.921	0.96
Ruminococcus2	1.42 x 10^7 (5.57 x 10^6)	1.02 x 10^8 (4.34 x 10^7)	**0.029**	0.101
Coprococcus	2.78 x 10^6 (3.44 x 10^5)	2.09 x 10^6 (7.51 x 10^5)	0.399	0.487
Blautia	2.98 x 10^7 (7.82 x 10^6)	1.35 x 10^8 (7.38 x 10^7)	0.372	0.682
Lactobacillus/Enterococcus	9.02 x 10^6 (6.81 x 10^6)	3.70 x 10^6 (3.49 x 10^6)	0.13	0.96
Lactococcus	2.23 x 10^6 (2.24 x 10^6)	2.24 x 10^5 (2.24 x 1^5)	0.333	0.807
Dorea	2.76 x 10^6 (1.23 x 10^6)	2.17 x 10^6 (1.63 x 10^6)	0.585	0.683
Anaerostipes	1.88 x 10^6 (1.00 x 10^6)	9.44 x 10^6 (6.76 x 10^6)	0.487	0.853
Lachnospiraceae incertae sedi	3.47 x 10^6 (1.42 x 10^6)	1.91 x 1+0^7 (9.38 x 10^6)	0.62	0.764
Collinsella	1.05 x 10^6 (3.80 x 10^5)	8.90 x 10^7 (7.80 x 10^7)	0.059	0.138

OF quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Abbreviations:** OF = oligofructose

Substrates: OFI (oligofructose inulin)				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	1.49×10^7 (4.67×10^6)	3.08×10^8 (8.15×10^7)	0.029	0.066
Bacteroides	5.06×10^5 (3.48×10^5)	4.79×10^6 (2.85×10^6)	0.066	0.309
Prevotella	1.98×10^5 (1.20×10^5)	1.53 x 105 (83253)	0.804	0.96
Alistipes	8.12×10^5 (7.02×10^5)	2.16×10^5 (9.89×10^5)	0.457	0.96
Roseburia	3.02×10^6 (1.52×10^6)	6.93×10^5 (1.85×10^5)	0.921	0.96
***Clostridium* cluster IVXA + IVXB**	1.21×10^6 (735683)	4.10×10^5 (98245)	0.655	0.96
Faecalibacterium prausnitzii and relatives	1/52 x 1+0^7 (9.56×10^6)	3.63×10^7 (1.47×10^7)	0.112	0.702
***Ruminococcaceae* (excluding Fprau)**	1.37×10^7 (9.50×10^6)	1.34×10^7 (3.63×10^6)	0.691	0.927
Ruminococcus2	9.58×10^6 (6.48×10^6)	1.28×10^7 (3.34×10^6)	0.882	0.992
Coprococcus	2.67×10^6 (1.89×10^6)	6.13 x 105 (2.28×10^5)	0.346	0.487
Blautia	2.14×10^7 (1.30×10^7)	1.50×10^8 (1.29×10^8)	0.655	0.764
Lactobacillus/Enterococcus	1.53×10^6 (8.83×10^5)	9.64×10^5 (3.29×10^5)	0.96	0.96
Lactococcus	1.07×10^5 (75721)	1.38×10^5 (58877)	0.747	0.823
Dorea	3.65×10^6 (2.92×10^6)	1.38×10^6 (7.19×10^5)	0.297	0.52
Anaerostipes	9.56×10^5 (4.58×10^5)	7.01×10^5 (6338)	0.766	0.894
Lachnospiraceae incertae sedi	3.36 x 106 (2.74×10^6)	9.19×10^6 (6.43×10^6)	0.372	0.764
Collinsella	2.86×10^6 (2.67×10^6)	3.83×10^6 (3.22×10^6)	0.804	0.843

OFI quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Abbreviations:** OFI – oligofructose/inulin

Substrates: ITF-mix (Inulin-type fructans mix)				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	2.87×10^7 (1.38×10^7)	8.61×10^8 (2.85×10^8)	**0.017**	0.06
Bacteroides	3.32×10^6 (2.23×10^6)	5.29×10^7 (3.64×10^7)	**0.013**	0.12
Prevotella	2.87×10^6 (2.64×10^6)	1.12×10^7 (5.82×10^6)	0.346	0.96
Alistipes	872157 (712282)	397718 (147691)	0.655	0.96
Roseburia	2.97×10^7 (1.59×10^7)	2.77×10^8 (1.59×10^8)	**0.011**	0.159
***Clostridium* cluster IVXA + IVXB**	3.24×10^6 (1.81×10^6)	5.26×10^6 (1.520^6)	0.074	0.96
Faecalibacterium prausnitzii and relatives	3.24×10^7 (1.81×10^7)	8.44×10^7 (2.49×10^7)	0.053	0.702
***Ruminococcaceae* (excluding Fprau)**	2.20×10^7 (9.62×10^6)	2.81×10^7 (9.63×10^6)	0.254	0.867
Ruminococcus2	1.32×10^7 (7.24×10^6)	9.41×10^7 (5.09×10^7)	**0.02**	0.101
Coprococcus	1.97×10^6 (7.98×10^5)	3.73×10^6 (1.68×10^6)	0.427	0.487
Blautia	2.58×10^7 (1.15×10^7)	1.73×10^8 (5.10×10^7)	**0.026**	0.173
Lactobacillus/Enterococcus	9.07×10^6 (4.77×10^6)	8.91×10^6 (7.65×10^6)	0.766	0.96
Lactococcus	25894 (9474)	7634 (7634)	0.519	0.807
Dorea	1.67×10^6 (1.16×10^6)	2.07×10^6 (8.89×10^5)	0.457	0.639
Anaerostipes	1.67×10^6 (1.10×10^6)	4.90×10^6 (3.62×10^6)	0.053	0.741
Lachnospiraceae incertae sedi	2.25×10^6 (1.00×10^6)	1.58×10^7 (4.96×10^6)	**0.029**	0.406
Collinsella	7.64×10^5 (2.72×10^5)	1.00×10^8 (6.84×10^7)	**0.015**	0.08

ITF-mix quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Keyword:** ITF = inulin-type fructans

Substrates: 2'FL (2'fucosyllactose)				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	2.04×10^7 (4.43×10^5)	1.56×10^8 (7.00×10^7)	0.197	0.212
Bacteroides	6.99×10^5 (2.73×10^5)	1.18×10^6 (5.51×10^5)	0.728	0.728
Prevotella	1.09×10^5 (6.7×10^5)	3280 (3280)	0.691	0.96
Alistipes	7.57×10^5 (6.03×10^5)	1.20×10^6 (7.97×10^5)	**0.047**	0.66
Roseburia	6.08×10^6 (3.72×10^6)	1.37×10^6 (9.79×10^5)	0.427	0.88
***Clostridium* cluster IVXA + IVXB**	1.04×10^6 (7.28×10^5)	4.57×105 (2.74×10^5)	0.487	0.96
Faecalibacterium prausnitzii and relatives	1.64×10^6 (7.24×10^6)	1.89×10^8 (1.00×1^7)	0.551	0.702
***Ruminococcaceae* (excluding Fprau)**	1.45×10^7 (7.98×10^7)	1.32×10^7 (6.12×10^6)	0.691	0.927
Ruminococcus2	1.25×10^7 (6.94×10^6)	7.28×10^6 (2.19×10^6)	0.487	0.853
Coprococcus	2.77×10^6 (1.85×10^6)	8.09×10^5 (6.35×10^5)	0.092	0.355
Blautia	2.59×10^7 (1.12×10^7)	5.63×10^7 (2.64×10^7)	0.804	0.804
Lactobacillus/Enterococcus	1.29×10^6 (1.21×10^6)	1.07×10^7 (1.07×10^7)	0.487	0.96
Lactococcus	9.97×10^5 (7.43×10^5)	1.27×10^5 (1.10×10^5)	0.487	0.807
Dorea	4.32×10^6 (2.61×10^6)	2.04×10^6 (1.75×10^6)	0.197	0.52
Anaerostipes	1.22×10^6 (6.12×10^5)	5.53×10^5 (2.80×10^5)	0.321	0.798
Lachnospiraceae incertae sedi	3.42×10^6 (2.47×10^6)	4.83×10^6 (2.58×10^6)	0.519	0.764
Collinsella	4.29×10^6 (3.76×10^6)	3.29×10^6 (1.22×10^6)	0.766	0.843

2'FL quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Abbreviations:** 2'FL = 2'fucosyllactose

Substrates: β-glucan				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	3.55×10^7 (4.85×10^6)	9.30×10^8 (6.19×10^7)	0.124	0.158
Bacteroides	8.85×10^5 (3.96×10^5)	3.22×10^7 (2.80×10^7)	**0.017**	0.12
Prevotella	1.35×10^6 (7.76×10^5)	2.11×10^6 (1.14×10^6)	0.804	0.96
Alistipes	5.76×10^5 (2.97×10^5)	2.34×10^5 (1.14×10^5)	0.215	0.751
Roseburia	3.49×10^7 (2.22×10^7)	1.26×10^8 (6.42×10^7)	0.297	0.88
***Clostridium* cluster IVXA + IVXB**	3.43×10^6 (2.01×10^6)	2.12×10^6 (7.86×10^5)	0.655	0.96
Faecalibacterium prausnitzii and relatives	4.88×10^7 (9.46×10^6)	9.27×10^7 (3.96×10^7)	0.519	0.702
***Ruminococcaceae* (excluding Fprau)**	1.49×10^7 (9.46×10^6)	2.02×10^7 (4.35×10^6)	0.254	0.867
Ruminococcus2	1.71×10^7 (7.36×10^6)	2.96×10^7 (1.84×10^7)	0.804	0.992
Coprococcus	2.23×10^6 (5.23×10^5)	5.84×10^5 (2.14×10^5)	**0.022**	0.157
Blautia	3.54×10^7 (1.19×10^7)	7.66×10^7 (3.12×10^7)	0.457	0.682
Lactobacillus/Enterococcus	6.58×10^6 (2.89×10^6)	3.04×10^7 (2.74×10^7)	0.691	0.96
Lactococcus	3.14×10^5 (5747.3)	$2,89 \times 10^5$ (2.89×10^5)	0.385	0.807
Dorea	4.34×10^6 (2.25×10^6)	1.46×10^6 (7.69×10^5)	0.082	0.52
Anaerostipes	1.71×10^6 (1.10×10^6)	4.26×10^6 (3.68×10^6)	0.728	0.894
Lachnospiraceae incertae sedi	3.03×10^6 (1.45×10^6)	1.50×10^7 (1.22×10^7)	0.62	0.764
Collinsella	1.70×10^6 (1.29×10^5)	9.18×10^7 (6.12×10^7)	0.092	0.183

β-glucan quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns).

Substrates: OFI/2'FL (Oligofructose inulin/2'fucosyllactose): 50/50				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	2.14×10^7 (1.20×10^6)	1.66×10^8 (6.05×10^7)	0.066	0.115
Bacteroides	5.09×10^5 (1.87×10^5)	2.61×10^6 (1.69×10^6)	0.234	0.37
Prevotella	7.90×10^5 ($4.-5 \times 10^5$)	2.86×10^5 (1.59×10^5)	0.691	0.96
Alistipes	6.50×10^5 (4.47×10^5)	3.58×10^5 (2.80×10^5)	0.519	0.96
Roseburia	5.43×10^6 (3.00×10^6)	9.77×10^5 (7.79×10^5)	0.62	0.88
***Clostridium* cluster IVXA + IVXB**	8.58×10^5 (6.52×10^5)	5.08×10^5 (4.27×10^5)	0.96	0.96
Faecalibacterium prausnitzii and relatives	1.77×10^7 (9.32×10^6)	2.49×10^7 (1.43×10^7)	0.457	0.702
***Ruminococcaceae* (excluding Fprau)**	1.11×10^7 (4.77×10^6)	1.38×10^7 (9.51×10^6)	0.728	0.927
Ruminococcus2	9.94 x 106 (5.36×10^6)	7.81 x 106 (5.24×10^6)	>.999	>.999
Coprococcus	2.44×10^6 (1.63×10^6)	7.69×10^5 (5.49×10^5)	0.215	0.487
Blautia	2.33×10^7 (9.93×10^6)	5.21×10^7 (2.51×10^7)	0.728	0.784
Lactobacillus/Enterococcus	6.39×10^5 (2.70×10^5)	1.42×10^7 (1.41×10^7)	0.691	0.96
Lactococcus	1.06×10^5 (7.29×10^5)	1.42×10^5 (1.42×10^5)	0.413	0.807
Dorea	3.41×10^6 (2.26×10^6)	3.02×10^6 (2.91×10^6)	0.254	0.52
Anaerostipes	1.16×10^6 (6.92×10^5)	7.38×10^5 (6.24×10^5)	0.399	0.798
Lachnospiraceae incertae sedi	3.50×10^6 (2.90×10^6)	5.70×10^6 (2.90×10^6)	0.62	0.764
Collinsella	5.78×10^5 (3.48×10^5)	8.35×10^6 (4.20×10^6)	0.165	0.288

OFI/2'FL (50/50) quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Abbreviations:** OFI = oligofructose/inulin; 2'FL = 2'fucosyllactose

Substrates: OFI/2'FL (Oligofructose inulin/2'fucosyllactose): 85/15				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	1.98×10^7 (2.07×10^6)	1.49×10^8 (6.40×10^7)	0.15	0.175
Bacteroides	4.25×10^5 (2.36×10^5)	2.45×10^6 (6.12×10^7)	0.254	0.37
Prevotella	1.93×10^5 (1.93×10^5)	1.93×10^5 (1.93×10^5)	0.96	0.96
Alistipes	7.43×10^5 (1.97×10^5)	4.20×10^5 (1.97×10^5)	0.96	0.96
Roseburia	5.68×10^6 (3.26×10^6)	1.06×10^6 (6.64×10^5)	0.655	0.88
***Clostridium* cluster IVXA + IVXB**	1.03×10^6 (7.20×10^5)	4.88×10^5 (2.06×10^5)	0.804	0.96
Faecalibacterium prausnitzii and relatives	1.82×10^7 (8.26×10^6)	3.16×10^7 (1.44×10^7)	0.399	0.702
***Ruminococcaceae* (excluding Fprau)**	1.05×10^7 (5.56×10^6)	1.47×10^7 (9.08×10^6)	0.487	0.927
Ruminococcus2	1.00 x 107 (5.17×10^6)	9.55×10^6 (2.17×10^6)	0.843	0.992
Coprococcus	2.95×10^6 (2.21×10^6)	9.63×10^5 (5.84×10^5)	0.399	0.487
Blautia	2.53×10^7 (1.24×10^7)	1.40×10^7 (1.16×10^7)	0.551	0.702
Lactobacillus/Enterococcus	4.16×10^6 (3.32×10^6)	3.27×10^6 (2.98×10^5)	0.843	0.96
Lactococcus	9.20×10^5 (7.28×10^5)	1.50×10^5 (8.13×10^5)	0.823	0.823
Dorea	4.08×10^6 (2.71×10^6)	2.46×10^6 (2.21×10^6)	0.234	0.52
Anaerostipes	1.26×10^6 (6.87×10^5)	5.94×10^5 (1.39×10^5)	0.96	0.96
Lachnospiraceae incertae sedi	3.63×10^6 (2.70×10^6)	6.29×10^6 (4.67×10^6)	0.655	0.764
Collinsella	4.43×10^6 (4.08×10^6)	9.92×10^6 (8.63×10^6)	0.843	0.843

OFI/2'FL (85/15) quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Abbreviations:** OFI = oligofructose/inulin; 2'FL = 2'fucosyllactose

Substrates: OFI/2'FL (Oligofructose inulin/2'fucosyllactose): 95/5				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	1.63×10^7 (1.55×10^6)	1.67×10^8 (1.14×10^7)	**0.022**	0.063
Bacteroides	3.66×10^5 (1.29×10^5)	3.16×10^6 (1.53×10^6)	0.112	0.37
Prevotella	1.36×10^5 (82401)	2.66×10^5 (2.66×10^5)	0.921	0.96
Alistipes	6.34×10^5 (5.03×10^5)	2.61×10^5 (1.46×10^5)	0.921	0.96
Roseburia	4.78×10^6 (2.65×10^6)	1.81×10^6 (6.81×10^5)	0.691	0.88
***Clostridium* cluster IVXA + IVXB**	9.68×10^5 (6.49×10^5)	7.36×10^5 (4.31×10^5)	0.728	0.96
Faecalibacterium prausnitzii and relatives	1.73×10^7 (8.73×10^6)	3.48×10^7 (1.76×10^7)	0.297	0.702
***Ruminococcaceae* (excluding Fprau)**	1.18×10^7 (6.57×10^6)	1.06×10^7 (4.66×10^6)	0.96	0.96
Ruminococcus2	9.71×10^6 (5.25×10^6)	1.39×10^7 (3.66×10^6)	0.399	0.798
Coprococcus	2.15×10^6 (1.24×10^6)	8.93×10^5 ($.81 \times 10^5$)	0.254	0.487
Blautia	2.02×10^7 (9.07×10^6)	1.11×10^8 (8.69×10^7)	0.234	0.682
Lactobacillus/Enterococcus	3.80×10^6 (1.89×10^6)	4.84×10^6 (4.83×10^6)	0.766	0.96
Lactococcus	68911 (56130)	1.72×10^5 (1.50×10^5)	0.71	0.823
Dorea	3.27×10^6 (2.33×10^6)	2.53×10^6 (2.33×10^6)	0.843	0.907
Anaerostipes	1.21×10^6 (7.05×10^5)	4.13×10^5 (68083)	0.728	0.894
Lachnospiraceae incertae sedi	2.90×10^6 (2.17×10^6)	7.19×10^6 (5.18×10^6)	0.234	0.764
Collinsella	3.62×10^6 (3.10×10^6)	1.64×10^6 (1.36×10^6)	0.551	0.772

OFI/2'FL (95/5) quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Abbreviations:** OFI = oligofructose/inulin; 2'FL = 2'fucosyllactose

Substrates: OF/2'FL: (Oligofructose/2'fucosyllactose) 50/50				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	3.14×10^7 (7.77×10^6)	$1{,}53 \times 10^9$ (9.76×10^7)	**0.009**	**0.04**
Bacteroides	5.22×10^6 (2.51×10^6)	4.14×10^7 (3.04×10^7)	0.333	0.424
Prevotella	4.39×10^6 (2.52×10^6)	5.67×10^6 (2.52×10^6)	0.96	0.96
Alistipes	1.17×10^6 (9.76×10^5)	6.44×10^5 (5.29×10^5)	0.297	0.832
Roseburia	3.27×10^7 (1.65×10^7)	9.00×10^7 (4.07×10^7)	0.321	0.88
***Clostridium* cluster IVXA + IVXB**	3.96×10^6 (2.24×10^6)	2.73×10^6 (7.70×10^5)	0.96	0.96
Faecalibacterium prausnitzii and relatives	3.57×10^7 (1.20×10^7)	2.73×10^7 (3.62×10^6)	0.62	0.723
***Ruminococcaceae* (excluding Fprau)**	2.69×10^7 (1.00×10^7)	2.22×10^7 (5.29×10^6)	0.62	0.927
Ruminococcus2	1.29×10^7 (6.28×10^6)	6.28×10^7 (2.60×10^7)	**0.029**	0.101
Coprococcus	2.42×10^6 (6.72×10^5)	1.22×10^6 (5.21×10^5)	0.215	0.487
Blautia	2.37×10^7 (9.41×10^6)	1.85×10^8 (6.04×10^7)	**0.037**	0.173
Lactobacillus/Enterococcus	9.90 x 106 (4.97×10^6)	4.89 x 106 (3.66×10^6)	0.551	0.96
Lactococcus	1.76×10^6 (1.74×10^6)	59904 (41397)	0.519	0.807
Dorea	2.99×10^6 (1.33×10^6)	2.21×10^6 (1.33×10^6)	0.297	0.52
Anaerostipes	1.77×10^6 (1.14×10^6)	4.12×10^6 (3.07×10^6)	0.399	0.798
Lachnospiraceae incertae sedi	2.91×10^6 (1.27×10^6)	1.52×10^7 (6.62×10^6)	0.137	0.764
Collinsella	7.04×10^5 (81019)	6.32×10^7 (4.52×10^7)	**0.033**	0.115

OF/2'FL (50/50) quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Abbreviations:** OFI = oligofructose; 2'FL = 2'fucosyllactose

Substrates: ITF-mix/2'FL: (inulin-type fructans mix/2'fucosyllactose) 50/50				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	2.66×10^7 (1.05×10^7)	1.40×10^9 (1.45×10^8)	**0.004**	**0.04**
Bacteroides	5.67×10^6 (2.58×10^6)	4.76×10^7 (2.72×10^7)	0.264	0.37
Prevotella	2.73×10^6 (2.04×10^6)	9.00×10^6 (5.48×10^6)	0.457	0.96
Alistipes	6.31×10^5 (4.24×10^5)	2.76×10^5 (1.37×10^5)	0.96	0.96
Roseburia	3.68×10^7 (2.01×10^7)	8.29×10^7 (7.01×10^7)	0.372	0.88
***Clostridium* cluster IVXA + IVXB**	4.07×10^6 (2.32×10^6)	2.56×10^6 (7.13×10^5)	0.96	0.96
Faecalibacterium prausnitzii and relatives	3.11×10^7 (1.30×10^7)	4.97×10^7 (8.14×10^6)	0.275	0.702
***Ruminococcaceae* (excluding Fprau)**	2.26×10^7 (8.79×10^6)	2.29×10^7 (9.59×10^6)	0.882	0.96
Ruminococcus2	1.41×10^7 (6.96×10^6)	9.70×10^8 (5.91×10^7)	**0.02**	0.101
Coprococcus	2.63×10^6 (1.26×10^6)	1.02×10^6 (4.52×10^5)	0.487	0.487
Blautia	2.47×10^7 (9.96×10^6)	1.85×10^8 (6.25×10^7)	**0.009**	0.119
Lactobacillus/Enterococcus	1.2×10^7 (8.14×10^6)	9.08×10^6 (4.52×10^6)	0.843	0.96
Lactococcus	2.07×10^5 (1.95×10^5)	0	0.118	0.807
Dorea	2.38×10^6 (1.17×10^6)	1.81×10^6 (1.36×10^6)	0.585	0.683
Anaerostipes	1.72×106 (8.95×10^5)	1.81×10^6 (1.36×10^6)	0.585	0.894
Lachnospiraceae incertae sedi	3.23×10^6 (1.80×10^6)	5.12×10^6 (1.15×10^6)	0.457	0.764
Collinsella	4.30×10^5 (2.14×10^5)	$6..65 \times 10^7$ (4.97×10^7)	**0.01**	0.08

ITF-mix/2'FL (50/50) quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified

adjusted *P* (*Q*) values (grey columns). **Abbreviations:** ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Substrates: OF/β-glucan: (Oligofructose/β-glucan) 50/50				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	3.51×10^7 ($1.0^8 \times 10^7$)	1.60×10^9 (2.15×10^8)	**0.033**	0.066
Bacteroides	2.48×10^6 (1.53×10^6)	2.65×10^7 (2.31×10^7)	0.18	0.37
Prevotella	$3.0^4 \times 10^6$ (2.10×10^6)	3.88×10^6 (2.22×10^6)	0.96	0.96
Alistipes	3.53×10^5 (2.42×105)	1.96×10^5 (16887)	0.804	0.96
Roseburia	2.92×10^7 (1.79×10^7)	5.35×10^7 (1.79×10^7)	0.215	0.88
***Clostridium* cluster IVXA + IVXB**	4.31×10^6 (2.82×10^6)	2.79×10^6 (1.07×10^6)	0.691	0.96
Faecalibacterium prausnitzii and relatives	5.24×10^7 (2.00×10^7)	8.38×10^7 (2.52×10^7)	0.427	0.702
***Ruminococcaceae* (excluding Fprau)**	2.02×10^7 (9.05×10^6)	2.97×10^7 ($6,94 \times 10^6$)	0.18	0.867
Ruminococcus2	1.45×10^7 (7.25×10^6)	2.30×10^7 ($1.00E+0^7$)	0.234	0.654
Coprococcus	1.88×10^6 (5.59×10^5)	6.46×10^5 (2.44×10^5)	0.321	0.487
Blautia	2.72×10^7 (1.07×10^7)	5.68×10^7 (1.35×10^7)	0.427	0.682
Lactobacillus/Enterococcus	7.37×10^6 ($4.28 \times 1\ 0^6$)	7.41×0^6 (4.21×10^6)	0.568	0.96
Lactococcus	26288 (11875)	36828 (14386)	0.766	0.823
Dorea	2.45×10^6 (1.17×10^6)	1.05×10^6 (4.83×10^5)	0.921	0.921
Anaerostipes	1.30×10^6 (8.58×10^5)	5.20×10^6 (5.03×10^6)	0.321	0.798
Lachnospiraceae incertae sedi	2.71×10^6 (1.26×10^6)	2.37×10^7 (2.22×10^7)	0.275	0.764
Collinsella	3.98×10^5 (3.33×10^5)	1.80×10^7 (7.36×10^6)	**0.017**	0.08

OF/β-glucan (50/50) quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Abbreviations:** OFI = oligofructose

Substrates: OFI/β-glucan: (Oligofructose inulin/β-glucan) 50/50				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	3.62×10^7 (6.32×10^6)	1.11×10^9 (2.85×10^8)	0.101	0.142
Bacteroides	2.49×10^6 (1.08×10^6)	2.32×10^7 (2.85×10^7)	0.165	0.37
Prevotella	2.49×10^6 (1.51×10^6)	2.32×10^7 (1.87×10^7)	0.457	0.96
Alistipes	2.23×10^5 (1.38×10^5)	1.15×10^5 (65059)	0.585	0.96
Roseburia	3.75×10^7 (2.04×10^7)	7.36×10^7 (6.67×10^7)	0.96	0.96
***Clostridium* cluster IVXA + IVXB**	4.36×10^6 (2.57×10^6)	1.25×10^6 (7.41×10^5)	0.254	0.96
Faecalibacterium prausnitzii and relatives	5.10×10^7 (1.49×10^7)	3.98×10^7 (8.24×10^6)	0.551	0.702
***Ruminococcaceae* (excluding Fprau)**	1.67×10^7 (7.50×10^6)	1.91×10^7 (7.78×10^6)	0.346	0.867
Ruminococcus2	1.56×10^7 (6.93×10^6)	2.01×10^7 (1.32×10^7)	0.921	0.992
Coprococcus	2.61×10^6 (8.45×10^5)	2.66×10^5 (27944)	**0.004**	0.056
Blautia	2.71×10^7 (9.96×10^6)	7.30×10^7 (2.78×10^7)	0.487	0.682
Lactobacillus/Enterococcus	7.69×10^6 (4.80×10^6)	5.78×10^7 (4.67×10^7)	0.297	0.96
Lactococcus	19747 (6089)	41484 (20777)	0.503	0.807
Dorea	3.17×10^6 (1.61×10^6)	8.70×10^5 (5.03×10^5)	0.15	0.52
Anaerostipes	2.03×10^6 (1.47×10^6)	2.35×10^6 (2.18×10^6)	0.921	0.96
Lachnospiraceae incertae sedi	2.80×10^6 (1.28×10^6)	1.49×10^7 (1.43×10^7)	0.728	0.784
Collinsella	7.09×10^5 (5.92×10^5)	1.80×10^7 (1.57×10^7)	0.053	0.138

OFI/β-glucan (50/50) quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Abbreviations:** OFI = oligofructose/inulin

Substrates: ITF-mix/β-glucan: (Inulin-type fructan mix/β-glucan) 50/50				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	3.00×10^7 (7.50×10^6)	1.45×10^9 (1.37×10^8)	**0.007**	**0.04**
Bacteroides	2.45×10^6 (1.26×10^6)	1.91×10^7 (1.55×10^7)	0.254	0.37
Prevotella	2.87×10^6 (1.80×10^6)	1.07×10^6 (7.55×10^5)	0.585	0.96
Alistipes	2.88×10^5 (2.72×10^5)	93251 (78035)	0.165	0.751
Roseburia	3.97×10^7 (2.47×10^7)	7.14×10^7 (3.91×10^7)	0.551	0.88
***Clostridium* cluster IVXA + IVXB**	4.19×10^6 (2.46×10^6)	2.35×10^7 (3.91×10^7)	0.96	0.96
Faecalibacterium prausnitzii and relatives	5.16×10^7 (1.84×10^7)	6.17×10^7 (2.08×10^7)	0.921	0.921
***Ruminococcaceae* (excluding Fprau)**	1.87×10^7 (8.54×10^6)	2.47×10^7 (1.09×10^7)	0.18	0.867
Ruminococcus2	1.43×10^7 (6.67×10^6)	3.52×10^7 (2.48×10^7)	0.346	0.798
Coprococcus	2.10×10^6 (6.58×10^5)	6.31×10^5 (2.86×10^5)	0.101	0.355
Blautia	2.81×10^7 (1.05×10^7)	7.51×10^7 (2.00×10^7)	0.297	0.682
Lactobacillus/Enterococcus	5.48×10^6 (2.59×10^6)	2.66×10^7 (1.53×10^7)	0.234	0.96
Lactococcus	6.92×10^5 (6.78×10^5)	82124 (47746)	0.804	0.823
Dorea	2.91×10^6 (1.41×10^6)	1.03×10^6 (6.22×10^5)	0.234	0.52
Anaerostipes	1.44×10^6 (1.08×10^6)	7.11×10^6 (6.87×10^6)	0.346	0.798
Lachnospiraceae incertae sedi	2.69×10^6 (1.34×10^6)	1.76×10^7 (1.70×10^7)	0.585	0.764

Collinsella	1.25×10^6 (8.08×10^5)	1.94×10^7 (1.11×10^7)	0.197	0.306

ITF-mix/β-glucan (50/50) quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey columns). **Abbreviations:** ITF = inulin-type fructans

Substrates: Negative control				
Genus	**T0**	**T8**	**T0 vs T8**	
			P	***Q***
Bifidobacterium	3.51×10^7 (5.21×10^6)	5.24×10^7 (7.78×10^6)	0.62	0.62
Bacteroides	1.00×10^6 (7.63×10^5)	9.98×10^5 (3.73×10^5)	0.62	0.667
Prevotella	1.01×10^6 (8.29×10^5)	5.51×10^5 (4.77×10^5)	0.691	0.96
Alistipes	3.24×10^5 (2.35×10^5)	41392 (27568)	0.18	0.751
Roseburia	2.89×10^7 (1.58×10^7)	8.26×10^6 (4.06×10^6)	0.921	0.96
***Clostridium* cluster IVXA + IVXB**	2.54×10^6 (1.43×10^6)	1.08×10^6 (1.38×10^5)	0.728	0.96
Faecalibacterium prausnitzii and relatives	2.66×10^7 (1.16×10^7)	1.63×10^7 (6.36×10^6)	0.804	0.866
***Ruminococcaceae* (excluding Fprau)**	2.36×10^7 (1.14×10^7)	1.32×10^7 (6.40×10^6)	0.372	0.867
Ruminococcus2	1.11×10^7 (6.07×10^6)	8.00×10^6 (2.25×10^6)	0.766	0.992
Coprococcus	1.57×10^6 (6.00×10^5)	7.83×10^5 (2.56×10^5)	0.457	0.487
Blautia	3.03×10^7 (1.27×10^7)	1.98×10^7 (5.55×10^6)	0.457	0.682
Lactobacillus/Enterococcus	1.00×10^7 (2.80×10^6)	1.07×10^7 (4.38×10^6)	0.921	0.96
Lactococcus	30605 (7718)	1.51×10^5 (1.07×10^5)	0.297	0.807
Dorea	3.65×10^6 (1.74×10^6)	1.81×10^6 (7.77×10^5)	0.427	0.639
Anaerostipes	2.46×10^6 (1.30×10^6)	7.67×10^5 (6.36×10^5)	0.112	0.786

Lachnospiraceae incertae sedi	2.66×10^6 (1.21×10^6)	1.69×10^6 (4.63×10^5)	0.96	0.96
Collinsella	9.87×10^5 (5.45×10^5)	5.48×10^5 (4.07×10^6)	0.691	0.843

Negative control quantitative microbiome profiling (QMP) abundances measured by 16s rRNA sequencing T0 and T8 counts expressed as cells/mL mean and standard error (SE). Significant differences between respective T0 and T8 substrate sampling point are indicated by specified adjusted *P* (*Q*) values (grey Columns).

Appendix 2.6 Individual donor *Bifidobacterium* QMP values

***Individual donor Bifidobacterium* QMP data**			
Time (h)	**Donor 1**	**Donor 2**	**Donor 3**
OF T0	4.60×10^7	2.40×10^7	5.30×10^7
OF T8	1.33×10^9	1.25×10^9	9.99×10^8
OFI T0	5.82×10^6	2.13×10^7	1.76×10^7
OFI T8	4.24×10^8	3.47×10^8	1.51×10^8
ITF-mix T0	5.62×10^7	1.58×10^7	1.41×10^7
ITF-mix T8	1.35×10^8	3.60×10^8	8.76×10^8
2'FL TO	1.95×10^7	2.09×10^7	2.09×10^7
2'FL T8	3.04×10^7	1.67×10^8	2.72×10^7
β-glucan T0	4.48×10^7	3.31×10^7	1.85×10^7
β-glucan T8	1.00×10^9	9.81×10^8	8.07×10^8
OFI/2'FL 50/50 T0	2.03×10^7	2.01×10^7	2.38×10^7
OFI/2'FL 50/50 T8	6.31×10^7	1.63×10^8	2.73×10^8
OFI/2'FL 85/15 T0	1.82×10^7	2.39×10^7	1.73×10^7
OFI/2'FL 85/15 T8	2.07×10^7	2.19×10^8	2.06×10^8
OFI/2'FL 95/5 T0	1.83×10^7	1.74×10^7	1.33×10^7
OFI/2'FL 95/5 T8	1.79×10^8	1.77×10^8	1.44×10^8
OF/2'FL T0	4.65×10^7	2.06×10^7	2.73×10^7
OF/2'FL T8	1.70×10^9	1.53×10^9	1.36×10^9
ITF-mix/2'FL T0	4.76×10^7	1.58×10^7	1.64×10^7
ITF-mix/2'FL T8	1.21×10^9	1.68×10^9	1.31×10^9
OF/β-glucan T0	5.66×10^7	2.44×10^7	2.42×10^7
OF/β-glucan T8	1.99×10^9	1.57×10^9	1.25×10^9
OFI/β-glucan T0	4.88×10^7	3.08×10^7	2.90×10^7
OFI/β-glucan T8	1.48×10^9	5.49×10^8	1.30×10^9
ITF-mix/β-glucan T0	4.35×10^7	2.67×10^7	1.90×10^7
ITF-mix/β-glucan T8	1.72×10^9	1.29×10^9	1.35×10^9
Neg T0	4.55×10^7	2.92×10^7	3.06×10^7
Neg T8	5.58×10^7	3.75×10^7	6.38×10^7

Individual donor *Bifidobacterium* QMP data expressed as cells/mL. **Abbreviations:** OF = oligofructose; OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Appendix 2.7 SCFA molar ratios at 0, 4, 8, 24, 36 and 48 h across all interventions

Time (h)	Acid	OF	OFI	ITF-mix	2'FL	β-glucan	OFI/2'FL 50/50	OFI/2'FL 85/15	OFI/2'L 95/5	OF/2'FL	ITF-mix/2'FL	OF/β-glucan	OFI/β-glucan 50/50	ITF-mix/β-glucan	Negative
T0	Acetate	57.77 (3.51)	45.49 (3.09)	57.94 (2.68)	44.97 (3.12)	57.91 (2.72)	45.21 (3.1)	45.39 (2.98)	45.15 (2.92)	58.07 (2.77)	57.74 (2.72)	57.74 (2.71)	57.70 (2.62)	57.66 (2.63)	57.71 (2.70)
	Propionate	30.57 (2.79)	41.61 (6.23)	30.35 (3.38)	42.18 (6.44)	30.56 (3.44)	41.72 (6.30)	41.59 (6.15)	41.96 (6.20)	30.32 (3.46)	30.55 (3.40)	30.64 (3.37)	30.64 (3.35)	30.58 (3.32)	30.58 (3.40)
	Butyrate	11.67 (0.95)	12.93 (3.47)	11.67 (2.77)	12.82 (3.44)	11.53 (2.71)	13.04 (3.36)	12.97 (3.35)	12.88 (3.39)	11.61 (2.79)	11.71 (2.78)	11.64 (2.75)	11.76 (2.80)	11.80 (2.82)	11.65 (2.78)
T4	Acetate	67.72 (4.58)	51.82 (6.61)	69.63 (3.92)	55.51 (4.90)	64.82 (3.31)	53.83 (4.09)	53.77 (4.62)	50.61 (6.60)	71.52 (4.74)	71.01 (5.81)	68.43 (3.24)	69.66 (3.62)	70.87 (3.06)	61.17 (3.74)
	Propionate	27.26 (3.51)	39.20 (5.74)	24.64 (3.49)	36.68 (3.68)	25.93 (3.71)	38.12 (3.78)	37.51 (4.24)	40.69 (5.63)	23.09 (3.33)	23.78 (4.15)	24.03 (3.19)	23.34 (3.33)	22.34 (3.01)	26.97 (3.46)
	Butyrate	5.04 (1.07)	9.00 (0.94)	5.72 (0.85)	7.79 (1.23)	9.23 (0.39)	8.09 (0.86)	8.72 (0.55)	8.68 (0.97)	5.38 (1.45)	5.21 (1.75)	7.53 (0.56)	7.02 (0.44)	6.79 (0.30)	11.87 (0.28)
T8	Acetate	74.61 (1.39)	68.65 (4.44)	73.85 (1.61)	81.98 (2.54)	65.37 (3.77)	78.25 (3.17)	76.59 (2.74)	72.34 (5.29)	75.51 (0.79)	74.68 (0.63)	63.68 (4.65)	66.22 (2.19)	66.75 (3.20)	62.95 (2.93)
	Propionate	18.92 (1.13)	23.69 (0.54)	19.82 (1.70)	14.53 (1.86)	24.84 (2.90)	18.89 (2.36)	20.43 (2.07)	23.56 (4.93)	20.02 (1.33)	20.01 (1.08)	27.65 (2.35)	24.17 (2.45)	25.46 (1.53)	24.71 (2.62)
	Butyrate	6.46 (0.38)	7.67 (4.45)	6.32 (0.23)	3.49 (1.29)	9.78 (1.38)	2.86 (0.81)	2.97 (0.67)	4.11 (0.36)	4.47 (0.61)	5.32 (0.78)	8.68 (2.31)	9.61 (0.77)	7.79 (1.82)	12.33 (0.57)

Time (h)	Acid	OF	OFI	ITF-mix	2'FL	β-glucan	OFI/2'FL 50/50	OFI/2'FL 85/15	OFI/2'L 95/5	OF/2'FL	ITF-mix/2'FL	OF/β-glucan	OFI/β-glucan 50/50	ITF-mix/β-glucan	Negative
T24	Acetate	69.05 (2.30)	65.42 (3.74)	66.90 (3.05)	75.72 (2.34)	49.46 (2.73)	68.23 (0.96)	66.59 (3.12)	64.15 (2.77)	71.45 (2.19)	72.22 (1.27)	57.12 (3.42)	54.94 (1.29)	57.26 (3.25)	70.36 (1.24)
	Propionate	21.62 (3.43)	22.93 (1.79)	24.53 (4.51)	17.60 (1.23)	35.36 (1.83)	23.46 (0.91)	22.43 (1.81)	22.79 (1.07)	20.22 (3.95)	18.69 (1.78)	28.85 (2.70)	30.92 (1.12)	28.45 (2.80)	20.21 (1.22)
	Butyrate	9.34 (2.14)	11.64 (1.96)	8.57 (2.03)	6.68 (1.32)	15.18 (1.12)	8.31 (0.53)	10.98 (1.43)	13.07 (1.80)	8.33 (2.01)	9.09 (1.69)	14.03 (1.05)	14.14 (0.77)	14.30 (0.71)	9.45 (1.32)
T36	Acetate	68.81 (2.31)	66.58 (3.45)	66.10 (2.12)	74.41 (3.04)	49.75 (1.67)	68.23 (2.35)	67.10 (4.23)	65.28 (3.95)	69.45 (2.44)	71.06 (1.01)	57.25 (2.87)	54.64 (1.34)	57.99 (2.67)	70.29 (0.76)
	Propionate	21.35 (3.50)	21.63 (2.14)	23.82 (3.87)	18.21 (2.62)	34.00 (1.41)	23.52 (1.68)	21.67 (2.28)	22.83 (2.06)	21.76 (3.90)	19.86 (1.43)	28.41 (2.33)	31.05 (0.94)	27.56 (2.14)	19.97 (1.15)
	Butyrate	9.84 (1.78)	11.79 (1.46)	10.08 (1.84)	7.39 (0.81)	16.25 (1.28)	8.24 (0.71)	11.24 (1.95)	11.89 (2.14)	8.79 (1.87)	9.09 (1.62)	14.34 (0.83)	14.31 (1.45)	14.44 (0.93)	9.74 (1.30)
T48	Acetate	68.94 (2.47)	64.56 (3.69)	66.28 (2.70)	73.66 (3.46)	51.71 (1.63)	69.38 (2.97)	66.25 (4.37)	64.49 (3.69)	69.23 (2.92)	70.21 (1.67)	55.13 (3.49)	54.75 (2.30)	56.07 (2.94)	69.85 (1.69)
	Propionate	21.27 (3.54)	23.89 (2.54)	23.76 (4.41)	17.97 (2.66)	32.56 (1.85)	22.26 (2.47)	22.49 (2.56)	23.44 (2.37)	22.05 (4.40)	20.76 (1.71)	30.51 (3.26)	30.51 (1.43)	28.96 (2.61)	20.06 (1.39)
	Butyrate	9.79 (1.69)	11.55 (1.23)	9.97 (1.77)	8.37 (1.66)	15.74 (0.94)	8.37 (0.55)	11.25 (1.99)	12.07 (1.59)	8.73 (1.64)	9.03 (1.63)	14.37 (1.00)	14.74 (1.60)	14.97 (1.40)	10.11 (1.47)

SCFA molar ratios (mean and standard error) at 0, 4, 8, 24, 36 and 48 h fermentation. **Abbreviations:** OF = oligofructose, OFI = oligofructose/inulin; ITF = inulin-type fructans; 2'FL = 2'fucosyllactose

Appendix 3.1 Mean neurotransmitter concentrations using pH-controlled *in vitro* batch culture fermentation at 0, 10, 24, and 48 h.

GABA	T0	T10		T24		T48	
	Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
OF	3605.01 (1347.34)	22521.68 (11129.67)	**0.04**	648363.68 (196093.75)	**0.01**	836187.28 (303309.74)	**0.006a**
2'FL	3442.02 (1215.50)	10600.01 (5438.81)	1.00	259205.61 (86167.27)	0.78	173619.59 (62499.15)	1.00b
OF/2'FL	3620.14 (1330.43)	11033.90 (4680.11)	1.00	398177.21 (239084.58)	0.15	648721.89 (271926.65)	**0.04a**
Negative	3616.91 (1128.03)	2684.10 (765.47)	1.00	24783.72 (10623.96)	1.00	22208.95 (10595.85)	1.00c
***P* (b)**	1.00	0.25		0.09		**0.05**	

Serotonin	T0	T10		T24		T48	
	Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
OF	1187.13 (331.21)	1205.84 (347.58)	1.00	1307.19 (374.12)	**0.02**	1269.80 (357.09)	0.39a
2'FL	1188.22 (334.62)	1215.08 (355.87)	1.00	1246.12 (361.25)	0.64	1130.60 (332.18)	1.00a
OF/2'FL	1172.09 (334.56)	1205.94 (345.19)	1.00	1236.77 (349.36)	0.44	1207.17 (346.64)	1.00a
Negative	1202.60 (340.33)	1182.57 (335.46)	1.00	1170.84 (327.32)	1.00	1208.59 (320.75)	1.00a
***P* (b)**	1.00	1.00		0.99		0.99	

LC-MS analysis of GABA and serotonin concentrations in the supernatant of effluents collected from vessel 1-4 at 0, 10 24 and 48 h representing the mean (*n* = 5) and standard error (SE) of the data. Concentration reported in (ng/mL). **(a)** Significant differences compared with respective 0 h sampling are identified with specified *P* values (grey columns). **(b)** Significant differences between substrates at 0, 10, 24 and 48 h are indicated by specified *P* values (orange column). Significant differences between substrates at 48h are indicated by differing letters (grey column). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Dopamine	T0	T10		T24		T48	
	Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
OF	259.20 (174.26)	483.47 (144.86)	0.88	515.67 (141.38)	0.57	492.78 (140.66)	0.39a
2'FL	260.44 (172.92)	504.36 (144.53)	0.7	472.31 (134.94)	0.98	463.51 (134.07)	1.00a
OF/2'FL	252.00 (168.45)	506.93 (142.81)	0.61	491.46 (138.97)	0.71	509.03 (138.64)	1.00a
Negative	260.51 (172.80)	505.69 (142.90)	0.69	490.17 (139.80)	0.79	489.82 (140.48)	1.00a
***P* (b)**	1.00	0.99		0.98		0.97	

Tryptophan	T0	T10		T24		T48	
	Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
OF	231829.66 (5043.73)	248312.00 (15012.54)	1.00	279613.74 (8319.84)	**0.01**	258392.15 (15163.45)	0.39a
2'FL	220519.04 (4664.84)	228039.90 (7593.53)	1.00	217513.88 (10389.75)	1.00	208840.54 (16385.77)	1.00a
OF/2'FL	238282.03 (9598.23)	239076.92 (3742.07)	1.00	240143.72 (12042.85)	1.00	223110.55 (16564.48)	1.00a
Negative	230615.23 (4034.57)	209996.53 (16263.66)	0.57	189245.02 (5742.87)	0.02	165878.40 (19420.11)	1.00b
***P* (b)**	1.00	0.165		**≤ 0.001**		0.012	

LC-MS analysis of dopamine and tryptophan concentrations in the supernatant of effluents collected from vessel 1-4 at 0, 10 24 and 48 h representing the mean (*n* = 5) and standard error (SE) of the data. Concentration reported in (ng/mL). **(a)** Significant differences compared with respective 0 h sampling are identified with specified *P* values (grey columns). **(b)** Significant differences between substrates at 0, 10, 24 and 48 h are indicated by specified *P* values (orange row). Significant differences between substrates at 48h are indicated by differing letters (grey column). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Appendix 3.2 Individual neurotransmitter concentrations using pH-controlled *in vitro* batch culture fermentation at 0, 10, 24, and 48 h.

GABA		T 0	T 10	T 24	T 48
OF	D1	0.00	0.00	0.00	0.00
	D2	8321.51	26452.49	1201822.56	1871902.65
	D3	2582.76	63631.19	822092.78	958973.30
	D4	3651.03	13626.42	676310.88	697401.34
	D5	3469.78	8898.30	541592.22	652659.12
2'FL	D1	226870.90	256768.12	195666.71	206978.00
	D2	213229.43	226184.19	248452.11	224037.82
	D3	211748.14	215851.22	235932.38	236802.94
	D4	235805.84	226533.55	201910.08	146354.07
	D5	214940.89	214862.43	205608.10	230029.89
OF/2'FL	D1	0.00	0.00	0.00	23631.03
	D2	8225.82	26150.80	575892.04	1374958.14
	D3	2586.21	6501.57	80218.58	298903.10
	D4	3563.56	17053.34	1261969.41	1224407.69
	D5	3725.12	5463.82	72806.03	321709.53
Negative	D1	235554.80	250838.50	211434.81	201325.51
	D2	229958.87	227712.55	189234.79	176458.16
	D3	242622.28	156486.47	183276.24	91655.06
	D4	225924.82	222171.96	180821.41	191547.31
	D5	219015.38	192773.18	181457.88	168405.98

Individual volunteer GABA concentrations across 0, 10, 24, and 48 h fermentation. Concentration in (ng/mL). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Serotonin		T 0	T 10	T 24	T 48
OF	D1	1828.543	1891.399	1967.226	1938.207
	D2	1777.178	1894.009	2089.146	1957.425
	D3	1054.951	1073.992	1108.638	1141.57
	D4	1274.958	1169.802	1370.916	1311.815
	D5	0	0	0	0
2'FL	D1	1821.85	1866.099	1926.757	1757.88
	D2	1813.422	1899.155	1962.383	1797.386
	D3	1024.94	874.4826	984.1845	852.496
	D4	1280.865	1435.652	1357.258	1245.26
	D5	0	0	0	0
OF/2'FL	D1	1821.92	1862.844	1892.053	1834.004
	D2	1826.322	1886.344	1919.713	1932.08
	D3	1052.394	1026.947	1113.632	1042.507
	D4	1159.836	1253.587	1258.467	1227.245
	D5	0	0	0	0
Negative	D1	1827.556	1819.004	1768.697	1678.534
	D2	1846.128	1810.73	1770.456	1813.248
	D3	991.7268	984.6516	992.3396	1247.946
	D4	1347.575	1298.477	1322.696	1303.235
	D5	0	0	0	0

Individual volunteer serotonin concentrations across 0, 10, 24, and 48 h fermentation. Concentration in (ng/mL). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Tryptophan		T 0	T 10	T 24	T 48
OF	D1	1828.543	1891.399	1967.226	1938.207
	D2	1777.178	1894.009	2089.146	1957.425
	D3	1054.951	1073.992	1108.638	1141.57
	D4	1274.958	1169.802	1370.916	1311.815
	D5	0	0	0	0
2'FL	D1	1821.85	1866.099	1926.757	1757.88
	D2	1813.422	1899.155	1962.383	1797.386
	D3	1024.94	874.4826	984.1845	852.496
	D4	1280.865	1435.652	1357.258	1245.26
	D5	0	0	0	0
OF/2'FL	D1	1821.92	1862.844	1892.053	1834.004
	D2	1826.322	1886.344	1919.713	1932.08
	D3	1052.394	1026.947	1113.632	1042.507
	D4	1159.836	1253.587	1258.467	1227.245
	D5	0	0	0	0
Negative	D1	1827.556	1819.004	1768.697	1678.534
	D2	1846.128	1810.73	1770.456	1813.248
	D3	991.7268	984.6516	992.3396	1247.946
	D4	1347.575	1298.477	1322.696	1303.235
	D5	0	0	0	0

Individual volunteer tryptophan concentrations across 0, 10, 24, and 48 h fermentation. Concentration in (ng/mL). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Dopamine		T 0	T 10	T 24	T 48
OF	D1	875.4	864.102	850.9363	836.0207
	D2	0	675.7037	655.9527	651.2717
	D3	0	445.85	557.827	558.683
	D4	0	0	0	0
	D5	420.6117	431.7067	513.6395	417.9245
2'FL	D1	862.447	861.6107	809.4443	772.3153
	D2	0	661.6107	619.8304	629.4703
	D3	0	573.3697	519.1633	561.9866
	D4	0	0	0	0
	D5	439.7643	425.2434	413.1043	353.7967
OF/2'FL	D1	843.5437	858.521	845.238	817.4137
	D2	0	655.4917	608.374	672.3763
	D3	0	561.388	555.026	565.3753
	D4	0	0	0	0
	D5	416.4767	459.234	448.6927	489.9617
Negative	D1	861.3027	858.1283	842.6837	846.2063
	D2	0	664.7643	633.3073	636.363
	D3	0	542.4397	541.599	538.9684
	D4	0	0	0	0
	D5	441.2233	463.1307	433.266	427.541

Individual volunteer dopamine concentrations across 0, 10, 24, and 48 h fermentation. Concentration in (ng/mL). **Abbreviations:** OF = oligofructose; 2’FL = 2’fucosyllactose

Appendix 3.3 Mean organic acid concentrations using pH-controlled *in vitro* batch culture fermentation at 0, 10, 24, and 48 h.

Acetate	Time (h)	T0	T10		T24		T48	
		Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
Substrate	OF	1.43 (0.10)	23.16 (1.35)	**≤ 0.001**	67.02 (4.28)	**≤ 0.001**	93.37 (3.36)	**≤ 0.001a**
	2'FL	1.44 (0.10)	13.59 (4.08)	**0.004**	34.41 (4.85)	**≤ 0.001**	44.42 (3.92)	**≤ 0.001b**
	OF/2'FL	1.43 (0.10)	16.89 (3.68)	**≤ 0.001**	55.94 (4.67)	**≤ 0.001**	83.83 (5.56)	**≤ 0.001a**
	Negative	1.43 (0.10)	1.58 (0.10)	1.00	2.01 (0.23)	1.00	2.51 (0.43)	1.00c
***P* (b)**		1.00	**≤ 0.001**		**≤ 0.001**		**≤ 0.001**	

Propionate	Time (h)	T0	T10		T24		T48	
		Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
Substrate	OF	0.89 (0.11)	4.20 (0.46)	**≤ 0.001**	22.72 (1.59)	**≤ 0.001**	34.72 (2.06)	**≤ 0.001a**
	2'FL	0.90 (0.11)	1.35 (0.47)	**≤ 0.001**	5.77 (0.77)	**≤ 0.001**	13.55 (1.91)	**≤ 0.001b**
	OF/2'FL	0.90 (0.12)	5.08 (0.52)	**≤ 0.001**	14.06 (1.40)	**≤ 0.001**	32.11 (1.39)	**≤ 0.001a**
	Negative	0.90 (0.12)	1.03 (0.13)	1.00	1.14 (0.15)	1.00	1.25 (0.15)	1.00c
***P* (b)**		1.00	**≤ 0.001**		**≤ 0.001**		**≤ 0.001**	

GC-FID analysis of acetate and propionate concentrations in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing the mean (n = 5) and standard error (SE) of the data. Concentration reported in (mmol l^{-1}). **(a)** Significant differences compared with respective 0 h sampling are identified with specified *P* values (grey columns). **(b)** Significant differences between substrates at 0, 10. 24 and 48 h are indicated by specified *P* values (orange row). Significant differences between substrates at 48 h are indicated by differing letters (grey column). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Butyrate	Time (h)	T0	T10		T24		T48	
		Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
Substrate	**OF**	0.63 (0.20)	4.78 (0.93)	**≤ 0.001**	16.95 (1.08)	**≤ 0.001**	22.85 (1.62)	**≤ 0.001a**
	2'FL	0.62 (0.20)	1.47 (0.87)	1.00	4.65 (0.71)	**≤ 0.001**	7.2 (1.28)	**≤ 0.001b**
	OF/2'FL	0.63 (0.20)	4.43 (0.85)	**≤ 0.001**	12.94 (1.12)	**≤ 0.001**	19.28 (1.74)	**≤ 0.001a**
	Negative	0.63 (0.20)	0.66 (0.22)	1.00	0.75 (0.21)	1.00	0.90 (0.22)	1.00c
***P* (b)**		1.00	**0.003**		**≤ 0.001**		**≤ 0.001**	

Lactate	Time (h)	T0	T10		T24		T48	
		Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
Substrate	**OF**	0	5.96 (0.24)	**≤ 0.001**	4.16 (0.23)	**≤ 0.001**	1.29 (0.12)	**≤ 0.001a**
	2'FL	0	4.01 (0.40)	**≤ 0.001**	2.72 (0.75)	**≤ 0.001**	0.54 (0.15)	**≤ 0.001a**
	OF/2'FL	0	4.77 (0.16)	**≤ 0.001**	3.06 (0.22)	**≤ 0.001**	0.94 (0.14)	**≤ 0.001a**
	Negative	0	0	1.00	0	1.00	0	1.00a
***P* (b)**		1.00	**≤ 0.001**		**≤ 0.001**		**≤ 0.001**	

GC-FID analysis of butyrate and lactate concentrations in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing the mean (*n* = 5) and standard error (SE) of the data. Concentration reported in (mmol l^{-1}). **(a)** Significant differences compared with respective 0 h sampling are identified with specified *P* values (grey columns). **(b)** Significant differences between substrates at 0, 10. 24 and 48 h are indicated by specified *P* values (orange row). Significant differences between substrates at 48 h are indicated by differing letters (grey column). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Succinate	Time (h)	T0	T10		T24		T48	
		Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
Substrate	**OF**	0	0.13 (0.04)	**≤ 0.001**	0.31(0.06)	**≤ 0.001**	0.73 (0.08)	**≤ 0.001a**
	2'FL	0	0.07 (0.01)	**0.06**	0.13 (0.04)	**0.06**	0.27 (0.07)	**0.002b**
	OF/2'FL	0	0.10 (0.02)	**≤ 0.001**	0.24 (0.05)	**≤ 0.001**	0.6 (0.06)	**≤ 0.001a**
	Negative	0	0	1.00	0	1.00	0	1.00c
***P* (b)**		1.00	**0.005**		**≤ 0.001**		**≤ 0.001**	

Total organic acid	Time (h)	T0	T10		T24		T48	
		Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
Substrate	**OF**	2.94 (0.17)	37.83 (5.19)	**≤ 0.001**	97.95 (5.60)	**≤ 0.001**	152.96 (5.55)	**≤ 0.001a**
	2'FL	2.95 (0.16)	20.68 (4.46)	**0.01**	47.88 (4.74)	**≤ 0.001**	64.19 (4.10)	**≤ 0.001b**
	OF/2'FL	2.96 (0.16)	29.02 (6.82)	**≤ 0.001**	80.84 (4.70)	**≤ 0.001**	136.76 (6.48)	**≤ 0.001a**
	Negative	2.96 (0.17)	3.26 (0.28)	1.00	3.90 (0.40)	1.00	4.65 (0.58)	1.00c
***P* (b)**		1.00	**0.005**		**≤ 0.001**		**≤ 0.001**	

GC-FID analysis of succinate and total organic acid concentrations in the supernatant of effluents collected from vessel 1-4 at 0, 10, 24 and 48 h representing the mean (*n* = 5) and standard error (SE) of the data. Concentration reported in (mmol l^{-1}). **(a)** Significant differences compared with respective 0 h sampling are identified with specified *P* values (grey columns). **(b)** Significant differences between substrates at 0, 10. 24 and 48 h are indicated by specified *P* values (orange row). Significant differences between substrates at 48 h are indicated by differing letters (grow column). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Appendix 3.4 Individual organic acid data for all 5 donors used during *in vitro* fermentation

Acetate																				
Time (h)	**OF**					**2'FL**					**OF/2'FL**					**Negative**				
T0	1.51	1.41	1.51	1.66	1.05	1.52	1.42	1.52	1.66	1.05	1.52	1.42	1.51	1.67	1.06	1.52	1.41	1.51	1.67	1.06
T10	22.80	18.90	24.70	27.03	22.35	3.39	13.41	21.70	5.77	23.68	11.96	18.75	5.28	23.73	24.72	1.86	1.43	1.57	1.72	1.31
T24	76.39	77.23	58.59	65.98	56.91	38.24	37.59	48.89	22.19	25.14	45.46	73.08	54.82	55.99	50.34	2.79	2.23	1.70	1.86	1.47
T48	102.76	94.43	91.50	82.15	96.03	53.24	44.02	50.25	30.45	44.14	102.76	84.85	83.31	68.16	80.07	2.98	1.99	3.98	1.87	1.72

Individual donor organic acid data – acetate concentrations across 0, 10, 24, and 48 h fermentation. Concentration in (mmol l^{-1}). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Propionate																				
Time (h)	**OF**					**2'FL**					**OF/2'FL**					**Negative**				
T0	0.78	1.19	0.82	0.56	1.09	0.78	1.20	0.83	0.58	1.09	0.79	1.23	0.83	0.57	1.10	0.78	1.23	0.83	0.57	1.09
T10	5.17	3.04	3.48	3.98	5.35	3.02	0.35	1.03	0.67	1.69	5.15	5.65	4.18	3.75	6.64	1.31	1.24	0.84	0.63	1.11
T24	20.42	21.54	22.51	20.26	28.86	8.04	5.18	5.50	3.44	6.68	15.71	10.66	11.66	13.87	18.42	1.68	1.11	0.97	0.78	1.15
T48	31.04	33.48	37.11	30.46	41.50	10.64	15.21	15.69	7.75	18.43	30.98	30.40	33.76	28.76	36.65	1.79	1.23	1.25	0.87	1.11

Individual donor organic acid data – propionate concentrations across 0, 10, 24, and 48 h fermentation. Concentration in (mmol l^{-1}). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Butyrate																				
Time (h)	**OF**					**2'FL**					**OF/2'FL**					**Negative**				
T0	0.84	0.27	0.50	0.23	1.30	0.80	0.27	0.50	0.22	1.31	0.82	0.26	0.51	0.23	1.31	0.83	0.27	0.50	0.23	1.31
T10	3.11	3.68	4.02	4.71	8.37	0.70	0.56	0.73	0.42	4.94	3.05	2.80	4.03	4.68	7.57	0.81	0.27	0.52	0.26	1.43
T24	16.94	15.08	18.20	14.26	20.25	3.69	3.75	3.50	5.05	7.28	10.85	12.10	12.54	11.93	17.30	0.84	0.39	0.80	0.26	1.48
T48	22.11	20.25	21.92	20.79	29.19	3.78	5.33	6.61	10.18	10.12	18.65	16.13	18.73	16.95	25.94	0.87	0.39	1.09	0.53	1.61

Individual donor organic acid data – butyrate concentrations across 0, 10, 24, and 48 h fermentation. Concentration in (mmol l^{-1}). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Lactate																				
Time (h)	**OF**					**2'FL**					**OF/2'FL**					**Negative**				
T0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T10	6.29	6.37	5.11	6.32	5.71	3.72	3.21	4.72	5.18	3.23	4.78	4.21	5.19	4.82	4.84	0.00	0.00	0.00	0.00	0.00
T24	4.16	3.42	4.79	4.45	3.97	1.41	1.32	5.22	3.62	2.05	3.03	2.40	3.80	3.05	3.02	0.00	0.00	0.00	0.00	0.00
T48	1.41	0.92	1.19	1.32	1.62	0.20	0.30	0.56	0.63	1.03	0.90	0.57	0.87	0.95	1.42	0.00	0.00	0.00	0.00	0.00

Individual donor organic acid data – lactate concentrations across 0, 10, 24, and 48 h fermentation. Concentration in (mmol l^{-1}), **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactosse

Succinate																				
Time (h)	**OF**					**2'FL**					**OF/2'FL**					**Negative**				
T0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T10	0.28	0.09	0.10	0.10	0.10	0.10	0.05	0.02	0.07	0.10	0.20	0.06	0.08	0.09	0.09	0.00	0.00	0.00	0.00	0.00
T24	0.45	0.43	0.16	0.17	0.33	0.27	0.10	0.08	0.09	0.09	0.35	0.30	0.10	0.15	0.28	0.00	0.00	0.00	0.00	0.00
T48	0.80	0.74	0.53	0.60	0.96	0.39	0.21	0.20	0.11	0.47	0.64	0.57	0.47	0.49	0.83	0.00	0.00	0.00	0.00	0.00

Individual donor organic acid data – succinate concentrations across 0, 10, 24, and 48 h fermentation. Concentration in (mmol l^{-1}). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Total organic acids																				
	OF					**2'FL**					**OF/2'FL**					**Negative**				
T0	3.12	2.87	2.84	2.44	3.44	3.11	2.89	2.84	2.47	3.45	3.13	2.91	2.84	2.46	3.46	3.13	2.90	2.84	2.46	3.46
T10	35.65	28.07	32.41	35.14	57.89	10.92	18.58	28.23	12.09	33.60	23.14	25.28	12.76	30.07	53.85	3.99	2.93	2.93	2.61	3.86
T24	104.37	97.70	84.25	88.13	115.32	51.65	47.94	63.19	35.39	41.23	71.40	91.54	77.91	73.99	89.36	5.31	3.73	3.46	2.91	4.11
T48	158.12	149.82	152.25	135.31	169.31	69.25	65.07	73.31	49.12	64.20	153.94	132.52	137.13	115.31	144.91	5.63	3.61	6.32	3.27	4.44

Individual donor organic acid data – total SCFA concentrations across 0, 10, 24, and 48 h fermentation. Concentration in (mmol l^{-1}). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Appendix 3.5 Mean bacterial populations using pH-controlled *in vitro* batch culture fermentation at 0, 10, 24, and 48 h

Total Bacteria (Eub I-II-III)	Time (h)	T0	T10		T24		T48	
		Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
Substrate	OF	8.24 (0.10)	8.84 (0.11)	**0.003**	8.95 (0.06)	**0.003**	8.50 (0.03)	0.62
	2'FL	8.25 (0.1)	8.67 (0.6)	**0.046**	8.76 (0.04)	**0.034**	8.31 (0.07)	1.00
	OF/2'FL	8.25 (0.1)	8.73 (0.05)	**0.017**	8.80 (0.06)	**0.021**	8.42 (0.10)	1.00
	Negative	8.24 (0.1)	7.97 (0.12)	0.56	7.97 (0.02)	0.67	7.81 (0.13)	0.08
***P* (b)**		1.00	**≤ 0.001**		**≤ 0.001**		**≤ 0.001**	

Bifidobacterium spp. (Bif164)	Time (h)	T0	T10		T24		T48	
		Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
Substrate	OF	7.37 (0.10)	8.67 (0.15)	**≤ 0.001**	8.85 (0.07)	**≤ 0.001**	8.25 (0.07)	**≤ 0.001**
	2'FL	7.37 (0.10)	8.50 (0.10)	**≤ 0.001**	8.60 (0.07)	**≤ 0.001**	8.06 (0.04)	**≤ 0.001**
	OF/2'FL	7.36 (0.10)	8.51 (0.08)	**0.002**	8.63 (0.06)	**0.002**	8.21 (0.08)	**0.002**
	Negative	7.37 (0.10)	7.37 (0.16)	1.00	7.42 (0.15)	1.00	7.33 (0.18)	**1.00b**
***P* (b)**		1.00	**≤ 0.001**		**≤ 0.001**		**≤ 0.001**	

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probe: Total bacteria (Eub I-II-II) and *Bifidobacterium* (Bif164) collected at 0, 10, 24 and 48 h representing the mean (*n* = 5) and standard error (SE) of the data. **(a)** Significant differences compared with respective 0 h sampling are identified with specified *P* values (grey columns). **(b)** Significant differences between substrates at 0, 10. 24 and 48 h are indicated by specified *P* values (orange rows). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Most *Bacteroidaceae* and *Prevotellaceae*, some *Porphyromonadaceae* (Bac303)	Time (h)	T0	T10		T24		T48	
		Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
Substrate	OF	6.19 (0.18)	7.35 (0.15)	**≤ 0.001**	7.83 (0.07)	**≤ 0.001**	7.56 (0.09)	**≤ 0.001a**
	2'FL	6.20 (0.16)	7.05 (0.08)	**0.01**	7.15 (0.15)	**0.01**	6.87 (0.19)	0.09b
	OF/2'FL	6.17 (0.17)	7.38 (0.07)	**≤ 0.001**	7.49 (0.20)	**≤ 0.001**	7.24 (0.13)	**0.003ab**
	Negative	6.18 (0.16)	6.19 (0.18)	1.00	6.12 (0.14)	1.00	5.79 (0.18)	0.81c
***P* (b)**		1.00	**≤ 0.001**		**≤ 0.001**		**≤ 0.001**	

Lactobacillus/Enterococcus (Lab158)	Time (h)	T0	T10		T24		T48	
		Mean (SE)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)	Mean (SE)	*P* (a)
Substrate	OF	5.97 (0.09)	6.78 (0.14)	**≤ 0.001**	6.67 (0.14)	**0.006**	6.25 (0.22)	1.00a
	2'FL	5.96 (0.07)	6.72 (0.18)	**0.002**	6.70 (0.23)	**0.004**	6.34 (0.22)	0.43a
	OF/2'FL	5.91 (0.09)	6.33 (0.21)	**0.013**	6.61 (0.21)	**0.006**	6.39 (0.21)	0.18a
	Negative	5.92 (0.07)	5.96 (0.05)	1.00	5.94 (0.08)	1.00	5.65 (0.15)	1.00a
***P* (b)**		1.00	**0.006**		**0.02**		0.69	

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probe: most *Bacteroidacae* and *Prevotellaceae* (Bac303) and Lactobacillus/*Enterococcus* (Lab158) collected at 0, 10, 24 representing the mean (*n* = 5) and standard error (SE) of the data. **(a)** Significant differences compared with respective 0 h sampling are identified with specified *P* values (grey columns). **(b)** Significant differences between substrates at 0, 10. 24 and 48 h are indicated by specified *P* values (orange column). Significant differences between substrates at 48h are indicated by differing letters (grey column). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Appendix 3.6 Individual bacterial populations using pH-controlled *in vitro* batch culture fermentation at 0, 10, 24, and 48 h

Total Bacteria (Eub338 I-II-III)

Time (h)	OF					2'FL					OF/2'FL					Negative				
T0	8.57	8.34	8.12	8.09	8.06	8.58	8.34	8.16	8.1	8.06	8.59	8.33	8.16	8.09	8.06	8.58	8.33	8.15	8.1	8.06
T10	8.75	8.58	8.72	9.15	9.02	8.68	8.8	8.51	8.57	8.79	8.66	8.6	8.78	8.77	8.86	8.08	8.23	7.68	7.77	8.22
T24	8.82	8.91	8.86	9.16	9.01	8.78	8.67	8.75	8.7	8.92	8.8	8.62	8.84	8.77	8.96	7.93	8.13	7.63	7.51	8.66
T48	8.4	8.45	8.52	8.57	8.57	8.23	8.11	8.43	8.31	8.48	8.19	8.23	8.62	8.4	8.66	7.81	8.11	7.55	7.51	8.08

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probe: total bacteria (Eub338 I-II-III) at 0, 10 , 24 and 48 h. **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

***Bifidobacterium* spp. (Bif164)**

Time (h)	OF					2'FL					OF/2'FL					Neg				
T0	7.45	7.65	7.31	7.04	7.38	7.46	7.65	7.32	7.05	7.38	7.46	7.65	7.29	7.04	7.38	7.45	7.65	7.31	7.05	7.38
T10	8.68	8.90	8.66	9.01	8.12	8.52	8.80	8.46	8.57	8.17	8.52	8.50	8.73	8.56	8.24	7.34	7.85	7.20	6.92	7.54
T24	8.79	8.99	8.86	9.01	8.62	8.62	8.67	8.75	8.60	8.36	8.75	8.59	8.76	8.59	8.45	7.40	7.85	7.46	6.90	7.52
T48	8.21	8.32	8.19	8.47	8.08	8.20	8.03	8.05	7.98	8.02	8.14	8.16	8.52	8.08	8.15	7.32	7.83	7.34	6.73	7.44

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probe: *Bifidobacterium* spp. (Bif164) at 0, 10 , 24 and 48 h. **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Most *Bacteroidaceae* and *Prevotellaceae*, some *Porphyromonadaceae* (Bac303)

Time (h)	OF					2'FL					OF/2'FL					Neg				
T0	6.76	6.44	5.94	5.94	5.87	6.75	6.38	6.06	5.95	5.87	6.74	6.41	5.88	5.95	5.87	6.71	6.36	6.03	5.95	5.87
T10	6.99	7.09	7.35	7.79	7.55	7.01	7	6.81	7.08	7.33	7.13	7.37	7.5	7.47	7.42	6.66	6.29	5.56	6.3	6.12
T24	7.75	7.65	7.92	7.79	8.04	6.99	6.93	7.72	6.99	7.12	7.12	7.04	7.88	7.37	8.05	6.65	6.16	5.91	5.85	6.01
T48	7.65	7.55	7.72	7.2	7.68	6.46	6.44	7.45	6.92	7.1	7.03	7.01	7.7	7.13	7.35	6.09	6.15	5.56	5.18	5.98

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: most *Bacteroidacae* and *Prevotellaceae* (Bac303) at 0, 10 , 24 and 48 h.

Abbreviations: OF = oligofructose; 2'FL = 2'fucosyllactose

***Lactobacillus/Enterococcus* (Lab158)**

Time (h)	**OF**					**2'FL**					**OF/2'FL**					**Neg**				
T0	5.71	6.2	5.81	6.11	6.02	5.7	5.96	5.99	6.11	6.02	5.67	6.04	5.7	6.11	6.02	5.7	5.93	5.86	6.11	6.02
T10	6.31	6.65	6.84	7.17	6.92	6.23	6.39	6.93	6.89	7.17	5.74	6.47	6.16	7	6.27	5.78	6.06	5.98	5.98	6
T24	6.94	6.31	6.66	7.02	6.41	6.39	6.24	6.86	7.5	6.49	6.15	6.26	6.6	7.37	6.65	5.79	5.97	6.2	5.73	6.01
T48	6.22	5.45	6.46	6.82	6.3	6.52	5.88	6.18	7.13	6.01	6.04	6.15	6.2	7.19	6.35	5.25	5.95	5.43	5.61	6.00

Individual bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probe: *Lactobacillus/Enterococcus* spp. (Lab158) at 0, 10 , 24 and 48 h.

Abbreviations: OF = oligofructose; 2'FL = 2'fucosyllactose

Appendix 4.1 Research ethics application

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Professor Bob Rastall
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Dear Bob, Anisha and Peter

I am pleased to inform you that Professor Richard Frazier has given a favourable opinion for conduct for your study, 'Randomised four-arm trial investigating the effects of different food matrices on the prebiotic efficacy of inulin-type fructans from chicory root' via the in-School exceptions route. This email constitutes your permission to proceed with the studies as described in your application. The following study number has been assigned to your study and you should quote this number in any correspondence you undertake about your studies.

STUDY Number – 36/2020

If you feel that you need to make changes to the way your studies are run, please let us know at the earliest opportunity and we can advise you of whether a formal amendment to your proposal is required or not.
I wish you the best of luck with the projects and finish by reminding you of the need for safe custody of project data at all times (a service that Barbara Parr, copied in, can provide if you require it).

Kind regards
Professor Julie Lovegrove
SCFP Research Ethics Chair

University of Reading

Prof. Bob Rastall
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Consent Form for prebiotic, anxiety, and depression study

Please initial boxes

7. I have read and had explained to me by …….. the accompanying Information Sheet relating to the project on: …….. ☐

8. I have had explained to me the purposes of the project and what will be required of me, and any questions I have had have been answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation. ☐

9. I have had explained to me what information will be collected about me, what it will be used for, who it may be shared with, how it will be kept safe, and my rights in relation to my data. ☐

10. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason. ☐

11. (a). I understand that the data collected from me in this study will be preserved and made available in anonymised form, so that they can be consulted and re-used by others. ☐

5. (b). I understand that the data collected from me in this study will be preserved, and subject to safeguards will be made available to other authenticated researchers. ☐

6. I understand that this study has been reviewed by the University of Reading Research Ethics Committee and has been given a favourable ethical opinion for conduct. ☐

7. I have received a copy of this Consent Form and of the accompanying Information Sheet. ☐

Accordingly, I consent as indicated below:

- I consent to my contact details being stored on the Nutrition Unit Volunteer Database. ☐ Yes ☐ No

- I consent to my screening information (including date of birth, height, weight, dietary habits, smoking status, long-term use of medication and pre and probiotics, being stored on the Nutrition Unit Volunteer Database. ☐ Yes ☐ No

- I consent as part of this human intervention trial to providing 2 urine, 2 stool and 10 saliva samples. ☐ Yes ☐ No

- I am happy to be included on a register of research participants for the purposes of being contacted about further studies by…… Please mark with your initials ☐ Yes ☐ No

Name: ……

Date of birth: ……

Signed: ……

Date: ……

Participant details

Name of Participant: _____ Date of Birth: _____

Signature: _____ Date: _____

Address of Participant:

(*Please add if you consent to be part of the Hugh Sinclair Unit of Human Nutrition Volunteer Database*)

_____

_____

Telephone number _____

General Practitioner (GP) details

Name: _____

Address: _____

Telephone: _____

Witnessed by

Name of researcher taking consent: _____

Signature: _____ Date: _____

PARTICIPANT INFORMATION SHEET

Impact of prebiotics on the gut microbiota and anxiety and depression

You are being invited to take part in a research study. Before you decide if you want to take part it is important that you understand what is involved. Please read the following information and discuss with others if you wish. Please ask us if there is anything you do not understand or if you would like any additional information. Take time to decide whether or not you wish to take part.

This human study has been subject to ethical review, according to the procedures specified by the School of Chemistry, Food and Pharmacy Research Ethics Committee and University Research Ethics Committee has been given a favourable opinion.

Aim

The aim of this study is to evaluate how functional foods (inulin-type fructans and 2'fucsoyllactose) alters microbial (gut bacteria) composition and anxiety and depression in a working and university (staff and students) population. 92 volunteers will take part in this study who will be randomised into 4 different groups. One group will consume inulin-type fructans, one group will consume 2'fuscyllactose, one group will consume a combination of both inulin-type fructans and 2'fucsyllactose and one group will consume a placebo in the form of maltodextrin. Neither volunteer nor investigator will know what product is issued.

Before you decide whether to take part in the study, please read the following information carefully. If you want to know anything about the study, which is not written here, please ask the investigator.

What are prebiotics?

- Prebiotics are non-digestible fibre that exert positive bacterial changes in the intestine and bring about health benefits
- They have been found to improve the intestinal bacterial composition of the general population
- They are safe for human consumption

- They have been consumed by humans for hundreds of years

Proposed benefits of prebiotics

- Reduce the number/activities of disease-causing bacteria
- Influence satiety
- Improve immune response
- Improve gut transit time
- Reduce risk of gastrointestinal illness such as travellers' diarrhoea, irritable bowel syndrome, infections
- Inhibit pathogens
- Repress inflammation
- Increase absorption of minerals and vitamin synthesis

Why is this study being carried out?

*A*nxiety and depression are the two most common mental health disorders recorded worldwide costing health services billions of pounds per year. Several reports have suggested that poor mental health amongst working professionals and university students is on the increase, becoming something of a global concern. Factors driving this; including pressure, overdemanding workloads, financial concerns, and peer pressure; can adversely affect both working and academic performance and self-worth. Thus, there is becoming an increasing need to develop new strategies to help tackle these modalities. In recent years there has been increasing interest in the bi-directional relationship that exists between the gut and the brain and is suggested to play a role in influencing mood (anxiety and depression) via the production of chemical messengers. As diet is key manipulator of the gut microbiota one way to influence the composition of the gut is via diet and the use of functional foods including inulin-type fructans, produced from chicory and 2'fucosyllactose a major carbohydrate component of breastmilk which is currently commercially produced as a novel food ingredient.

The idea that functional foods may help to reduce anxiety and depression holds particular appeal due to being relatively free of side effects, readily accessible and have been shown to have additional health benefits including improving bowel transit function, reducing infections and increasing satiety amongst others. Yet, to date, previous research on the potential for prebiotics to reduce anxiety and depression has produced mixed results due to differences in the population tested, dosages and types of prebiotics used, and means of assessing changes in anxiety and depression suggesting further work in this area would be highly beneficial. Therefore, this present study aims to address the question can manipulation of the gut microbiota using inulin-type fructans and 2'fucosyllactose reduce anxiety and depression in a working and university population.

Inclusion criteria/exclusion criteria

Inclusion criteria- If the following applies to you, you will be considered for participation in the trial:

1. Healthy at the time of pre-examination
2. Possess mild/moderately elevated levels of stress and anxiety as measured via PHQ-9 and GAD-7 (PHQ-9 range: 7-14 and GAD-7 range: 8-15). Volunteer must meet both criteria to be considered eligible.
3. Aged ≥ 18 to ≤ 45 years at the time of pre-examination
4. BMI is ≥ 18.5 and ≤ 29.9
5. Has a stool frequency of at least 3 bowel movements per week
6. Able and willing to comply with the study instructions
7. Suitable for participation in the study according to the investigator/study personnel
8. Written informed consent is given by volunteer

Exclusion Criteria

1. No command of any local language
2. Previously or currently diagnosed neurological or psychiatric disorders
3. Previous history of renal, hepatic, cardiovascular disease, or clinically significant diabetes
4. Gastrointestinal disorders including IBS, IBD or other conditions that might affect the gut environment
5. Food allergies or intolerances
6. Using drugs (e.g. antibiotics, aspirin, proton pump inhibitors) influencing gastrointestinal function (8 weeks before intervention)
7. Use of laxatives and labelled pre-and probiotics in the previous 4 weeks before the beginning of intervention
8. Currently involved or will be involved in another clinical or food study
9. History of drug (recreational) or alcohol abuse.
10. Use of medication including anti-depressants i.e. selective serotonin receptor inhibitors or Amitriptyline for 3 months prior to commencing the trial
11. Have received bowel preparation for investigative procedures in the 4 weeks prior to the study
12. If you have undergone surgical resection of any part of the bowel.
13. If participants are pregnant or are lactating

What will I be asked to do?

- All participants will be asked to fill out a health screening questionnaire, anxiety and depression questionnaire and inclusion/exclusion criteria will be reviewed for volunteer eligibility
- Informed consent from yourself will be required
- On giving consent and passing initial screening, participants' height and weight will be measured
- Once the study begins, participants will be randomly allocated into 1 of 4 groups each assigned a different prebiotic or placebo containing 10 g of test compound(s). Volunteers will be asked to consume their assigned product at the same time each day (either with breakfast or within 2 to 3 hours of dissolving in water). The length of the intervention is 5 weeks (1 week run-in and 4-week intervention).

- Participants will provide two stool samples: one on day 0 and on week 4 (Day 28) to identify changes in gut microbiota composition.
- Urine samples (*n* = 2) will also be provided at day 0 and at Week 4 (Day 28) looking for changes in urinary metabolites
- Saliva samples (*n* = 10) will also be collected on Day 0 and Week 4 (Day 28) looking for changes in cortisol levels.
- Volunteers will be given containers and specimen pots to take home for initial and final faecal, urine and saliva collections. No treatment will be issued until initial stool, urine and saliva samples has been provided
- **Maintenance of normal dietary patterns throughout the study is essential** and participants will be required to complete food and drink logs throughout the study via a web-based app (MyFitnessPal).
- Any adverse medical events which occur during the trial (e.g. headache, gut symptoms) should be recorded in a diary along with medication taken.
- Any drastic changes in anxiety and depression levels should be reported immediately to the researcher.
- All incidence of respiratory infections, colds or other illness occurring during participation on the study should also be reported.
- Anxiety and depression questionnaires will be completed on Day 0 and week 4 (Day 28) of the intervention period.
- Daily stool habit should be recorded in a diary throughout the whole 5 weeks of the intervention.
- Daily sleep diary will also be completed.
- Please note that participants will be removed from the study if they develop acute gastrointestinal illness (e.g. food poisoning) or intolerance to the supplement/food product, if the researcher should suspect that drastic increases in anxiety and depression have

occurred and the researcher feels the participant may be at risk or if they do not comply to above stated restrictions.

Are there any risks?

The main risk associated with this study is causing an increase anxiety and depression in the participant as a result of pre-screening. To help avoid this, potential participants will undergo pre-screening to determine their initial levels of anxiety and depression. Only those who meet the strict criteria will be accepted. Any potential participant who is considered at risk of severe levels of anxiety and depression (as defined by PHQ-9 and GAD-7 cut-off criteria) or if the investigator suspects that a potential participant is not to be considered eligible, they will be excluded from participation. Any student who shows severe signs of depression and anxiety and are deemed to be at risk by the investigator will be given access to list of resources including the student welfare team (Appendix D).

Inulin is a fermentable dietary fibre and is used world-wide as a food ingredient and as a supplement. Inulin does not pose any risk to participants and has been safely used in our own research and that of many others for several years. 2'fucosyllactose is a human milk oligosaccharide and is currently produced synthetically and used world-wide as a supplement and novel food ingredient with a maximum daily intake of 3 g/day being deemed the safe daily maximum intake by the European Food Safety Authority (EFSA).

Dietary restrictions during testing

Participants must not consume pre- or probiotic (live culture) supplements or food products containing them during the study. Participants must not consume pro- or prebiotic supplements or food products for a minimum of 4 weeks prior to starting the intervention.

Examples of these food products are:

- Danone Actimel yoghurt drink
- Yakult milk drink
- Danone Activia yoghurt
- Kellogg's Rice Krispies multigrain
- Kellogg's all-bran and all-bran prebiotic oaty clusters
- Goodbelly Oat Flakes with Banana & Blueberry
- Weetabix
- Muller Vitality Yoghurt/Drinks
- Bio&me yoghurt, granola or muesli

If a participant is unsure if a food product should or should not be consumed during this study they may contact the investigator Peter Jackson at any time on either p.p.j.jackson@pgr.reading.ac.uk or by mobile or via Prebiotics on Health and Wellbeing on Teams

Data Protection

The organisation responsible for protection of your personal information is the University of Reading (the Data Controller). Queries regarding data protection and your rights should be directed to the University Data Protection Officer at imps@reading.ac.uk, or in writing to: University of Reading, Information Management & Policy Services, Whiteknights House, Pepper Lane, Whiteknights, Reading , RG6 6UR, UK.

The University of Reading collects, analyses, uses, shares and retains personal data for the purposes of research in the public interest. Under data protection law we are required to inform you that this use of the personal data we may hold about you is on the lawful basis of being a public task in the public interest and where it is necessary for scientific or historical research purposes. If you withdraw from a research study, which processes your personal data, dependant on the stage of withdrawal, we may still rely on this lawful basis to continue using your data if your withdrawal would be of significant detriment to the research study aims. We will always have in place appropriate safeguards to protect your personal data.

If we have included any additional requests for use of your data, for example adding you to a registration list for the purposes of inviting you to take part in future studies, this will be done only with your consent where you have provided it to us and should you wish to be removed from the register at a later date, you should contact Prof Bob Rastall and Peter Jackson at p.p.j.jackson@pgr.reading.ac.uk and/or r.a.rastall@reading.ac.uk

You have certain rights under data protection law which are:

- Withdraw your consent, for example if you opted in to be added to a participant register
- Access your personal data or ask for a copy
- Rectify inaccuracies in personal data that we hold about you
- Be forgotten, that is your details to be removed from systems that we use to process your personal data
- Restrict uses of your data
- Object to uses of your data, for example retention after you have withdrawn from a study

Some restrictions apply to the above rights where data is collected and used for research purposes.
You can find out more about your rights on the website of the Information Commissioners Office (ICO) at https://ico.org.uk

You also have a right to complain the ICO if you are unhappy with how your data has been handled. Please contact the University Data Protection Officer in the first instance.

Confidentiality

Consent will be taken prior to volunteer commencing study using REDCap and private Teams channel. Data will be collected for each volunteer using REDCap. Datasets generated on REDCap will be downloaded (CSV) deidentified and each volunteer will be pseudonymised with each being given a unique pseudo volunteer identifier (i.e IN00_1, 2, 3 etc). Volunteer's names will not be used in any reports or publications. All data generated from the study will be held securely within a password protected file, only the study investigators will have access. A record of the names of the volunteers will not be held on the same file.

Information matching volunteer names with identification codes will be kept in a password protected folder on the Universities storage cloud (OneDrive), investigators will only use identification codes. All pseudonymised datasets will be held separately from volunteer identity data i.e. name and email address. The data will be held in the strictest confidence

The only time data will be matched with volunteer names is for those volunteers that request to have their personal results discussed with them or if they wish to leave the study and want their data to be destroyed. A request for individual results to be discussed will include a review of all sample results for each volunteer. A list of the names and addresses of the subjects in this project will be compiled, this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years following project completion.

All quantitative data will be stored in the simplest format of comma separated values (CSV) on the University cloud (OneDrive). Sharable data will be anonymised data and in a format suitable for preservation. This will involve removing any linked ID codes so that data cannot be relinked by either the investigators or anyone else i.e., REDCap has a built-in function able to hash record IDs when exporting data. At the end of the project records all anonymised datasets will be published in the University of Reading's Research Data Archive and made available in order to support research findings, and so they can be consulted and re-used by others.

#

General Information

- You will receive £75 for completing the trial and a £20 if you are a reserve volunteer who does not receive a treatment. Volunteers that drop out will have their payment pro-rated to cover the part of the study completed.
- Stool, urine and saliva sample pots will be provided and advice on how to take stool, urine and saliva samples at home will be given
- Analysis of faecal samples will occur at the University of Reading
- You will be provided with enough prebiotic/placebo to last the duration of the study.
- If at any time you wish to withdraw from the study, you are completely free to do so without giving a reason.
  The information collected will be used for research purposes only. All information will be confidential, and individuals' names will not be used in any reports resulting from this work.
- Once the study has been completed you can request your results
- All unused samples will be destroyed after the completion of the study and sample analysis.

Contact details for further questions, or in the event of a complaint

The University has appropriate insurance and is well used to carrying out these types of trials.

In the event of a complaint, please contact the Principal Investigator, Professor Bob Rastall: r.a.rastall@reading.ac.uk
If he cannot resolve the issue to your satisfaction, the complaint will be taken up with the Head of Department of Food and Nutritional Sciences, Prof. Colette Fagan, c.c.fagan@reading.ac.uk

The investigators thank you for taking time to read this. If you have any queries, please feel free to contact:

Peter Jackson p.p.j.jackson@pgr.reading.ac.uk

Teams account: Prebiotics on Health and Wellbeing:

Study Timeline

Dates	Stage of Study	Treatment
Day -14 to -7	V0: Pre-screening visit	• Study briefing: consent will be taken • Medical and lifestyle questionnaire will be taken • Inclusion and exclusion criteria are evaluated by examiner by means of questionnaires. • Explaining and dispensing of dietary app information and run-in questionnaires • Dispense sample pots for Day-0 faecal, urine and saliva collection • Daily bowel habit and anxiety and depression questionnaire to be completed
Day 0	V1: Start of study	• Provide baseline stool sample • Provide baseline urine sample • Provide baseline salvia samples • Baseline height and weight taken • Dispense bowel habit diaries and web-app food diary information • Dispense food product/inulin sachets. • Dispense sample pots for 4 week faecal, urine and saliva collection • During the 4 week study each study nutrient sachet will be consumed twice daily. • Baseline anxiety and depression questionnaires to be completed.
Week 4 (Day 28)	V2: End of study	• Final anxiety and depression questionnaires to be submitted. • Bowel habit diary to be submitted • Final body weight taken • Confirm food diaries have been submitted. • Provide final faecal, urine and saliva samples • Return any unused study nutrient sachets

Appendix C

Pre-screening Questionnaire

Public Health Questionnaire 9: PHQ-9

		Not at all	Several Days	More than half the days	Nearly every day
Over the last two weeks, how often have you been bothered by any of the following problems?					
1.	**Little interest or pleasure in doing things**				
2.	**Feeling down, depressed, or hopeless**				
3.	**Trouble falling asleep or staying awake, or sleeping too much**				
4.	**Feeling tired or having little energy**				
5.	**Poor appetite or overeating**				
6.	**Feeling bad about yourself - or that you are a failure or have let yourself or your family down?**				
7.	**Trouble concentrating on things, such as reading the newspaper or watching television?**				
8.	**Moving or speaking so slowly that other people could have noticed? Or the opposite - being so fidgety or restless that you have been moving around a lot more than usual?**				
9.	**Thoughts that you would be better off dead, or of hurting yourself in some way?**				

Pre-screening questionnaire

General anxiety disorder assessment: GAD-7

		Not at all	Several Days	More than half the days	Nearly every day
Over the last 2 weeks, how often have you been bothered by any of the following problems?					
1.	Feeling nervous, anxious or on edge?				
2.	Not being able to stop or control worrying?				
3.	Worrying too much about different things?				
4.	Trouble relaxing?				
5.	Being so restless that it is hard to sit still?				
6.	Becoming easily annoyed or irritable?				
7.	Feeling afraid as if something awful might happen?				

Useful welfare links

Student welfare team:

Email: studentwelfare@reading.ac.uk

Telephone: **0118 378 4777** - Monday – Friday between 10:00 and 16:00

Your Doctor/GP Surgery: - you can request an emergency appointment

NHS non-emergency advice: call 111 (available 24/7)

Emergency Services (Police, Ambulance, Fire Service) - 999 (only call this in an emergency).

University Security - 0118 378 7799 (non-emergency)/ 0118 378 6300 (emergency).

The Samaritans - 116 123 (available 24/7)

Berkshire NHS mental health crisis number - 0300 365 0300 (available 24/7)

Prebiotic, anxiety, and depression study

Medical and Lifestyle Questionnaire

Name:		Title:
Address:		Date of Birth: Age:
Daytime Telephone:	Evening Telephone:	Best time to call:
Weight (kg):	Height (m):	BMI (kg/m^2):
E-mail: Do you use emails on a regular basis? YES/NO		

How did you hear about the study? _____

Please circle as appropriate

<u>Medical questions</u>

16. Have you been diagnosed as having any of the following?

a) High blood cholesterol YES/NO

b) High blood pressure YES/NO

c) Thyroid disorder YES/NO

d) Diabetes or other endocrine disorders YES/NO

e) Heart problems, stroke or any vascular disease in the past 12 months YES/NO

f) Inflammatory diseases (e.g. rheumatoid arthritis) YES/NO

h) Renal, gastrointestinal, respiratory or liver disease? YES/NO

g) Cancer YES/NO

h) Blood disorders YES/NO

i) Haemochromatosis YES/NO

Have you been diagnosed as suffering from any other illness?

YES/NO

If 'YES', please give details

17. Are you currently suffering from or have ever been diagnosed with any neurological or psychiatric disorders?
    YES/NO

    If yes, please give details

18. Within the past 3 months, have you taken any medication (prescription or non-prescription) including anti-depressants?
    YES/NO
    If 'YES', what are they and for what reasons?

19. Have you had any surgery within the past 3 months or do you have surgery planned? YES/NO
    If 'YES', please give details

20. Have you ever suffered from a pulmonary embolism, deep vein thrombosis, blood clots or had a blood transfusion?
    YES/NO
    If 'YES', please give details

21. Do you have a pacemaker?
    YES/NO

22. (Women only) Are you premenopausal, perimenopausal or postmenopausal? *Please circle as appropriate*

If you are premenopausal:

c) Are you using contraception? YES/NO
If 'YES', please give details (including the name of the contraceptive pill or device)

Do you have regular menstrual cycles?
YES/NO

d) Are you pregnant, lactating or planning a pregnancy in the next year?
YES/NO

<u>Lifestyle questions</u>

23. Are you currently taking part in or within the last 3 months been involved in a clinical trial or a research study?
    YES/NO
    If 'YES', please give details:

24. Have you been screened or contacted recently about a study?
    YES/NO
    If 'YES', please give details

25. Do you follow any specific diets (e.g. ketogenic, vegan, vegetarian, carnivore, intermittent fasting)
    YES/NO
    If 'YES', which diet do you follow.

26. Do you have any food allergies (e.g. gluten, dairy, nuts, soya) or intolerances (e.g. lactose)?
    YES/NO
    If 'YES', what are they?

27. Do you use any of the following:
    e) Dietary supplements, e.g. fish oils, evening primrose oil, vitamins or minerals? (such as iron or calcium)
       YES/NO
    f) Probiotics, e.g. Actimel, Yakult, Activia yoghurts or capsules?
       YES/NO
    g) Cholesterol-lowering products, e.g. Flora Pro-Activ or Benecol?
       YES/NO
    h) Prebiotics, e.g. inulin, Bimuno?
       YES/NO

If 'YES' to any, please give details

28. Are you following or planning to start a restricted diet, e.g. to lose weight?
    YES/NO

    If 'YES', would you be willing to postpone this until after your final study visit?
    YES/NO

29. Do you drink alcohol? YES/NO
    If 'YES', approximately how many units do you drink per week? _____*Units*

    One unit of alcohol is half a pint of beer/lager, a single pub measure of spirits e.g. gin/vodka, or a small glass of wine (125 ml).

30. Do you exercise more than three times a week, including walking?
    YES/NO
    If 'YES', please specify the type of exercise, frequency and intensity

31. Do you smoke?
    YES/NO
    If 'YES, please give details

This is the end of the questionnaire - thank you for your time.

All information provided will remain confidential at all time

Volunteer Diary

Volunteer No. _____

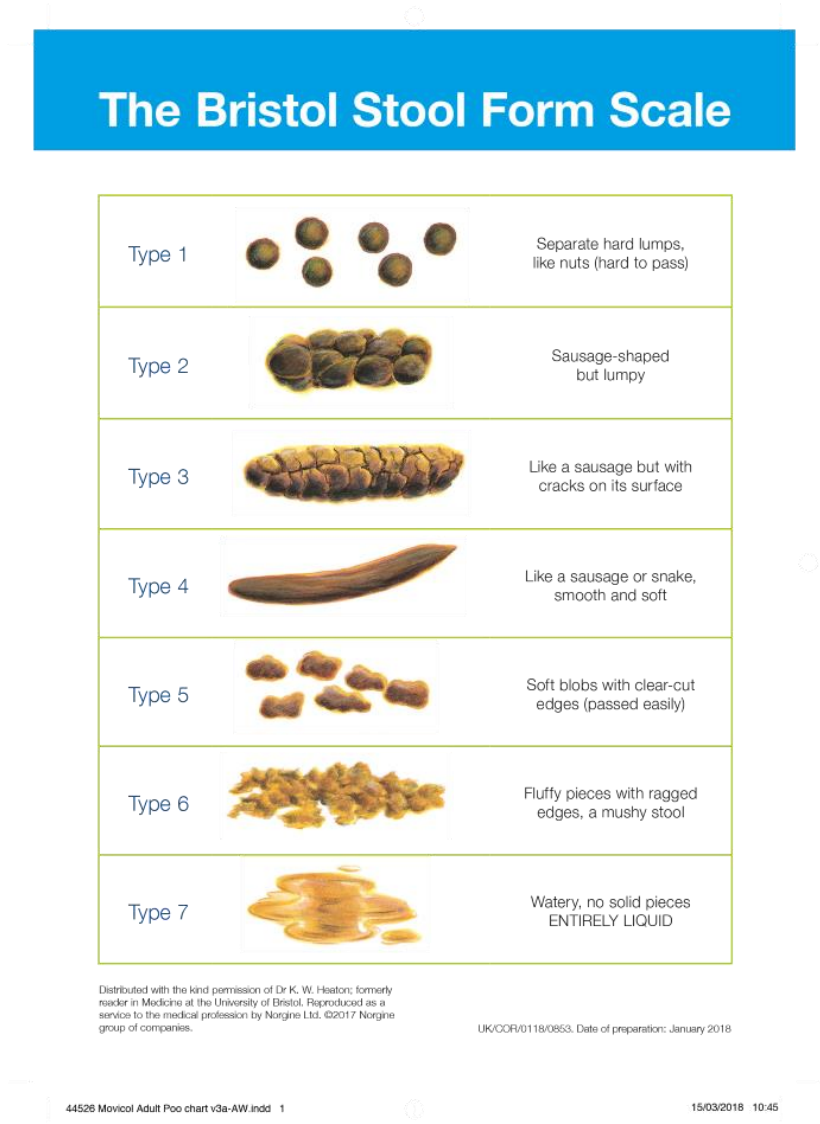
Period No. _____ Day No. to Day No.

Please fill in the diary carefully and completely for each day. If you are unsure how to answer, please give the best information you can. Please return completed diary to Peter Jackson on your next visit.

To be filled in by investigator only!

Date started at:

Next visit at:



The Bristol Stool Form Scale provides a scale relating to stool consistency, please use this chart to rate your stool consistency 1-7 (solid – liquid) in your daily diary.

E.g. a rating of 4 – used in the diary example would relate to “like a sausage or snake, smooth and soft”)

Volunteer number:

Study day	Date d/m	Number of stools (if 0, please include)	Stool consistency as per Bristol chart (page 2)			Study product consumption	Flatulence	Intestinal bloating	Abdominal pressure	Abdominal pain	Feeling of fullness	Comments
e.g	15/01	2	3	4	X	Yes	1	1	1	1	1	
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												

Positive and Negative Affect Schedule (PANAS-SF)

Indicate the extent you have felt this way over the past week (please only tick one box per feeling)		Very Slightly or not at all	A little	Moderately	Quite a bit	Extremely
1	Interested					
2	Distressed					
3	Excited					
4	Upset					
5	Strong					
6	Guilty					
7	Scared					
8	Hostile					
9	Enthusiastic					
10	Proud					
11	Irritable					
12	Alert					
13	Ashamed					
14	Inspired					
15	Nervous					
16	Determined					
17	Attentive					
18	Jittery					
19	Active					
20	Afraid					

Form for Adverse Events

Study name and REC ref number	
Volunteer number	
Principal Investigator	
Study Researcher	

Description of AE	**Category of AE ***	**Date of start**	**Date of end**	**Grading ****	**Date/ time reported**	**Measures taken including medical/ nurse advice/study withdrawal**
				Intensity: Frequency: Relation to study product:		

NB: This form must be completed on the day of the adverse event and sent to all research nurses and unit managers at time of event. This will enable logging of the adverse event and follow up with the volunteer by a nurse.

Form sent to unit managers and nurses: YES / NO completed by: _____ Date: _____
To be completed by a nurse Followed up by (name): _____ Date: _____ **Outcome:**

Category of AE*: **1**.Cannula related AE (pain, erythema or swelling)

2a. Upper respiratory **2b**. Lower respiratory

3. Allergy- skin reactions **4**. Gastro – intestinal reactions **5.** Other

Grading**: **Intensity:** light=1; moderate=2; severe=3

Frequency: rare=1; frequent=2; often=3; non applicable=4

Relation to study product: unrelated=1; unlikely=2; probable=3, definitely related = 4

How to take urine sample protocol

Note: Urine samples must be collected as the urination after waking

Procedure

1. You will be provided with a sterile, screw top container.
2. Before commencing urine collection, it is essential to ensure that you thoroughly wash your hands with soap and hot water.
3. Start to pee and collect urine sample at mid-stream into the sterile, screw top container.
4. Place urine sample into cool bag.
5. Thoroughly wash your hands
6. Deliver urine sample to the Harry Nursten building at the University (if you cannot hand your urine sample in within 1 hour of voiding please store at 4 °C for as short as time as possible.

Note: What does mid-stream urine mean?

A mid-stream urine sample means you don't collect the first or last of your urine samples which reduces the risk of the sample being contaminated with bacteria from your hands or the skin around the urethra.

Date and time of collection: Please fill out once completed

Date	**Time**

How to collect stool sample protocol

Note: Stool samples should ideally be collected as the first voiding after waking but this is not essential.

1. You will be provided with an anaerobic chamber pot housing an anaerobic sachet and a sterile pot. Along if needed a wooden stick for collection and stool catcher
2. Thoroughly wash your hands with hot soap and water.
3. At time of voiding either void directly into the provided plastic pot or onto the stool catcher
4. If voiding onto the stool catcher use the wooden stick to transfer the stool sample into the provided plastic container
5. Seal the plastic container with the lid.
6. Place plastic tub containing stool sample in the anaerobic chamber and open the anaerobic sachet and place next to the plastic pot in the anaerobic chamber.
7. Place lid on anaerobic chamber and seal.
8. If you use the stool catcher then place in a plastic bag, tie up and discard in the bin.
9. Deliver sample to the university

Date and time of collection: Please fill out once completed

Date	**Time**

How to collect saliva samples

Note: Saliva samples must be collected every 15 min (0, 15, 30, 45 and 60 min) for the first hour upon waking in separately labelled sterile tubes. (**it is important that you don't spit but drool**)

Procedure

1. You will be provided with 5 labelled sterile, screw top containers to collect your salvia samples.
2. Saliva samples will need to be collected every 15 min (i.e. 0 min, 15 min, 30 min, 45 min and 60min) for 1 hour immediately upon waking.
3. Before commencing saliva samples, it is essential to ensure that you thoroughly wash your hands with soap and hot water.
4. To collect the saliva samples, take one of the labelled sterile test tubes (corresponding to respective time point., 0 min, 15 min etc) and unscrew the lid. Start to drool into the sterile tube for around 1 min. Immediately screw top tube (**it is important that you don't spit but drool**)
5. Place saliva samples into cool bag.
6. Thoroughly wash your hands.
7. Deliver saliva samples to the Harry Nursten building at the University (if you cannot delivery your saliva samples within 1 hour of collection, please store at 4 °C for as short as time as possible.

Date and time of collection: Please fill out once completed

Date	**Time**	
	0 min	
	15 min	
	30 min	
	45 min	
	60 min	

Recruitment Poster

University of Reading

HUGH SINCLAIR Unit of Human Nutrition

Volunteers Required

Help us investigate the effects of dietary carbohydrates on microbial composition, anxiety, and depression

We are looking for healthy non-smoking adults:

- Aged 18-45 students (BSc, MSc, and PhD) with elevated levels of anxiety and depression (to be determined via completion of pre-screening questionnaires) who are currently not taking antidepressants.
- Willing to study nutrient sachets for 4 weeks
- Willing to provide 2 stool 2 urine and 10 salvia samples.

You will be reimbursed for reasonable expenses

If you are interested in taking part or wish to find out more information about this study, please contact Peter Jackson at p.p.j.jackson@pgr.reading.ac.uk (Scan QR code) or via mobile or Teams at Prebiotics on health and wellbeing:

Note: Due to the nature of ongoing Covid-19 requirements collection/delivery of study products and samples will be conducted at your either place of residence or on campus (to be discussed at pre-screening).

This study has been approved by University Research Ethics Committee (UREC): Study Number – UREC 21/43

Social Media Advert

Social media ad

Would you like to help us investigate how dietary carbohydrates impact on gut microbiota composition and anxiety and depression

We are currently recruiting healthy non-smoking adults aged 18-45 with mild/moderate levels of anxiety and depression who are willing to study nutrient sachets for 4 weeks, along with providing 2 urine, stool and 10 samples.

You will be reimbursed for your time.

If this sounds interesting please email us on p.p.j.jackson@pgr.reading.ac.uk or via teams

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Teams account

Prebiotics on Health and Wellbeing :

Appendix 4.2 Mean bacterial populations measured using FISH-FLOW at D0 and D28 of the intervention phase

	Intervention												
	OF (*n* = 23)			Maltodextrin (*n* = 23)			OF/2'FL (*n* = 23)			2'FL (*n* = 23)			*P* (b)
Probe	**D0**	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	
Eub I-II-III	10.09 (0.07)	10.35 (0.07)	**≤ 0.001**	9.96 (0.06)	9.97 (0.07)	0.97	10.03 (0.06)	10.25 (0.06)	**≤ 0.001**	10.01 (0.06)	10.28 (0.06)	**≤ 0.001**	**0.048**
Bif164	8.57 (0.13)	9.52 (0.12)	**≤ 0.001**	8.55 (0.13)	8.37 (0.15)	0.14	8.69 (0.10)	9.41 (0.13)	**≤ 0.001**	8.63 (0.14)	8.93 (0.12)	**0.01**	**≤ 0.001**

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: total bacteria (Eub338 I-II-III) and Bifidobacterium (Bif164) collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between interventions at Day 28 are indicted by specified *P* values (orange column) and are the result of employing a general linear model (GLM). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Appendix 4.3 Quantitative Microbiome Profiling (QMP) 16S rRNA data measured at D0 and D28 of the intervention phase

Phylum	Intervention											
	OF (*n* = 23)			Maltodextrin (*n* = 23)			OF/2'FL (*n* = 23)			2'FL (*n* = 23)			*P* (b)
	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	
Actinomycetota (Actinobacteria)	1.43×10^9 (4.06×10^8)	4.50×10^9 (9.82×10^8)	**≤ 0.001**	9.18×10^9 (1.65×10^8)	9.45×10^9 (2.73×10^8)	0.94	9.57×10^8 (1.76×10^8)	4.42×10^9 (1.24×10^9)	**≤ 0.001**	1.25×10^8 (2.56×10^8)	2.10×10^9 (5.50×10^8)	0.31	**0.003**
Bacteroidota (Bacteroidetes)	2.32×10^9 (4.93×10^8)	5.49×10^9 (1.18×10^9)	**≤ 0.001**	2.32×10^9 (5.75×10^8)	1.88×10^9 (3.85×10^8)	0.55	2.88×10^9 (6.36×10^8)	4.22×10^9 (7.93×10^8)	0.10	2.35×10^9 (4.7×10^8)	3.30×10^9 (7.40×10^8)	0.25	**0.016**
Pseudomonadota (Proteobacteria)	8.49×10^7 (3.56×10^7)	1.80×10^8 (6.65×10^7)	0.13	9.86×10^7 (3.12×10^7)	1.64×10^8 (6.59×10^7)	0.30	1.87×10^8 (8.73×10^7)	1.59×10^8 (6.14×10^7)	0.65	2.96×10^7 (6.09×10^6)	1.00×10^8 (2.92×10^7	0.26	0.767
Verrucomicrobiota (Verrucomicrobia)	1.06×10^8 (3.11×10^7)	3.61×10^8 (1.72×10^8)	0.19	1.82×10^8 (7.34×10^7)	1.20×10^8 (4.81×10^7)	0.23	8.35×10^7 (3.09×10^7)	1.72×10^8 (9.93×10^7)	0.27	1.30×10^8 (4.66×10^7)	1.94×10^8 (6.38×10^7)	0.94	0.933
Euryarchaeota	9.79×10^7 (5.66×10^7)	1.65×10^8 (6.36×10^7)	0.09	8.23×10^7 (3.13×10^7)	5.99×10^7 (2.56×10^7)	0.57	4.12×10^7 (1.85×10^7)	5.21×10^7 (2.98×10^7)	0.78	8.51×10^7 (6.51×10^7)	1.90×10^7 (1.04×10^7)	0.10	**0.045**
Mycoplasmatota (Tenericutes)	5.24×10^8 (1.71×10^8)	5.60×10^8 (1.25 x 108)	0.73	6.49×10^8 (2.41×10^8)	6.46×10^8 (2.81×10^8)	0.98	4.57×10^8 (1.44×10^8)	4.22×10^8 (1.50×10^8)	0.75	3.24×10^8 (7.36×10^7)	3.81×10^8 (9.93×10^7)	0.60	0.701

Quantitative Microbiome Profiling 16S rRNA sequencing data (phylum level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between interventions at Day 28 are indicted by specified *P* values (orange column) and are the result of employing a general linear model (GLM). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Phylum	Intervention												
	OF (*n* = 23)			Maltodextrin (*n* = 23)			OF/2'FL (*n* = 23)			2'FL (*n* = 23)			*P* (b)
	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	
Fusobacteria	9.12×10^5 (9×10^5)	0	**≤ 0.001**	0	0	1.00	4.03×10^5 (4.03×10^5)	2.59×10^7 (2.59×10^7)	0.05	4.03×10^5 (4.03×10^5)	2.09×10^5 (2.09×10^5)	0.99	0.399
Bacillota (firmicutes)	8.48×10^9 (1.36×10^9)	1.61×10^{10} (2.11×10^9)	**≤ 0.001**	6.84×10^9 (1.07×10^9)	8.35×10^9 (1.23×10^9)	0.39	8.47×10^9 (1.21×10^9)	1.23×10^{10} (1.91×10^9)	**0.01**	8.36×10^9 (9.86×10^8)	1.25×10^{10} (1.55×10^9)	0.004	**0.027**

Quantitative Microbiome Profiling 16S rRNA sequencing data (phylum level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between interventions at Day 28 are indicted by specified *P* values (orange column) and are the result of employing a general linear model (GLM). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Genus	Intervention											P (b)
	OF (*n* = 23)			Maltodextrin (*n* = 23)			OF/2'FL (*n* = 23)			2'FL (*n* = 23)		
	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	
Bifidobacterium	1.15×10^9 (3.75×10^8)	4.28×10^9 (9.03×10^8)	**≤ 0.001**	6.96×10^8 (1.67×10^8)	7.69×10^8 (2.55×10^8)	0.90	8.18×10^8 (1.62×10^8)	4.05×10^9 (1.23×10^9)	**≤ 0.001**	9.41×10^8 (2.19×10^8)	1.72×10^9 (5.14×10^8)	0.34	**0.003**
Bacteroides	1.68×10^9 (3.98×10^8)	4.21×10^9 (9.41×10^8)	**≤ 0.001**	1.53×10^9 (4.55×10^8)	1.32×10^9 (3.08×10^8)	0.71	2.22×10^9 (5.54×10^8)	3.18×10^9 (7.12×10^8)	0.15	1.78×10^9 (4.11×10^8)	2.23×10^9 (5.75×10^8)	0.50	**0.021**
Prevotella	1.12×10^8 (5.71×10^7)	5.55×10^8 (2.01×10^8)	**0.001**	4.24×10^8 (3.43×10^8)	3.31×10^8 (2.48×10^8)	0.67	1.12×10^8 (6.79×10^7)	4.78×10^8 3.37×10^8)	**0.04**	7.38×10^7 (3.00×10^7)	2.65×10^8 (1.00×10^8)	0.28	0.829
Alistipes	3.49×10^8 (7.61×10^7)	6.99×10^8 (2.09×10^8)	**0.004**	2.42×10^8 (6.50×10^7)	1.46×10^8 (2.99×10^7)	0.38	3.20×10^8 (7.08×10^7)	3.31×10^8 (8.17×10^7)	0.93	3.05×10^8 (7.03×10^7)	3.60×10^8 (9.21×10^7)	0.65	**0.017**
Roseburia	9.37×10^8 (2.42×10^8)	1.74×10^9 (4.01×10^8)	**0.01**	8.22×10^8 (2.52×10^8)	1.10×10^9 (2.61×10^8)	0.33	8.55×10^8 (1.67×10^8)	1.84×10^9 (4.11×10^8)	**0.001**	6.23×10^8 (1.24×10^8)	1.31×10^8 (2.92×10^8)	0.03	0.412
Faecalibacterium prausnitzii	5.82×10^8 (9.94×10^7)	1.93×10^9 (4.16×10^8)	**≤ 0.001**	7.65×10^8 (2.75×10^8)	9.85×10^8 (2.15×10^8)	0.44	1.12×10^9 (3.30×10^8)	1.80×10^9 (4.48×10^8)	**0.02**	6.20×10^8 (1.43×10^8)	1.20×10^9 (1.76×10^8)	0.047	0.142
Ruminococcus	7.73×10^8 (1.52×10^8)	2.24×10^9 (5.48×10^8)	**≤ 0.001**	4.76×10^8 (7.72×10^7)	6.34×10^8 (1.28×10^8)	0.60	8.10×10^8 (1.89×10^8)	1.21×10^9 (4.14×10^8)	0.24	7.06×10^8 (1.51×10^8)	1.20×10^9 (3.73×10^8)	0.14	0.05

Quantitative Microbiome Profiling 16S rRNA sequencing data (genus level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between interventions at Day 28 are indicted by specified *P* values (orange column) and are the result of employing a general linear model (GLM). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Genus (continued)	OF (*n* = 23)			Maltodextrin (*n* = 23)			OF/2'FL (*n* = 23)			2'FL (*n* = 23)			*P* (b)
	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	
Ruminococcus2	2.94×10^8 (6.11×10^7)	4.36×10^8 (1.30×10^8)	0.16	3.11×10^8 (6.50×10^7)	3.11×10^8 (9.87×10^7)	0.43	3.09×10^8 (7.08×10^7)	3.90×10^8 (9.92×10^7)	0.42	4.39×10^8 (1.24×10^8)	6.44×10^8 (1.41×10^8)	0.004	0.387
Clostridium Cluster 14A&B	2.27×10^8 (5.40×10^7)	3.06×10^8 (4.12×10^7)	0.16	1.71×10^8 (3.42×10^7)	2.48×10^8 (5.23×10^7)	0.42	3.35×10^8 (9.03×10^7)	2.30×10^8 (4.55×10^7)	0.07	2.65×10^8 (6.55×10^7)	3.36×10^8 (5.82×10^7)	0.08	0.12
Eubacterium	1.17×10^8 (2.20×10^7)	2.33×10^8 (6.57×10^7)	**0.003**	8.70×10^7 (1.62×10^7)	9.75×10^7 (2.01×10^7)	0.70	9.94×10^7 (2.13×10^7)	1.13×10^8 (2.51×10^7)	0.71	9.73×10^7 (1.87×10^7)	1.90×10^8 (4.19×10^7)	0.02	0.098
Coprococcus	7.01×10^8 (2.75×10^8)	9.12×10^8 (1.59×10^8)	0.16	3.56×10^8 (5.18×10^7)	4.64×10^8 (1.16×10^8)	0.40	4.63×10^8 (8.23×10^7)	4.89×10^8 (9.99×10^7)	0.86	7.09×10^8 (1.47×10^8)	8.27×10^8 (1.34×10^8)	0.42	**0.037**
Lactobacillus/Enterococcus	9.99×10^7 (6.30×10^7)	3.80×10^8 (1.80×10^8)	**0.01**	7.78×10^6 (6.92×10^6)	3.19×10^6 (2.62×10^6)	0.97	9.61×10^7 (6.99×10^7)	1.43×10^8 (1.20×10^8)	0.64	2.91×10^7 (1.54×10^7)	5.25×10^7 (3.19×10^7)	0.81	0.085
Lactococcus	6.93×10^6 (4.60×10^6)	1.13×10^6 (6.73×10^5)	0.10	1.48×10^6 (1.23×10^6)	1.77×10^6 (8.34×10^5)	0.93	2.36×10^6 (1.17×10^6)	8.72×10^5 (3.48×10^5)	0.67	6.26×10^6 (4.67×10^6)	3.09×10^6 (1.29×10^6)	0.34	0.268
Anaerostipes	5.50×10^8 (2.86×10^8)	6.19×10^8 (1.60×10^8)	0.66	1.99×10^8 (3.91×10^7)	3.42×10^8 (9.34×10^7)	0.33	2.37×10^8 (4.62×10^7)	2.78×10^8 (6.70×10^7)	0.79	4.34×10^8 (1.26×10^8)	5.17×10^8 (1.11×10^7)	0.60	0.146
Akkermansia	1.06×10^8 (3.11×10^7)	3.60×10^8 (1.71×10^8)	**0.02**	1.91×10^8 (7.63×10^7)	1.20×10^8 (4.81×10^7)	0.54	8.35×10^7 (3.09×10^7)	1.84×10^8 (9.91×10^7)	0.33	1.31×10^8 (4.66×10^7)	1.91×10^8 (6.41×10^7)	0.56	0.462

Quantitative Microbiome Profiling 16S rRNA sequencing data (genus level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between interventions at Day 28 are indicted by specified *P* values (orange column) and are the result of employing a general linear model (GLM). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Genus (continued)	OF (*n* = 23)			Maltodextrin (*n* = 23)			OF/2'FL (*n* = 23)			2'FL (*n* = 23)			*P* (b)
	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	
Blautia	1.59×10^9 (3.57×10^8)	1.83×10^9 (3.25×10^8)	0.47	1.54×10^9 (3.70×10^8)	1.39×10^9 (4.10×10^8)	0.69	1.31×10^9 (2.41×10^8)	1.62×10^9 (3.53×10^8)	0.37	1.78×10^9 (2.87×10^8)	2.42×10^9 (4.72×10^8)	0.06	0.309
Desulfovibrio	1.31×10^7 (7.38×10^7)	4.57×10^7 (1.66×10^7)	**0.02**	2.02×10^7 (9.94×10^6)	3.25×10^7 (1.61×10^7)	0.34	3.64×10^7 (3.32×10^7)	7.02×10^7 (5.46×10^7)	**0.02**	7.69×10^6 (3.86×10^6)	1.66×10^7 (9.17×106)	0.52	0.646
Lachnospiraceae incertae sedis	3.41×10^8 (7.36×10^7)	6.23×10^8 (1.14×10^8)	**0.004**	2.81×10^8 (4.47×10^7)	5.40×10^8 (1.34×10^8)	0.07	4.47×10^8 (9.36×10^7)	5.45×10^8 (1.03×10^8)	0.34	4.90×10^8 (9.48×10^7)	6.10×10^8 (1.06×10^8)	0.24	0.728
Dorea	2.97×10^8 (5.54×10^7)	4.67×10^8 (9.64×10^7)	0.09	3.01×10^8 (6.37×10^7)	2.88×10^8 (7.38×10^7)	0.99	3.44×10^8 (5.87×10^7)	4.30×10^8 (9.78×10^7)	0.38	5.54×10^8 (1.09×10^8)	4.75×10^8 (9.67×10^7)	0.44	0.516
Collinsella	1.52×10^8 (4.39×10^7)	2.89×10^8 (6.29×10^7)	**0.01**	7.69×10^7 (1.87×10^7)	9.43×10^7 (2.90×10^7)	0.67	7.33×10^7 (1.91×10^7)	2.14×10^8 (4.68×10^7)	0.01	1.83×10^8 (5.09×10^7)	2.19×10^8 (5.83×10^7)	0.47	0.08
Flavonifractor	2.75×10^7 (7.82×10^6)	7.82×10^7 (1.58×10^7)	**0.01**	4.77×10^7 (1.32×10^7)	5.32×10^7 (1.76×10^7)	0.66	7.09×10^7 (2.00×10^7)	8.22×10^7 (2.15×10^7)	0.56	5.62×10^7 (1.10×10^7)	7.52×10^7 ($1. 68x 10^7$)	0.34	0.234
Gemmiger	4.14×10^8 (1.09×10^8)	5.21×10^8 (8.79×10^7)	0.13	2.81×10^8 (7.30×10^7)	2.38×10^8 (6.44×10^7)	0.66	2.81×10^8 (5.97×10^7)	4.31×10^8 (1.05×10^8)	**0.03**	2.81×10^8 (5.69×10^7)	2.83×10^8 (4.45×10^7)	0.98	0.061

Quantitative Microbiome Profiling 16S rRNA sequencing data (genus level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between interventions at Day 28 are indicted by specified *P* values (orange column) and are the result of employing a general linear model (GLM). **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Appendix 4.4 Mean bowel habit diary data: Stool consistency, Frequency, Flatulence, Intestinal bloating, Abdominal Pressure, Abdominal Pain and Feeling of Fullness measured at run-in week and last of the intervention phase

Gastrointestinal sensation and bowel habit	Intervention												*P* (b)
	OF (*n* = 23)			Maltodextrin (*n* = 23)			OF/2'FL (*n* = 23)			2'FL (*n* = 23)			
	Run-in	D22-D28	*P* (a)	Run-in	D22-D28	*P* (a)	Run-in	D22-D28	*P* (a)	Run-in	D22-D28	*P* (a)	
Stool Frequency	1.44 (0.12)	1.45 (0.13)	0.90	1.21 (0.08)	1.21 (0.08)	0.95	1.45 (0.14)	1.82 (0.17)	**≤ 0.001**	1.28 (0.10)	1.26 (0.09)	0.81	**0.03**
Stool Consistency	3.88 (0.15)	4.36 (0.15)	**0.01**	3.83 (0.17)	4.02 (0.19)	0.29	3.89 (0.19)	4.35 (0.17)	**0.01**	3.73 (0.21)	3.46 (0.17)	0.17	**0.001**
Flatulence	0.42 (0.08)	0.73 (0.10)	**0.001**	0.63 (0.10)	0.72 (0.10)	0.36	0.68 (0.09)	0.78 (0.09)	0.33	0.52 (0.08)	0.55 (0.10)	0.80	0.404
Intestinal Bloating	0.32 (0.08)	0.58 (0.11)	**0.01**	0.55 (0.12)	0.57 (0.12)	0.82	0.47 (0.12)	0.44 (0.12)	0.74	0.34 (0.11)	0.29 (0.08)	0.60	0.182
Abdominal Pain	0.137 (0.04)	0.21 (0.07)	0.29	0.22 (0.08)	0.21 (0.05)	0.77	0.20 (0.06)	0.21 (0.06)	0.92	0.23 (0.06)	0.14 (0.05)	0.15	0.779
Abdominal Pressure	0.21 (0.07)	0.35 (0.09)	0.10	0.42 (0.10)	0.40 (0.10)	0.83	0.40 (0.10)	0.35 (0.011)	0.58	0.29 (0.09)	0.24 (0.08)	0.68	0.654
Feeling of Fullness	0.44 (0.12)	0.45 (0.12)	0.92	0.63 (0.12)	0.65 (0.12)	0.87	0.53 (0.13)	0.53 (0.14)	1.00	0.33 (0.10)	0.36 (0.10)	0.77	0.38

Gastrointestinal sensation and bowel habit scores at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences between respective run-in phase and last week of the intervention phase (Day 22-28) interventions are identified with specified *P* values (grey columns). **(b)** Significant differences between substrates at Day 28 are indicted by specified *P* values (orange column) and are the result of employing a linear marginal model (LLM) integrating run-in phase data as a baseline covariate. **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Appendix 4.5 Mean mood state scores across all four interventions at D0 and D28 of the intervention phase

Mood state	Intervention												*P* (b)
	OF (*n* = 23)			Maltodextrin (*n* = 23)			OF/2'FL (*n* = 23)			2'FL (*n* = 23)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	
Becks Depression Inventory	18.96 (0.96)	8.52 (0.92)	≤ **0.001**	18.04 (0.89)	16.04 (1.14)	0.07	19.09 (0.83)	9.52 (0.92)	≤ **0.001**	17.83 (0.75)	13.13 (1.41)	≤ **0.001**	≤ **0.001**
State Trait Anxiety Inventory Y1	54.91 (1.11)	41.61 (1.62)	≤ **0.001**	55.91 (1.73)	52.30 (1.28)	0.06	55.35 (1.10)	42.04 (1.82)	≤ **0.001**	52.91 (1.19)	46.43 (1.64)	≤ **0.001**	≤ **0.001**
State Trait Anxiety Inventory Y2	55.70 (1.05)	42.35 (1.85)	**0.002**	55.17 (1.42)	52.65 (1.68)	0.06	56.00 (1.145)	45.26 (1.80)	≤ **0.001**	53.52 (0.71)	49.00 (1.52)	≤ **0.001**	≤ **0.001**
Positive Affect	26.30 (1.14)	34.17 (1.29)	≤ **0.001**	24.91 (1.22)	26.78 (1.06)	0.18	24.61 (0.93)	29.39 (1.20)	≤ **0.001**	24.48 (1.26)	31.91 (1.25)	≤ **0.001**	≤ **0.001**
Negative Affect	32.09 (1.21)	23.48 (0.99)	≤ **0.001**	28.91 (0.98)	28.74 (1.24)	0.88	28.65 (1.15)	23.78 (0.76)	≤ **0.001**	29.96 (0.95)	25.00 (1.02)	≤ **0.001**	≤ **0.001**
Pittsburgh Sleep Quality Index	6.61 (0.46)	5.26 (0.45)	≤ **0.001**	6.91 (0.45)	5.83 (0.50)	0.004	6.61 (0.48)	5.48 (0.47)	0.003	6.00 (0.32)	4.65 (0.38)	≤ **0.001**	0.293
Cortisol Awakening Response	22.31 (0.68)	20.32 (0.76)	≤ **0.001**	21.89 (0.60)	22.26 (0.69)	0.30	22.82 (0.92)	20.89 (0.95)	≤ **0.001**	22.13 (0.61)	21.17 (0.70)	**0.008**	≤ **0.001**

Mood state scores and cortisol awakening response values at Day 0 and Day 28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences within respective D0 and Day 28 scores/values are identified with specified P values (grey columns). (**b)** Significant differences between substrates at D28 are indicted by specified *P* values (orange column) and are the result of employing a linear marginal model (LMM) integrating run-in phase data as a baseline covariate. **Abbreviations:** OF = oligofructose; 2'FL = 2'fucosyllactose

Appendix 4.6 Bacterial taxa -taxa and taxa– mood state correlation matrix

Bacterial Taxa – mood state correlation matrix

	BDI	STAI Y1	STAI Y2	PANAS PA	PANAS NA	PSQI	CAR
BDI	1.00	0.72	0.66	-0.36	0.336	0.203	0.283
	1.00	$6.44x10^{-16}$	$9.42x10^{-13}$	$4.61x10^{-04}$	0.001	0.052	0.006
STAI Y1	0.72	1.00	0.86	-0.40	0.440	0.290	0.282
	$6.44x10^{-16}$	1.00	$1.14x10^{-28}$	$7.73x10^{-05}$	$1.12x10^{-05}$	0.005	0.006
STAI Y2	0.66	0.86	1.00	-0.36	0.514	0.187	0.243
	$9.42x10^{-13}$	$1.14x10^{-28}$	1.00	$3.57x10^{-04}$	$1.64x10^{-07}$	0.074	0.019
PANAS PA	-0.36	-0.40	-0.36	1.00	-0.205	-0.273	-0.208
	$4.61x10^{-04}$	$7.73x10^{-05}$	$3.57x10^{-04}$	1.00	0.050	0.008	0.046
PANAS NA	0.34	0.440	0.514	-0.21	1.00	0.18	0.16
	0.00	$1.12x10^{-05}$	$1.64x10^{-07}$	0.05	1.00	0.09	0.12
PSQI	0.20	0.29	0.19	-0.273	0.18	1.00	0.41
	0.05	0.005	0.074	0.008	0.09	1.00	$5.0x10^{-05}$
Car	0.28	0.28	0.24	-0.21	0.16	0.41	1.00
	0.006	0.006	0.019	0.05	0.12	$5.0x10^{-05}$	1.00
Bifidobacterium	-0.37	-0.33	-0.42	0.17	-0.32	-0.01	-0.22
	$2.91x10^{-04}$	0.001	$3.1x10^{-05}$	0.11	0.03	0.94	0.04
Bacteroides	-0.15	-0.18	-0.20	0.05	-0.073	-0.121	-0.201
	0.15	0.08	0.06	0.61	0.486	0.252	0.055
Prevotella	0.24	0.08	0.03	-0.06	-0.04	0.08	0.21
	0.02	0.46	0.79	0.54	0.68	0.44	0.05
Alistipes	-0.17	-0.18	-0.17	0.15	-0.06	-0.04	-0.07
	0.10	0.09	0.10	0.15	0.58	0.68	0.52
Roseburia	-0.09	-0.14	-0.13	0.21	-0.02	-0.15	-0.26
	0.42	0.17	0.20	0.05	0.84	0.14	0.01
Faecalibacterium prausnitzii	-0.25	-0.20	-0.18	0.17	0.06	-0.22	-0.20
	0.02	0.05	0.08	0.11	0.57	0.04	0.06
Ruminococcus	-0.15	-0.18	-0.14	0.20	-0.21	-0.10	-0.08
	0.16	0.08	0.19	0.06	0.04	0.36	0.45
Ruminococcus2	-0.07	-0.22	-0.22	-0.01	0.03	0.12	0.06
	0.52	0.04	0.03	0.89	0.79	0.24	0.58
***Clostridium* cluster XIV A&B**	-0.15	-0.03	-0.01	-0.01	0.07	-0.05	0.02
	0.16	0.77	0.92	0.94	0.50	0.64	0.86

	BDI	STAI Y1	STAI Y2	PANAS PA	PANAS NA	PSQI	CAR
Eubacterium	-0.21	-0.25	-0.24	0.09	-0.04	0.04	-0.06
	0.04	**0.01**	**0.02**	0.39	0.68	0.70	0.58
Coprococcus	-0.20	-0.23	-0.21	0.12	-0.01	-0.01	-0.08
	0.06	**0.03**	**0.04**	0.26	0.95	0.96	0.42
Lactobacillus/Enterococcus	-0.18	-0.24	-0.22	-0.02	-0.14	-0.03	-0.13
	0.09	**0.02**	**0.03**	0.84	0.19	0.81	0.21
Lactococcus	-0.17	-0.23	-0.19	0.17	-0.23	-0.05	-0.02
	0.10	**0.03**	**0.07**	0.11	**0.03**	0.64	0.86
Anaerostipes	-0.22	-0.24	-0.19	0.00	-0.05	-0.01	-0.01
	0.04	**0.02**	**0.06**	0.99	0.61	0.95	0.95
Akkermansia	-0.06	-0.21	-0.23	0.00	-0.18	-0.04	-0.11
	0.54	**0.05**	**0.03**	0.98	**0.08**	0.69	0.30
Blautia	-0.22	-0.28	-0.27	0.14	-0.09	0.04	-0.02
	0.03	**0.01**	**0.01**	0.18	0.37	0.69	0.82
Desulfovibrio	-0.03	-0.04	-0.13	0.22	-0.19	-0.09	-0.06
	0.80	0.67	0.22	**0.04**	**0.07**	0.37	0.55
Lachnospiraceae incertae sedis	-0.22	-0.09	-0.11	0.03	-0.14	-0.14	-0.20
	0.04	0.38	0.31	0.77	0.19	0.17	**0.05**
Dorea	-0.22	-0.26	-0.25	0.15	-0.08	-0.03	-0.09
	0.04	**0.01**	**0.02**	0.14	0.46	0.76	0.41
Collinsella	-0.15	-0.09	-0.14	-0.07	-0.11	0.06	-0.13
	0.15	0.37	0.19	0.49	0.29	0.58	0.21
Flavonifractor	-0.21	-0.17	-0.14	0.12	0.03	-0.10	-0.12
	0.04	0.11	0.19	0.27	0.77	0.34	0.27
Gemmiger	-0.18	-0.16	-0.16	0.14	-0.07	-0.02	-0.21
	0.09	0.14	0.13	0.18	0.51	0.85	**0.05**

Bacterial taxon-mood state interactions from the entire cohort. Pairwise correlations between bacterial taxon and mood states fold change data were calculated using a Spearman's rank correlation (two sided adjusted for using FDR). Taxa- mood state correlations ranged from -1 to 1 (negative to positive). Red text indicates adjusted *P* (*Q*) values ≤ 0.05; Blue text indicates adjusted *P* (*Q*) values ≥ 0.05 - ≤ 0.07; Green text indicates adjusted *P* (*Q*) values ≥ 0.07 - ≤ 0.09 (grey rows). **Abbreviations:** BDI = Beck's Depression Inventory; STAI Y1 and Y2 = State Trait Anxiety Inventory; PANAS = Positive and Negative Affect Scores – PA = Positive Affect; NA = Negative Affect; CAR = Cortisol Awakening Response; PSQI = Pittsburgh Sleep Quality Index

Bacterial taxa-taxa correlation matrix

	Bifidobacterium	*Bacteroides*	*Prevotella*	*Alistipes*	*Roseburia*	*Faecalibacterium prausnitzii*	*Ruminococcus*	*Ruminococcus2*	*Clostridium* Cluster XIV A&B	*Eubacterium*	*Coprococcus*	*Lactobacillus/Enterococcus*	*Lactococcus*	*Anaerostipes*	*Akkermansia*	*Blautia*	*Desulfovibrio*	*Lachnospiraceae incertae sedis*	*Dorea*	*Collinsella*	*Flavonifractor*	*Gemmiger*
Bifidobacterium	1.00	-0.10	-0.16	0.05	0.18	0.19	0.14	0.27	0.10	0.57	0.55	0.20	0.12	0.49	0.15	0.48	0.07	0.26	0.54	0.53	-0.04	0.53
	1.00	0.35	0.14	0.66	0.09	0.07	0.18	0.01	0.33	4.03×10^{-09}	1.76×10^{-08}	0.05	0.26	9.43×10^{-07}	0.16	1.59×10^{-06}	0.51	0.01	2.34×10^{-08}	4.70×10^{-08}	0.72	6.00×10^{-08}
Bacteroides	-0.098	1.00	-0.04	0.746	0.324	0.376	0.29	-0.03	0.507	-0.11	-0.14	0.13	0.34	-0.14	0.09	-0.17	0.08	0.24	0.00	-0.03	0.71	0.16
	0.351	1.00	0.74	1.14×10^{-14}	0.002	2.12×10^{-04}	0.005	0.79	2.51×10^{-07}	0.29	0.18	0.20	0.001	0.18	0.39	0.11	0.44	0.02	0.99	0.78	3.28×10^{-15}	0.13
Prevotella	-0.16	-0.04	1.00	-0.10	-0.12	-0.210	-0.176	0.079	-0.096	-0.143	-0.103	0.045	-0.154	-0.131	0.110	-0.104	-0.093	-0.120	-0.094	-0.122	-0.053	-0.236
	0.14	0.74	1.00	0.33	0.24	0.045	0.094	0.454	0.365	0.175	0.331	0.673	0.142	0.215	0.298	0.324	0.378	0.253	0.374	0.248	0.615	0.024
Alistipes	0.05	0.75	-0.10	1.00	0.26	0.302	0.428	0.034	0.461	0.079	0.057	0.016	0.482	0.080	0.204	0.022	0.270	0.247	0.066	0.005	0.649	0.307
	0.66	1.14×10^{-14}	0.33	1.00	0.01	0.003	2.08×10^{-05}	0.75	3.82×10^{-06}	0.46	0.59	0.88	1.15×10^{-06}	0.45	0.05	0.83	0.01	0.02	0.53	0.97	2.76×10^{-12}	0.003

	Bifidobacterium	*Bacteroides*	*Prevotella*	*Alistipes*	*Roseburia*	*Faecalibacterium prausnitzii*	*Ruminococcus*	*Ruminococcus2*	*Clostridium* Cluster XIV A&B	*Eubacterium*	*Coprococcus*	*Lactobacillus/Enterococcus*	*Lactococcus*	*Anaerostipes*	*Akkermansia*	*Blautia*	*Desulfovibrio*	*Lachnospiraceae incertae sedis*	*Dorea*	*Collinsella*	*Flavonifractor*	*Gemmiger*
Roseburia	0.18	0.32	-0.12	0.26	1.00	0.42	0.47	0.17	0.40	0.23	0.18	-0.08	0.39	0.12	0.04	0.28	0.16	0.26	0.30	0.01	0.14	0.46
	0.09	0.002	0.24	0.01	1.00	2.42×10^{-05}	2.65×10^{-05}	0.11	7.24×10^{-05}	0.03	0.09	0.42	0.0001	0.24	0.68	0.01	0.14	0.01	0.004	0.89	0.19	4.76×10^{-06}
Faecalibacterium prausnitzii	0.19	0.38	-0.21	0.30	0.42	1.00	0.38	0.18	0.48	0.18	0.14	0.00	0.32	0.09	-0.12	0.22	0.00	0.34	0.15	-0.06	0.28	0.38
	0.07	2.12×10^{-04}	0.04	0.00	2.42×10^{-05}	1.00	0.0002	0.09	1.10×10^{-06}	0.09	0.18	0.97	0.002	0.39	0.24	0.04	0.96	0.0001	0.15	0.59	0.01	0.0002
Ruminococcus	0.14	0.29	-0.18	0.43	0.47	0.38	1.00	0.18	0.34	0.23	0.19	-0.06	0.91	0.04	0.03	0.36	0.23	0.38	0.23	-0.03	0.25	0.44
	0.18	0.01	0.09	2.08×10^{-05}	2.65×10^{-05}	0.0002	1.00	0.09	0.00	0.03	0.07	0.54	1.14×10^{-35}	0.72	0.77	0.0004	0.03	0.0000	0.03	0.81	0.02	1.31×10^{-05}
Ruminococcus2	0.27	-0.03	0.08	0.03	0.17	0.177	0.18	1.0	0.176	0.61	0.59	0.01	0.23	0.38	0.19	0.68	0.15	0.20	0.49	0.28	0.11	0.39
	0.01	0.79	0.45	0.75	0.11	0.092	0.09	1.0	0.09	1.38×10^{-10}	6.15×10^{-10}	0.93	0.03	0.00002	0.08	8.33×10^{-14}	0.16	0.05	6.98×10^{-07}	0.01	0.30	0.00

	Bifidobacterium	*Bacteroides*	*Prevotella*	*Alistipes*	*Roseburia*	*Faecalibacterium prausnitzii*	*Ruminococcus*	*Ruminococcus2*	*Clostridium* Cluster XIV A&B	*Eubacterium*	*Coprococcus*	*Lactobacillus/Enterococcus*	*Lactococcus*	*Anaerostipes*	*Akkermansia*	*Blautia*	*Desulfovibrio*	*Lachnospiraceae incertae sedis*	*Dorea*	*Collinsella*	*Flavonifractor*	*Gemmiger*
***Clostridium* cluster XIV A&B**	0.10	0.51	-0.10	0.46	0.40	0.483	0.34	0.2	1.00	0.13	0.12	0.02	0.29	0.06	-0.05	0.15	0.12	0.45	0.22	0.11	0.40	0.27
	0.33	**2.51×10^{-07}**	0.36	**3.82×10^{-06}**	**7.24×10^{-05}**	**1.10×10^{-06}**	**0.001**	0.1	1.00	0.22	0.26	0.85	**0.00**	0.60	0.63	0.15	0.24	**7.64×10^{-06}**	**0.03**	0.31	**8.55×10^{-05}**	**0.01**
Eubacterium	0.57	-0.11	-0.14	0.08	0.23	0.18	0.23	0.6	0.13	1.00	0.98	0.09	0.22	0.66	0.21	0.77	0.16	0.25	0.60	0.42	0.04	0.61
	4.03×10^{-09}	0.29	0.17	0.46	**0.03**	0.09	**0.03**	**1.38×10^{-10}**	0.22	1.00	**1.30×10^{-64}**	0.37	**0.03**	**6.19×10^{-13}**	**0.05**	**1.54×10^{-19}**	0.12	**0.02**	**2.03×10^{-10}**	**3.18×10^{-05}**	0.68	**1.29×10^{-10}**
Coprococcus	0.55	-0.14	-0.10	0.06	0.18	0.14	0.19	0.59	0.12	0.98	1.00	0.10	0.18	0.67	0.18	0.75	0.19	0.23	0.60	0.40	0.04	0.55
	1.76×10^{-08}	0.18	0.33	0.59	0.09	0.18	**0.07**	**6.15×10^{-10}**	0.26	**1.30×10^{-64}**	1.00	0.35	**0.08**	**2.42×10^{-13}**	**0.08**	**7.96×10^{-18}**	**0.08**	**0.03**	**2.85×10^{-10}**	**7.68×10^{-05}**	0.70	**1.85×10^{-08}**
Lactobacillus/Enterococcus	0.20	0.13	0.04	0.02	-0.08	0.00	-0.06	0.01	0.02	0.09	0.10	1.00	-0.01	0.09	0.03	0.07	-0.12	-0.03	0.14	0.07	0.10	0.06
	0.05	0.20	0.67	0.88	0.42	0.97	0.54	0.93	0.85	0.37	0.35	1.00	0.93	0.37	0.80	0.52	0.25	0.74	0.17	0.52	0.34	0.56

	Bifidobacterium	*Bacteroides*	*Prevotella*	*Alistipes*	*Roseburia*	*Faecalibacterium prausnitzii*	*Ruminococcus*	*Ruminococcus2*	*Clostridium* Cluster XIV A&B	*Eubacterium*	*Coprococcus*	*Lactobacillus/Enterococcus*	*Lactococcus*	*Anaerostipes*	*Akkermansia*	*Blautia*	*Desulfovibrio*	*Lachnospiraceae incertae sedis*	*Dorea*	*Collinsella*	*Flavonifractor*	*Gemmiger*
Lactococcus	0.12	0.34	-0.15	0.48	0.39	0.32	0.91	0.23	0.29	0.22	0.18	-0.01	1.00	0.06	0.04	0.32	0.23	0.36	0.24	-0.06	0.30	0.43
	0.26	**0.001**	0.14	**1.15×10^{-06}**	**0.0001**	**0.002**	**1.14×10^{-35}**	**0.03**	**0.004**	**0.03**	**0.08**	0.93	1.00	0.56	0.71	**0.00**	**0.03**	**0.00**	**0.02**	0.54	**0.004**	**2.35×10^{-05}**
Anaerostipes	0.49	-0.14	-0.13	0.08	0.12	0.09	0.04	0.38	0.06	0.66	0.67	0.09	0.06	1.00	0.15	0.52	0.07	0.16	0.40	0.27	-0.01	0.36
	9.43×10^{-07}	0.18	0.21	0.45	0.24	0.39	0.72	**0.0002**	0.60	**6.19×10^{-13}**	**2.42×10^{-13}**	0.37	0.56	1.00	0.14	**1.45×10^{-07}**	0.48	0.13	**6.67×10^{-05}**	**0.01**	0.89	0.54
Akkermansia	0.15	0.09	0.11	0.20	0.04	-0.12	0.03	0.19	-0.05	0.21	0.18	0.03	0.04	0.15	1.00	0.14	0.23	0.05	0.05	0.12	0.14	0.06
	0.16	0.39	0.30	**0.05**	0.68	0.24	0.77	**0.08**	0.63	**0.05**	0.08	0.80	0.71	0.14	1.00	0.19	**0.02**	0.61	0.61	0.25	0.18	0.54
Blautia	0.48	-0.17	-0.10	0.02	0.28	0.22	0.36	0.68	0.15	0.77	0.75	0.07	0.32	0.52	0.14	1.00	0.24	0.19	0.70	0.40	0.02	0.57
	1.59×10^{-06}	0.11	0.32	0.83	**0.01**	**0.04**	**0.0004**	**8.33×10^{-14}**	0.15	**1.54×10^{-19}**	**7.96×10^{-18}**	0.52	**0.002**	**1.45×10^{-07}**	0.19	1.00	**0.02**	**0.07**	**9.82×10^{-15}**	**7.32×10^{-05}**	0.88	**2.39×10^{-09}**

	Bifidobacterium	*Bacteroides*	*Prevotella*	*Alistipes*	*Roseburia*	*Faecalibacterium prausnitzii*	*Ruminococcus*	*Ruminococcus2*	*Clostridium* Cluster XIV A&B	*Eubacterium*	*Coprococcus*	*Lactobacillus/Enterococcus*	*Lactococcus*	*Anaerostipes*	*Akkermansia*	*Blautia*	*Desulfovibrio*	*Lachnospiraceae incertae sedis*	*Dorea*	*Collinsella*	*Flavonifractor*	*Gemmiger*
Desulfovibrio	0.07	0.08	-0.09	0.27	0.16	0.00	0.23	0.15	0.12	0.16	0.19	-0.12	0.23	0.07	0.23	0.24	1.00	0.12	0.09	0.01	0.08	0.19
	0.51	0.44	0.38	**0.01**	0.14	0.96	**0.03**	0.16	0.24	0.12	**0.08**	0.25	**0.03**	0.48	**0.02**	**0.02**	1.00	0.26	0.39	0.95	0.48	**0.07**
Lachnospiraceae incertae sedis	0.26	0.24	-0.12	0.25	0.26	0.34	0.38	0.20	0.45	0.25	0.23	-0.03	0.36	0.16	0.05	0.19	0.12	1.00	0.22	0.10	0.16	0.24
	0.01	**0.02**	0.25	**0.02**	**0.01**	**0.00**	**0.00002**	**0.05**	**7.64x 10^{-06}**	**0.02**	**0.03**	0.74	**0.00004**	0.13	0.61	**0.07**	0.26	1.00	**0.04**	0.36	0.14	**0.02**
Dorea	0.54	0.00	-0.09	0.07	0.30	0.15	0.23	0.49	0.22	0.60	0.60	0.14	**0.24**	0.40	0.05	0.70	0.09	0.22	1.00	0.45	0.10	0.56
	2.34x 10^{-08}	0.99	0.37	0.53	**0.004**	0.15	**0.03**	**6.98x 10^{-07}**	**0.03**	**2.03x 10^{-10}**	**2.85x 10^{-10}**	0.17	**0.02**	**6.67x 10^{-05}**	0.61	**9.82x 10^{-15}**	0.39	**0.04**	1.00	**6.49x 10^{-06}**	0.34	**6.26x 10^{-09}**
Collinsella	0.53	-0.03	-0.12	0.00	0.01	-0.06	-0.03	0.28	0.11	0.42	0.40	0.07	-0.06	0.27	0.12	0.40	0.01	0.10	0.45	1.00	0.09	0.33
	4.70x 10^{-08}	0.78	0.25	0.97	0.89	0.59	0.81	**0.01**	0.31	**3.18x 10^{-05}**	**7.68x 10^{-05}**	0.52	0.54	**0.01**	0.25	**7.32x 10^{-05}**	0.95	0.36	**6.49x 10^{-06}**	1.00	0.38	**0.001**

	Bifidobacterium	*Bacteroides*	*Prevotella*	*Alistipes*	*Roseburia*	*Faecalibacterium prausnitzii*	*Ruminococcus*	*Ruminococcus2*	*Clostridium* Cluster XIV A&B	*Eubacterium*	*Coprococcus*	*Lactobacillus/Enterococcus*	*Lactococcus*	*Anaerostipes*	*Akkermansia*	*Blautia*	*Desulfovibrio*	*Lachnospiraceae incertae sedis*	*Dorea*	*Collinsella*	*Flavonifractor*	*Gemmiger*
Flavonifractor	-0.04	0.71	-0.05	0.65	0.14	0.28	0.25	0.11	0.40	0.04	0.04	0.10	0.30	-0.01	0.14	0.02	0.08	0.16	0.10	0.09	1.00	0.19
	0.72	**3.28x10^{-15}**	0.62	**2.76x10^{-12}**	0.19	**0.01**	**0.02**	0.30	**8.55x10^{-05}**	0.68	0.70	0.34	**0.004**	0.89	0.18	0.88	0.48	0.14	0.34	0.38	1.00	**0.07**
Gemmiger	0.53	0.16	-0.24	0.31	0.46	0.38	0.44	0.39	0.27	0.61	0.55	0.06	0.43	0.36	0.06	0.57	0.19	0.24	0.56	0.33	0.19	1.00
	6.00x10^{-08}	0.13	**0.02**	**0.003**	**4.76x10^{-06}**	**0.00002**	**1.31x10^{-05}**	**0.00**	**0.01**	**1.29x10^{-10}**	**1.85x10^{-08}**	0.56	**2.35x10^{-05}**	**0.00**	0.54	**2.39x10^{-09}**	0.07	**0.02**	**6.26x10^{-09}**	**0.001**	**0.07**	1.00

Bacterial taxa-taxa interactions from the entire cohort. Pairwise correlations between bacterial taxon using fold change data were calculated using a Spearman's rank correlation (two sided adjusted for using FDR). Taxa- mood state correlations ranged from -1 to 1 (negative to positive). Red text indicates adjusted *P* (*Q*) values ≤ 0.05; Blue text indicates adjusted *P* (*Q*) values ≥ 0.05 - ≤ 0.07; Green text indicates adjusted *P* (*Q*) values ≥ 0.07 - ≤ 0.09 (grey rows).

Appendix 5.1 Mean bacterial populations via FLOW-FISH across the entire 2'FL cohort and segregated by RES/NR status at D0 and D28 of the intervention phase

	Responder Status									
	Overall (*n* = 23)			NR (*n* = 11)			RES (*n* = 11)			
Probe	**D0**	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	***P* NR vs RES (b)**
Eub I-II-III	10.01 (0.06)	10.28 (0.06)	**0.001**	10.05 (0.09)	10.08 (0.06)	0.601	9.97 (0.08)	10.30 (0.08)	**≤ 0.001**	**0.004**
Bif164	8.64 (0.14)	8.94 (0.12)	**0.01**	8.93 (0.16)	8.76 (0.14)	0.064	8.45 (0.19)	9.19 (0.17)	**≤ 0.001**	0.067

Bacterial groups measured by FISH-FLOW (Log10 cells/mL) using probes: total bacteria (Eub338 I-II-III) and Bifidobacterium (Bif164) collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column) and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responders; RES = responders

Appendix 5.2 Mean quantitative microbiome profiling genus level 16S rRNA data across the entire 2'FL cohort and segregated by RES/NR status at D0 and D28 of the intervention phase

	Responder Status									
	Overall (*n* = 23)			NR (*n* = 11)			RES (*n* = 11)			
Genus	**D0**	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	***P* NR vs RES (b)**
Bifidobacterium	9.41×10^8 (2.19×10^8)	1.72×10^9 (5.14×10^8)	**≤ 0.001**	1.20×10^9 (2.53×10^8)	7.65×10^8 (3.72×10^8)	0.33	7.03×10^8 (3.45×10^8)	2.63×10^9 (9.15×10^8)	**≤ 0.001**	**0.008**
Bacteroides	1.78×10^9 (4.11×10^8)	2.23×10^9 (5.75×10^8)	**0.034**	1.72×10^9 (6.10×10^8)	2.05×10^9 (6.92×10^8)	0.74	1.84×10^9 (5.79×10^8)	2.40×10^9 (9.30×10^8)	0.54	0.663
Prevotella	7.38×10^7 (3.00×10^7)	2.65×10^8 (1.00×10^8)	0.36	2.31×10^8 (1.17×10^8)	3.62×10^8 (1.13×10^8)	0.43	7.37×10^7 (4.36×10^7)	2.96×10^8 (1.75×10^8)	0.75	0.563
Alistipes	3.05×10^8 (7.03×10^7)	3.60×10^8 (9.21×10^7)	0.80	3.62×10^8 (1.13×10^8)	3.96×10^8 (1.37×10^8)	0.77	2.53×10^8 (8.78×10^7)	3.26×10^8 (1.29×10^8)	0.50	0.832
Roseburia	6.23×10^8 (1.24×10^8)	1.31×10^8 (2.92×10^8)	**0.001**	8.08×10^8 (2.13×10^8)	7.32×10^8 (1.52×10^8)	0.43	4.54×10^8 (1.27×10^8)	1.82×10^9 (5.06×10^8)	**≤ 0.001**	**0.03**
Faecalibacterium prausnitzii	6.20×10^8 (1.43×10^8)	1.20×10^9 (1.76×10^8)	0.79	5.78×10^8 (1.85×10^8)	9.82×10^8 (1.68×10^8)	0.15	2.53×10^8 (8.78×10^7)	3.26×10^8 (1.29×10^8)	**0.02**	0.308
Ruminococcus	7.06×10^8 (1.51×10^8)	1.20×10^9 (3.73×10^8)	**0.02**	7.26×10^8 (1.57×10^8)	6.79×10^8 (2.18×10^8)	0.90	6.88×10^8 (2.59×10^8)	1.68×10^9 (6.71×10^8)	**0.02**	0.298

Quantitative Microbiome Profiling 16S rRNA sequencing data (genus level) from samples collected at D0 and D28 of the intervention phase expressed as cells/g wet faeces. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column) and are the result of employing a general linear model (GLM).

Abbreviations: NR = non-responder; RES = responder

Genus (continued)	Overall (*n* = 23)			Responder Status						
				NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Ruminococcus2	4.39×10^8 (1.24×10^8)	6.44×10^8 (1.41×10^8)	0.33	3.43×10^8 (5.02×10^7)	4.60×10^8 (1.01×10^8)	0.29	5.27×10^8 (2.36×10^8)	8.13×10^8 (2.50×10^8)	**0.05**	0.327
Clostridium Cluster 14A&B	2.65×10^8 (6.55×10^7)	3.36×10^8 (5.82×10^7)	0.74	1.83×10^8 (6.04×10^7)	1.83×10^8 (4.52×10^7)	0.88	3.32×10^8 (1.12×10^8)	4.76×10^8 (8.68×10^7)	0.28	**0.01**
Eubacterium	9.73×10^7 (1.87×10^7)	1.90×10^8 (4.19×10^7)	0.39	7.96×10^8 (2.08×10^8)	6.69×10^8 (1.72×10^8)	0.42	8.15×10^8 (2.62×10^8)	1.28×10^9 (2.32×10^8)	**0.01**	0.05
Coprococcus	7.09×10^8 (1.47×10^8)	8.27×10^8 (1.34×10^8)	0.57	7.06×10^8 (1.82×10^8)	5.77×10^8 (1.44×10^8)	0.39	7.11×10^8 (2.36×10^8)	1.06×10^9 (2.04×10^8)	**0.03**	0.05
Lactobacillus/Enterococcus	2.91×10^7 (1.54×10^7)	5.25×10^7 (3.19×10^7)	0.91	4.11×10^7 (2.84×10^7)	1.66×10^7 (1.38×10^7)	0.61	1.80×10^7 (1.45×10^7)	8.53×10^7 (5.95×10^7)	0.13	0.261
Lactococcus	6.26×10^6 (4.67×10^6)	3.09×10^6 (1.29×10^6)	0.99	5.75×10^5 (2.50×10^5)	2.61×10^6 (1.85×10^6)	0.77	1.14×10^7 (9.96×10^6)	3.53×10^6 (1.86×10^6)	0.27	0.681
Anaerostipes	4.34×10^8 (1.26×10^8)	5.17×10^8 (1.11×10^7)	0.69	3.40×10^8 (1.13×10^8)	3.03×10^8 (7.27×10^7)	0.71	5.19×10^8 (2.21×10^8)	7.13×10^8 (1.88×10^8)	0.05	**0.03**
Akkermansia	1.31×10^8 (4.66×10^7)	1.91×10^8 (6.41×10^7)	0.77	1.83×10^8 (8.22×10^7)	1.47×10^8 (8.85×10^7)	0.71	8.24×10^7 (4.76×10^7)	2.31×10^8 (9.34×10^7)	0.11	0.461

Quantitative Microbiome Profiling 16S rRNA sequencing data (genus level) from samples collected at D0 and D28 of the intervention phase expressed as cells/g wet faeces. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column) and are the result of employing a general linear model (GLM).
Abbreviations: NR = non-responder; RES = responder

Genus (continued)	Responder Status									
	Overall (*n* = 23)			NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	D28	*P* (b)
Blautia	1.78×10^9 (2.87×10^8)	2.42×10^9 (4.72×10^8)	**0.002**	1.95×10^9 (2.90×10^8)	1.97×10^9 (4.85×10^8)	0.55	1.61×10^9 (4.91×10^8)	2.93×10^9 (7.81×10^8)	**≤ 0.001**	**0.02**
Desulfovibrio	7.69×10^6 (3.86×10^6)	1.66×10^7 (9.17×106)	0.97	6.32×10^6 (5.99×10^6)	7.15×10^7 (6.80×10^7)	0.93	8.95×10^6 (5.17×10^6)	2.53×10^7 (1.64×10^7)	0.06	0.299
Lachnospiraceae incertae sedis	4.90×10^8 (9.48×10^7)	6.10×10^8 (1.06×10^8)	0.57	5.93×10^8 (1.55×10^8)	5.25×10^8 (1.34×10^8)	0.64	3.96×10^8 (1.13×10^8)	6.88×10^8 (1.65×10^8)	0.08	0.05
Dorea	5.54×10^8 (1.09×10^8)	4.75×10^8 (9.67×10^7)	0.71	7.19×10^8 (1.88×10^8)	4.32×10^8 (1.20×10^8)	0.61	4.02×10^8 (1.07×10^8)	5.15×10^8 (1.51×10^8)	0.15	0.697
Collinsella	1.83×10^8 (5.09×10^7)	2.19×10^8 (5.83×10^7)	0.86	2.44×10^8 (8.35×10^7)	1.42×10^8 (6.35×10^7)	0.39	1.27×10^8 (5.94×10^7)	2.88×10^8 (9.33×10^7)	**0.03**	0.05
Flavonifractor	5.62×10^7 (1.10×10^7)	7.52×10^7 (1.68×10^7)	0.93	5.06×10^7 (1.47×10^7)	8.22×10^7 (3.03×10^7)	0.26	6.14×10^7 (1.66×10^7)	6.14×10^7 (1.35×10^7)	0.90	0.697
Gemmiger	2.81×10^8 (5.69×10^7)	2.83×10^8 (4.45×10^7)	0.99	3.13×10^8 (8.50×10^7)	1.73×10^8 (2.03×10^7)	0.06	2.52×10^8 (7.86×10^7)	3.84×10^8 (7.29×10^7)	0.11	**0.02**

Quantitative Microbiome Profiling 16S rRNA sequencing data (genus level) from samples collected at D0 and D28 of the intervention phase expressed as cells/g wet faeces. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column) and are the result of employing a general linear model (GLM).
Abbreviations: NR = non-responder; RES = responder

Appendix 5.3 Mean quantitative microbiome profiling species level 16S rRNA data segregated by RES/NR status D0 and D28 of the intervention phase

Bifidobacterium	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* NR vs RES (b)
Bifidobacterium longum* subsp. *infantis	2.60×10^8 (6.86×10^7)	1.73×10^8 (4.00×10^7)	0.730	2.36×10^8 (1.01×10^8)	9.84×10^8 (4.07×10^8)	**0.001**	**0.03**
Bifidobacterium pseudocatenulatum	3.15×10^8 (1.02×10^8)	2.89×10^8 (1.12×10^8)	0.94	9.82×10^7 (5.74×10^7)	5.00×10^8 (2.65×10^8)	**0.04**	0.70
Bifidobacterium animalis* subsp. *lactis	0	2.33×10^8 (8.28×10^7)	**≤ 0.001**	3.33×10^5 (1.88×10^5)	7.64×10^6 (7.49×10^6)	0.90	**0.009**
Bifidobacterium saeculare	7.55×10^6 (7.55×10^6)	9.37×10^6 (9.37×10^6)	0.162	0	0	1.00	0.31
Bifidobacterium bifidum	5.25×10^6 (5.25×10^6)	1.11×10^8 (6.95×10^7)	0.10	1.01×10^8 (6.41×10^7)	5.72×10^8 (2.35×10^7)	0.169	0.55
***Bifidobacterium* (other)**	5.73×10^8 (2.35×10^8)	1.73×10^8 (4.81×10^7)	**0.057**	7.07×10^8 (5.79×10^8)	1.09×10^9 (5.77×10^8)	**0.030**	0.13

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Bacteroides	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Bacteroides fragilis	3.34×10^8 (2.79×10^8)	2.31×10^8 (1.41×10^8)	0.76	1.55×10^8 (7.32×10^7)	5.99×10^8 (3.82×10^8)	0.13	0.32
Bacteroides *plebeius*	2.37×10^8 (1.39×10^8)	4.44×10^8 (2.43×10^8)	**0.01**	1.43×10^7 (1.39×10^7)	1.45×10^7 (1.25×10^7)	1.00	0.08
Bacteroides clarus	9.20×10^6 (8.90×10^6)	9.14×10^6 (6.25×10^6)	0.99	8.13×10^6 (5.61×10^6)	1.47×10^6 (1.18×10^6)	0.18	0.22
Bacteroides dorei	8.84×10^7 (4.70×10^7)	1.17×10^8 (7.49×10^7)	0.83	4.24×10^8 (2.13×10^7)	2.98×10^8 (1.64×10^8)	0.34	0.34
Bacteroides ovatus	6.62×10^7 (2.15×10^7)	8.54×10^7 (2.88×10^7)	0.76	1.70×10^8 (8.66×10^7)	1.39×10^8 (4.46×10^7)	0.61	0.33
Bacteroides uniformis	2.97×10^8 (1.29×10^8)	2.30×10^8 (5.83×10^7)	0.75	4.68×10^8 (2.19×10^8)	4.72×10^8 (1.67×10^8)	0.84	0.20
Bacteroides stercoris	2.37×10^8 (1.37×10^8)	2.44×10^8 (1.21×10^8)	0.80	2.51×10^7 (1.59×10^7)	3.68×10^7 (2.85×10^7)	0.67	0.95
Bacteroides eggerthii	3.50×10^6 (3.38×10^6)	2.26×10^6 (2.10×10^6)	0.18	0 (0)	0 (0)	1.00	0.27
Bacteroides intestinalis	1.56×10^7 (1.43×10^7)	7.67×10^6 (7.30×10^6)	0.85	5.32×10^7 (3.38×10^7)	1.02×10^8 (7.24×10^7)	0.24	0.23

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
***Bacteroides* (continued)**	**D0**	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	***P* (b)**
Bacteroides cellulosilyticus	3.80×10^7 (3.56×10^7)	1.96×10^7 (1.79×10^7)	0.60	7.40×10^7 (3.77×10^7)	9.26×10^7 (5.79×10^7)	0.58	0.26
Bacteroides massiliensis	1.67×10^8 (1.45×10^8)	3.29×10^8 (2.43×10^8)	0.05	1.01×10^8 (4.63×10^7)	7.21×10^7 (3.95×10^7)	0.70	0.29
Bacteroides coprohilus	0 (0)	0 (0)	1.00	2.88×10^6 (2.88×10^6)	3.53×10^7 (2.44×10^7)	0.05	0.18
Bacteroides coprocola	9.45×10^7 (9.35×10^7)	2.53×10^8 (2.17×10^8)	0.09	5.92×10^7 (3.03×10^7)	5.48×10^7 (3.83×10^7)	0.96	0.36
Bacteroides xylanisolvens	4.48×10^7 (1.33×10^7)	3.82×10^8 (2.53×10^8)	0.07	1.12×10^8 (4.37×10^7)	1.13×10^8 (6.28×10^7)	0.99	0.30
Bacteroides finegoldii	1.89×10^7 (1.27×10^7)	7.57×10^6 (3.61×10^6)	0.18	1.04×10^6 (7.74×10^5)	7.68×10^5 (5.56×10^5)	0.97	0.07
Bacteroides nordi	5.16×10^5 (3.76×10^5)	7.02×10^5 (4.31×10^5)	0.79	1.76×10^6 (1.19×10^6)	1.63×10^6 (1.35×10^6)	0.84	0.54
Bacteroides caccae	6.92×10^7 (1.81×10^7)	7.04×10^7 (2.58×10^7)	0.99	4.78×10^7 (1.28×10^7)	1.81×10^8 (1.25×10^8)	0.15	0.42
Bacteroides salyersiae	1.94×10^7 (9.45×10^6)	3.96×10^7 (2.01×10^7)	0.30	8.53×10^6 (3.95×10^5)	1.66×10^7 (9.99×10^6)	0.62	0.36

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

***Bacteroides* (continued)**	**Responder Status**						
	NR (*n* = 11)			**RES (*n* = 12)**			
	D0	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	***P* (b)**
Bacteroides acidifaciens	0 (0)	0 (0)	1.00	0 (0)	8.67×10^6 (8.67×10^6)	0.18	0.35
Bacteroides (others)	3.46×10^8 (1.86×10^8)	4.41×10^8 (1.49×10^8)	0.80	3.53×10^8 (1.45×10^8)	7.17×10^8 (4.84×10^8)	0.31	0.61
Parabacteroides Johnsonii	9.00×10^5 (7.96×10^5)	2.26×10^6 (2.00×10^6)	0.99	3.64×10^6 (2.72×10^6)	2.41×10^7 (9.78×10^6)	0.10	0.22
Parabacteroides merdae	4.03×10^7 (1.56×10^7)	9.04×10^7 (2.58×10^7)	0.06	2.80×10^7 (1.27×10^7)	6.74×10^7 (3.21×10^7)	0.11	0.58
Parabacteroides goldsteinii	5.77×10^5 (5.62×10^5)	5.20×10^5 (5.20×10^5)	0.81	1.11×10^7 (6.78×10^6)	3.32×10^6 (2.06×10^6)	0.61	0.27

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Prevotella	Responder Status						
				RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P*(b)
Prevotella copri	5.54×10^7 (4.81×10^7)	1.44×10^8 (1.01×10^8)	0.424	7.12×10^7 (4.14×10^7)	2.93×10^8 (1.74×10^8)	**0.04**	0.48
Prevotella stercorea	2.04×10^6 (2.04×10^6)	4.09×10^6 (4.09×10^6)	0.16	0 (0)	0 (0)	1.00	0.31
Prevotella brevis	0 (0)	0 (0)	1.00	0	0 (0)	0.18	0.35
Prevotella bivia	0	1.36×10^5 (1.36×10^5)	0.16	0 (0)	0 (0)	1.00	0.31
Paraprevotella clara	1.43×10^7 (9.25×10^6)	3.40×10^7 (2.11×10^7)	**0.04**	7.37×10^5 (5.60×10^5)	1.92×10^5 (1.40×10^5)	0.86	0.10
Alloprevotella rava	1.45×10^5 (1.45×10^5)	0 (0)	0.71	1.66×10^5 (1.22×10^5)	1.08×10^5 (1.08×10^5)	0.13	0.35

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Alistipes	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Alistipes timonensis	1.97×10^8 (8.69×107)	2.83×10^8 (1.36×10^8)	0.20	7.99×10^7 (4.00×10^7)	1.22×10^8 (6.05×10^7)	0.61	0.25
Alistipes Shahii	6.76×10^7 (3.92×10^7)	6.70×10^7 (2.55×10^7)	0.99	3.16×10^7 (1.28×10^7)	7.71×10^7 (4.58×10^7)	0.19	0.85
Alistipes finegoldii	1.48×10^7 (8.00×10^6)	1.66×10^7 5.97×10^6)	0.84	3.49×10^7 (1.61×10^7)	3.66×10^7 (2.05×10^7)	0.84	0.38
Alistipes indistinctus	3.32×10^7 (1.51×10^7)	3.49×10^7 (9.27×10^6)	0.88	2.11×10^7 (9.88×10^6)	3.27×10^7 (1.08×10^7)	0.25	0.88
Alistipes onderdonkii	7.03×10^7 (4.51×10^7)	5.29×10^7 (3.04×10^7)	0.48	5.25×10^7 (3.11×10^7)	6.79×10^7 (5.20×10^7)	0.51	0.81

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Roseburia	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Roseburia inulinivorans	9.61×10^7 (5.09×10^7)	7.21×10^7 (2.65×10^7)	0.64	1.11×10^8 (3.25×10^7)	1.91×10^8 (6..42 $\times 10^7$)	0.12	0.11
Roseburia faecis	5.38×10^8 (1.28×10^8)	4.86×10^8 (8.93×10^7)	0.89	3.38×10^8 (7.79×10^7)	1.51×10^9 (4.98×10^8)	**0.006**	**0.03**
Roseburia hominis	3.03×10^7 (7.06×10^6)	3.22×10^7 (8.39×10^6)	0.85	7.22×10^7 (3.63×10^7)	9.24×10^7 (4.07×10^7)	**0.04**	0.18
Roseburia intestinalis	1.11×10^8 (6.36×10^7)	8.37×10^7 (6.20×10^7)	0.42	2.08×10^7 (6.42×10^6)	9.34×10^7 3.38×10^7	**0.03**	0.89

Eubacterium	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Eubacterium desmolans	8.74×10^6 (3.34×10^6)	9.97×10^6 (3.24×10^6)	0.92	2.03×10^7 (7.54×10^6)	1.98×10^7 (7.60×10^6)	0.95	0.26
Eubacterium coprostanoligenes	1.34×10^8 (2.70×10^7)	1.29×10^8 (2.88×10^7)	0.92	1.25×10^8 (2.75×10^7)	1.57×10^8 (5.10×10^7)	0.50	0.64

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Coprococcus	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	***P* (b)**
Coprococcus eutactus	5.86 x 10^8 (1.57 x 10^8)	4.26 x 10^8 (1.10 x 10^8)	0.26	6.72 x 10^8 (2.31 x 10^8)	9.55 x 10^8 (2.04 x 10^8)	**0.04**	**0.03**
***Coprococcus* (others)**	1E+0^8 (3.94 x 10^7)	9.40 x 10^7 (4.13 x 10^7)	0.83	2.86 x 10^7 (9.12 x 10^6)	9.88 x 10^7 (2.03 x 10^7)	**0.001**	0.92

Anaerostipes	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	***P* (b)**
Eubacterium hadrum	2.65 x 10^8 (1.06 x 10^8)	2.17 x 10^8 (6.42 x 10^7)	0.57	4.09 x 10^8 (1.69 x 10^8)	5.90 x 10^8 (1.66 x 10^8)	**0.03**	**0.04**
***Anaerostipes* (others)**	5.46 x 10^7 (2.10 x 10^7)	4.81 x 10^7 (1.75 x 10^7)	0.77	8.71^2 x 10^7 (3.87^6 x 10^7)	1.24 x 10^8 (3.73 x 10^7)	0.097	0.08

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Lachnospiraceae incertae sedis	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Eubacterium uniforme	0	6.89×10^5 (6.89×10^5)	0.16	9.93×10^5 (9.85×10^5)	1.25×10^6 (1.25×10^5)	0.58	0.70
Eubacterium ventriosum	1.41×10^7 (5.82×10^6)	4.10×10^7 (1.75×10^7)	0.28	1.55×10^7 (4.19×10^6)	5.94×10^7 (2.82×10^7)	0.07	0.59
Eubacterium eligens	4.59×10^6 (2.68×10^6)	1.18×10^7 (4.58×10^6)	0.70	2.64×10^6 (1.09×10^6)	3.05×10^7 (2.48×10^7)	0.13	0.47
Eubacterium ruminantium	3.77×10^7 (3.70×10^7)	1.60×10^7 (1.52×10^7)	0.44	1.86×10^7 (1.09×10^7)	6.11×10^7 (4.08×10^7)	0.12	0.33
Eubacterium xylanophilum	5.13×10^6 (4.10×10^6)	8.99×10^6 (3.40×10^6)	0.82	1.27×10^7 (5.82×10^6)	4.16×10^7 (2.46×10^7)	0.08	0.22
Eubacterium hallii	4.33×10^8 (1.12×10^8)	2.99×10^8 (1.04×10^8)	0.22	2.76×10^8 (1.03×10^8)	4.16×10^8 (9.59×10^7)	0.20	0.42
Ruminococcus gnavus	4.35×10^7 (2.85×10^7)	1.48×10^7 (7.90×10^6)	0.60	6.07×10^7 (4.75×10^7)	4.77×10^7 (4.00×10^7)	0.83	0.45
Lachnospiraceae incertae sedis (other)	3.21×10^7 (1.86×10^7)	5.96×10^7 (2.63×10^7)	0.09	1.65×10^7 (6.59×10^6)	5.14×10^7 (1.57×10^7)	0.02	0.79

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Ruminococcus	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Ruminococcus bromii	5.89×10^7 (1.36×10^7)	6.31×10^7 (1.88×10^7)	0.914	8.89×10^7 (2.16×10^7)	1.77×10^8 (5.01×10^7)	**0.02**	0.05
Ruminococcus champanellensis	9.64×10^6 (6.41×10^6)	1.01×10^7 (4.02×10^6)	0.95	3.12×10^7 (1.81×10^7)	4.83×10^7 (2.10×10^7)	0.035	0.10
Ruminococcus callidus	6.52×10^6 (4.03×10^6)	1.11×10^7 (6.49×10^6)	0.80	9.04×10^6 (4.86×10^5)	4.25×10^6 (2.69×10^6)	0.05	0.25
Ruminococcus flavefaciens	1.38×10^6 (1.28×10^6)	8.60×10^5 (6.92×10^5)	0.55	1.23×10^6 (8.11×10^5)	1.22×10^6 (8.57×10^5)	0.99	0.76

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Ruminococcus2	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Ruminococcus lactaris	1.09×10^8 (4.53×10^7)	1.29×10^8 (4.85×10^7)	0.61	3.28×10^7 (1.53×10^7)	6.55×10^7 (3.31×10^7)	0.30	0.42
Ruminococcus faecis	2.64×10^8 (4.62×10^7)	3.18×10^8 (9.66×10^7)	0.61	4.92×10^8 (2.56×10^8)	6.77×10^8 (2.58×10^8)	**0.02**	0.14

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Lactobacillus/ Enterococcus	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Lactobacillus ruminis	2.90×10^5 (2.10×10^5)	2.27×10^6 (1.76×10^6)	0.96	5.98×10^5 (5.53×10^5)	5.67×10^7 (5.66×10^7)	0.182	0.37
Lactobacillus salivarius	1.10×10^5 (1.10×10^5)	1.38×10^5 (1.38×10^5)	0.22	5.78×10^4 (3.36×10^4)	2.75×10^4 (2.75×10^4)	0.76	0.42
Lactobacillus mcosae	0	2.75×10^5 (2.75×10^5)	0.81	1.89×10^6 (1.85×10^6)	1.74×10^6 (1.74×10^6)	0.19	0.36
***Lactobacillus* (others)**	0	0	1.00	5.52×10^4 (3.77×10^4)	2.75×10^4 (3.00×10^4)	0.45	0.35
Enterococcus hermanniensis	4.11×10^7 (2.84×10^7)	1.41×10^7 (1.40×10^7)	0.18	1.52×10^7 (1.46×10^7)	2.69×10^7 (2.51×10^7)	0.54	0.67

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
Lactococcus	**D0**	**D28**	***P* (a)**	**D0**	**D28**	***P* (a)**	***P* (b)**
Lactococcus lactis subsp. lactis	5.83×10^5 (2.49×10^5)	2.62×10^6 (1.85×10^6)	0.76	1.09×10^7 (8.90×10^6)	1.95×10^6 (8.08×10^5)	0.168	0.74
Lactococcus garvieae	0	0	1.00	7420 (7420)	1.57×10^6 (1.57×10^6)	0.18	0.35

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Blautia	Responder Status						
	NR (*n* = 11)			RES (*n* = 11)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Blautia wexlerae	8.23×10^8 (1.27×10^8)	4.70×10^8 (1.14×10^8)	0.09	7.81×10^8 (3.76×10^8)	1.19×10^9 (4.19×10^8)	**0.001**	0.03
Blautia schinkii	3.62×10^8 (1.06×10^8)	4.32×10^8 (1.80×10^8)	0.49	2.23×10^8 (7.47×10^7)	3.30×10^8 (1.07×10^8)	0.32	0.62
Blautia faecis	1.47×10^8 (3.46×10^7)	1.12×10^8 (2.37×10^7)	0.55	2.03×10^8 (6.07×10^7)	2.42×10^8 (5.59×10^7)	0.49	0.05
Blautia cocoides	9.08×10^5 (7.19×10^5)	6.00×10^5 (4.0×10^5)	0.43	5.51×10^6 (2.66×10^6)	3.13×10^6 (2.23×10^6)	0.06	0.20
Blautia stercoris	4.85×10^6 (3.37×10^6)	2.81×10^6 (2.41×10^6)	0.78	5.02×10^6 (4.42×10^6)	1.76×10^7 (1.40×10^7)	0.89	0.33
Blautia glucerasea	1.87×10^5 (9.03×10^5)	1.32×10^6 (8.13×10^5)	0.85	2.19×10^5 (1.38×10^5)	5.86×10^5 (3.45×10^5)	0.19	0.23
Blautia hansenii	3.75×10^6 (1.54×10^6)	1.47×10^7 (6.93×10^6)	0.28	1.07×10^7 (4.65×10^7)	3.33×10^7 (1.25×10^7)	**0.03**	0.22
Blautia luti	4.27×10^8 (1.68×10^8)	4.60×10^8 (1.74×10^8)	0.74	1.79×10^8 (5.48×10^7)	3.96×10^8 (1.30×10^8)	**0.04**	0.77
Ruminococcus obeum	2.58×10^8 (6.13×10^7)	3.51×10^8 (1.15×10^8)	0.30	5.24×10^8 (3.87×10^8)	7.10×10^8 (3.88×10^8)	**0.04**	0.40

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Dorea	Responder Status						
	NR (*n* = 11)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	*P* (b)
Dorea formicigenerans	1.27×10^8 (3.26×10^7)	8.16×10^7 (2.19×10^7)	0.15	1.40×10^8 (4.62×10^7)	1.30×10^8 (3.60×10^7)	0.75	0.24
Dorea longicatena	5.77×10^8 (1.62×10^8)	3.50×10^8 (1.07×10^8)	0.18	2.31×10^8 (7.43×10^7)	3.82×10^8 (1.43×10^8)	0.34	0.86

Quantitative Microbiome Profiling 16S rRNA sequencing data (species level) from samples collected at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column), red text indicating a trends towards significance, and are the result of employing a general linear model (GLM). **Abbreviations:** NR = non-responder; RES = responder

Appendix 5.4 Mean bowel habit diary data: Stool consistency, Frequency, Flatulence, Intestinal bloating, Abdominal Pressure, Abdominal Pain and Feeling of Fullness across the entire cohort and segregated by RES/NR status measured at run-in week and last of the intervention phase

Gastrointestinal sensation and bowel habit	Responder Status									
	Overall D0 (*n* = 23)			NR (*n* = 1)			RES (*n* = 12)			
	Run-in week	Final week (D22-D28)	*P* (a)	Run-in week	Final week (D22-D28)	*P* (a)	Run-in week	Final week (D22-D28)	*P* (a)	*P* (b)
Stool Frequency	1.28 (0.10)	1.26 (0.09)	0.68	1.23 (0.13)	1.31 (0.17)	0.32	1.32 (0.14)	1.20 (0.09)	0.140	0.56
Stool Consistency	3.73 (0.21)	3.50 (0.71)	0.71	3.40 (0.31)	3.45 (0.23)	0.82	4.04 (0.27)	3.54 (0.27)	**0.045**	0.82
Flatulence	0.52 (0.08)	0.55 (0.10)	0.77	0.56 (0.12)	0.48 (0.14)	0.54	0.49 (0.12)	0.61 (0.15)	0.320	0.550
Intestinal Bloating	0.33 (0.11)	0.28 (0.09)	0.63	0.481 (0.65)	0.29 (0.44)	0.22	0.20 (0.09)	0.27 (0.12)	0.610	0.893
Abdominal Pain	0.23 (0.06)	0.14 (0.05)	0.16	0.31 (0.10)	0.16 (0.10)	0.12	0.15 (0.05)	0.11 (0.06)	0.69	0.74
Abdominal Pressure	0.29 (0.09)	0.24 (0.08)	0.60	0.27 (0.15)	0.26 (0.14)	0.93	0.30 (0.10)	0.21 (0.10)	0.54	0.783
Feeling of Fullness	0.33 (0.10)	0.32 (0.11)	0.90	0.40 (0.14)	0.40 (0.18)	1.00	0.26 (0.15)	0.24 (0.13)	0.87	0.454

Gastrointestinal sensation and bowel habit scores at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column) and are the result of employing a linear marginal model (LLM) integrating run-in phase data as a baseline covariate. **Abbreviations:** NR = non-responder; RES = responder

Appendix 5.5 Mean mood state scores across the entire cohort and segregated by RES/NR status ay D0 and D28 of the intervention phase

Mood state	Responder status									
	Overall (*n* = 23)			NR (*n* = 12)			RES (*n* = 12)			
	D0	D28	*P* (a)	D0	D28	*P* (a)	D0	D28	*P* (a)	P (b)
Becks Depression Inventory	17.83 (0.75)	13.13 (1.42)	**≤ 0.001**	17.64 (1.28)	14.00 (2.24)	0.08	18.00 (0.89)	11.5 (1.57)	**0.002**	0.29
State Trait Anxiety Inventory Y1	52.91 (1.19)	46.43 (1.64)	**0.01**	52.55 (2.02)	48.36 (2.56)	0.20	53.23 (1.43)	44.66 (2.07)	**0.009**	0.27
State Trait Anxiety Inventory Y2	53.52 (0.71)	49.00 (1.52)	**0.002**	53.82 (1.27)	51.36 (2.33)	0.21	53.25 (0.74)	46.17 (1.43)	**<0.001**	0.06
Positive Affect (scores)	24.48 (1.26)	31.91 (1.25)	**0.01**	25.91 (1.81)	31.00 (1.76)	0.07	23.16 (1.75)	32.75 (1.80)	**<0.001**	0.49
Negative Affect (scores)	29.96 (0.95)	25.00 (1.02)	**0.002**	30.00 (1.40)	26.55 (1.44)	0.10	29.91 (1.36)	23.58 (1.37)	**0.003**	0.13
Pittsburgh Sleep Quality Index	6.00 (0.32)	4.65 (0.38)	**0.01**	6.64 (0.41)	4.46 (0.47)	**0.001**	5.42 (0.43)	4.83 (0.60)	0.314	0.27
Cortisol awakening Response	63.58 (1.75)	60.83 (2.01)	**0.003**	62.32 (2.59)	59.89 (3.57)	0.064	64.74 (2.43)	61.70 (2.17)	**0.018**	0.66

Mood state scores and cortisol awakening response values at D0 and D28 of the intervention phase. Mean and Standard error (SE). **(a)** Significant differences compared with respective D0 and D28 samples are identified with specified *P* values (grey columns). **(b)** Significant differences between responder status at D28 are indicted by specified *P* values (orange column) and are the result of employing a linear marginal model (LMM) integrating D0 data as a baseline covariate. **Abbreviations:** NR = non-responder, RES = responder