University of Reading

Investigating Novel Prebiotic Blends and Their Impact on Markers of Satiety

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Commonly Used Abbreviations

Analysis of variance (ANOVA)

Arabinoxylan (ABX)

Area under the curve (AUC)

αlpha-glucooligosaccharide (GLOS)

βeta -glucan (β-GLU)

Body mass index (BMI)

Calcium chloride hexahydrate ($CaCl_2.6H_2$)

Degree of polymerization (DP)

Deoxyribonucleic acid (DNA)

Dipotassium phosphate (K_2HPO_4)

Dilution factor (DF)

Eubacteria I II III (EUB I II III)

Ethylenediaminetetraacetic acid (EDTA)

External standard (E/S)

Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$)

Fluorescent region for non-Eubacteria (NE-FL1)

Fluorescent *in situ* hybridisation/flow cytometry (FISH-FLOW)

Fluorometric Imaging Plate Reader (FLIPR)

Free fatty acid 2/3 (FFA2/3)

Food intake (FI)

Fructo-oligosaccharide *(*FOS)

Gastro-intestinal tract (GIT)

Glucagon-like-peptide-1 (GLP-1)

G protein-coupled receptor 41/43 (GPR41/43)

Galacto-oligosaccharide (GOS)

High amylose resistant starch2 (HAMRS-2)

High dietary fibre (HDF)

High performance liquid chromatography (HPLC)

High viscosity (HV)

Hybridisation buffer (HB)

Hydrochloric acid (HCl)

Hugh Sinclair unit of Human Nutrition (HSUHN)

Human Metabolome Database (HMDB)

Internal standard (I/S)

Inulin-propionate ester (IP)

Knock out (KO)

Long-chain fructo-oligosaccharide/inulin (LC-FOS)

Low fibre (LF)

Low dietary fibre (LDF)

Low viscosity (LV)

Low viscosity arabinoxylan (LV-ABX)

Magnesium sulfate ($MgSO_4$)

Mass spectrometry (MS)

Monopotassium phosphate (KH_2PO_4)

Medium viscosity arabinoxylan (MV-ABX)

NPY (Neuropeptide Y)

Nuclear magnetic resonance (NMR)

Oat fibre (OAT)

Oligofructose (OFS)

Orthogonal projections to latent structures discriminant analysis (OPLS-DA)

Peptide YY (PYY)

Phosphate buffered saline (PBS)

Polydextrose (PDX)

Polysaccharide utilising locus (PUL)

Principal component analysis (PCA)

Pro-opiomelanocortin (POMC)

Resistant maltodextrin (R-MLX)

Resistant starch (RS)

Realtime quantitative polymerase chain reaction (RTqPCR)

Quality control (QC)

Randomised control trial (RCT)

Relative Risk (RR)

Resistant starch (RS)

Ribonucleic acid (RNA)

Scientific Advisory Committee for Nutrition (SACN)

Short chain fatty acid (SCFA)

Simulator of the Human Intestinal Microbial Ecosystem (SHIME®)

Sodium bicarbonate ($NaHCO_3$)

Sodium dodecyl sulfate (SDS)

Statistical Package for the Social Sciences (SPSS)

Three-factor eating questionnaire (TFEQ)

Tris/HCl buffer (TE-FISH)

United Kingdom (UK)

Visual Analogue Scale (VAS)

Wheat bran (WB)

Wild type (WT)

Xylo-oligosaccharide (XOS)

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Thesis abstract

The purpose of this project was to develop a novel blend of prebiotics with the potential to influence perceptions of satiety and thus food intake. Prebiotics beneficially modulate the composition of the gut microbiota, they are fermented primarily in the ceacum resulting in the production of metabolites, including acetate and propionate. These metabolites are thought to impact appetite regulation by acting as a ligand, with a high affinity to G-coupled receptors (GPR42/3) located on L-cells, throughout the length of the colon. A subsequent stimulation of circulating satiety hormones triggers anorexogenic pathways involved in reducing food intake and increasing satiety. It is therefore hypothesised that using blends of prebiotics to increase SCFA production along the length of the colon might be of benefit in weight management.

Initially a literature review is presented, evaluating dietary intervention studies that have explored the satiety inducing effects of different prebiotics/prebiotic candidates on various human populations. In the reviewed 17/27 intervention studies prebiotics were associated with increased satiety. Variations in study design, dose and study food matrix might explain the disparity in the results.

The work of screening eleven commonly consumed prebiotics/prebiotic candidates for their fermentation characteristics is described in the first experimental chapter. Using an *in vitro* batch culture model of the gut (n=3), those prebiotics that stimulated the growth of acetate and propionate producing bacterial species, such as *Bifidobacterium* and *Propionibacterium* specifically at later fermentation time points (24-48h) (indicating a slower fermentation), were considered for further study. Inulin was identified as the most bifidogenic of all the substrates.

Three substrates that performed well in the first experimental section were blended with inulin to produce 3 novel prebiotic blends (inulin + resistant starch, inulin + α-gluco-oligosaccharides, and inulin + arabinoxylan). The fermentation characteristics of these blends were assessed in depth using a 3-stage continuous culture colonic model (n=3). The focus was on the influence of these prebiotic blends on the third fermentation vessel, which simulates the distal region of the colon. The hypothesis that residual prebiotic activity in this vessel might be associated with a flatter and more sustained SCFA spike following consumption, and that this might lead to better appetite control. Of the 3 blends, I+RS and I+ABX outperformed

I+GLOS exhibiting a sustained fermentation towards V3, however I+ABX was the frontrunner, due to the levels of propionate produced, including those in V3, which was desirable.

In a 9-week crossover, placebo controlled and double blinded human appetite study. I+ABX was consumed daily by healthy weight men. The primary endpoint was satiety and ad libitum energy intake on a study visit day. Secondary endpoints were prebiotic effects assessed in stool and urine. Subjective satiety scores were not influenced however there was a statistically significant reduction in energy intake during the *ad libitum* lunch of 34.28Kcal. If extended over three meals this might equate to a <100 Kcal reduction in energy intake per day, more than sufficient to elicit progressive weight loss or to contribute to weight maintenance. The intervention also induced significant increases in the abundance of *Bifidobacterium* (P=0.017) and *Propionibacterium* (P=0.021) in stool samples and an increase in the concentration of acetate. This research has demonstrated a pipeline for the development of prebiotics with the potential for use in weight management products.

Chronic consumption of LC-FOS with ABX significantly reduced food intake and therefore supplementation of the diet with I+ABX may be a useful tool in weight management. Further work is needed to understand the mechanism and to establish additional prebiotic benefits associated with consuming this blend. Our blended product is being patented by our industrial sponsor and may be explored in further clinical intervention study over extended time frames with weight control as the endpoint.

Chapter 1: General Introduction

Introduction

The overall aim of this research project was to develop a novel blend of functional food ingredients which might favour anorexigenic microbial metabolic activity in the colon. The influence of the gut microbiota on adiposity is topical and there is emerging evidence to suggest that colonic fermentation influences the satiety cascade. The rationale for our approach is based on good existing experimental data to suggest that microbial composition in the colon can be purposefully manipulated through dietary intervention. The development of anorexigenic functional food ingredients is commercially desirable and socially important in the context of the ongoing obesity crisis.

Obesity: metabolic and social consequences

In the UK, 90% of those with type 2 diabetes are overweight or obese (Ells *et al*., 2014) representing a large burden to public health with an estimated £6 billion spent on obesity related disorders in 2014 (PH England 2014). Increased consumption of energy dense, processed foods combined with a reduction in energy expenditure is characteristic of modern lifestyle and has contributed to rising obesity rates, which doubled between 1993 and 2014 (PH England 2016). As adiposity increases so too does the risk of developing metabolic conditions such as diabetes through stimulation of adipokines and raised triglyceride levels exacerbating insulin resistance (Rabe *et al*., 2008).

There are social and personal effects of obesity, along with growing rates among young people; a person is 57% more likely to be obese if their friend is obese. Social acceptance of the consumption of large amounts of processed foods that are high in fat and sugar can compound this (Leahey *et al*., 2011). Yet the economic spend on weight loss a product in the UK is increasing, with no slowing of obesity rates.

The risk of developing heart disease increases as BMI increases: by double in overweight people to more than 10 times in the morbidly obese when compared with lean counterparts (Hurt *et al*., 2010). Obesity is thought to be responsible for 6% of cancer cases in the UK**.** With the greatest risk linked to bowel, liver and oesophagal cancers (Brown *et al*., 2018). In men a 5 kg/m^2 increase in BMI was strongly associated with oesophageal cancer (RR 1.52, p<0·0001) and with thyroid (1.33, p=0·02), colon (1.24, p<0·0001), and renal (1.24, p <0.0001) cancers

(Renehan *et al*., 2008)**.** The risk of developing type 2 diabetes is strongly correlated with increasing BMI. The prevalence of diabetes is rising in the UK, with 6% (2.7 million people) of the population over 17 diagnosed in 2013. The overall relative risk (RR) an obese person has of developing diabetes compared to normal weight is 7.19, 95% CI: 5.74, 9.00 and for overweight was 2.99, 95% CI: 2.42, 3.72 (Gatineau *et al*., 2014).

Gut microbiota

The adult human microbiome comprises 100 trillion cells consisting of bacteria, bacteriophage, fungi, protozoa and viruses that, along with their collective genomes remains relatively stable throughout adulthood (Ursell *et al*., 2012). Between individuals, differences in the proportions of the ~1000 residential species and whole phylum are common (Turnbaugh *et al*., 2007).

Many factors are pivotal in the development of a healthy gut microbiota. They include diet during mode of delivery (caesarean vs vaginal) diet in early infancy (breastfed vs bottle), adulthood (high fibre vs low fibre) and antibiotic use. The commensal bacterial groups predominantly aid in nutrient metabolism, prevention of colonization of pathogenic microorganisms and they contribute to maintenance of intestinal barrier function (Evans *et al*., 2013). Much of this is due to bacterial groups such as bifidobacteria being strongly anti-pathogenic through competitive inhibition, but also due to lowering the pH, making it difficult for pathogens to take hold.

Due to the complexity of the microbiota, understanding what constitutes a healthy vs non-healthy microbiota, is challenging, particularly as there can be large intra-individual differences between healthy people. However, evidence is strengthening that there are distinct differences between those with obese vs. normal weight phenotypes, however overweight may be associated with reduced diversity. A gene count of <480,000 genes has been associated with obesity whilst higher diversity or higher gene count between 380,000-640,000 is associated with a leaner phenotype (Le Chatelier *et al*., 2013, The Human Microbiome Project, 2012) In a study of monozygotic twins Turnbaugh *et al*. demonstrated that whilst a core common microbiome exists, phylum level changes and an overall reduced bacterial diversity was associated with obesity (Turnbaugh *et al*., 2009). Ley *et al.* could distinguish the microbiome of obese (ob/ob) mice from their lean (ob/+ and +/+) siblings at a

phylum level. They found a 50% reduction in Bacteroidetes and increased prevalence of Firmicutes in obese mice (Ley *et al*., 2005). Turnbaugh *et al.* demonstrated that the microbiotas of obese mice exhibit modified metabolic potential through increased capacity for energy harvest. And importantly, transplanting the microbiota from obese mice into germ free mice induced obesity in the recipient (Turnbaugh *et al.*, 2006).

Microbial diversity may be strongly influenced by diet and environmental exposure, for example the saccharolytes: Prevotella and Xylanibacter, are more abundant in the stools of children from Burkina Faso, Africa than in European children (De Filippo *et al*., 2010). High fibre consuming Venezuelan and Malawians also show more diverse microbiota than their African American counterparts (Yatsunenko *et al*., 2012).

Prebiotics – structure and function

Prebiotics are substrates that can be selectively utilized by the host microbiota conferring a health benefit (Gibson *et al*., 2017). They must resist digestion in the upper GI tract and then selectively stimulate the growth and/or activity of specific commensal bacterial groups. bifidobacteria, for example can utilise fructo-oligosaccharides (DP>10) due to their *glycosidases* which can cleave specific linkages other bacterial groups cannot (Rios-Covian *et al*., 2015, Rios-Covian *et al*., 2013;). Polysaccharides such as resistant starch (RS), (DP >10) ferment more slowly (Zhou *et al*., 2013;), and has exhibited prebiotic potential in human randomised control trials (RCTS) (Hald *et al*., 2016, Maier *et al*., 2017). bifidobacteria can metabolise the outer oligosaccharide chains exposing the backbone for other bacterial groups such as *Bacteroides* to ferment. Metabolites such as acetate produced from the fermentation of these prebiotics may also be utilised by species such as *Faecalibacterium prausnitzii* to produce butyrate further along the colon, as observed in co-culture experiments by Rios-covian e*t al* (Rios-Covian *et al*., 2015). In the UK consumption of dietary prebiotics, from sources such as onion, banana and fortified products is low, at approximately 1-4g/d (Bonnema *et al*., 2010). It therefore may be desirable to supplement the diet with prebiotic enriched functional foods.

Appetite regulation and satiety

Appetite regulation and controlling food intake is complex and influenced by food structure and composition, internal physiological and biochemical responses to foods as well as external cues, learned social and cultural influences on eating behaviour. Satiation and satiety are pivotal in this regulation, whereas satiation refers to the amount of food and length of time spent during an eating episode, satiety denotes the length of time between meals, both of which are integral to appetite regulation. These physiological processes involve both short term signals that influence the amount of time between meals and long-term signals such as leptin and insulin which are concerned with the amount and distribution of fat Blundell *et al* developed the satiety cascade to describe the biological mechanisms in which food impacts satiation and satiety (Blundell and Hill, 1987). The cascade includes the underlying processes involved in satiation; the feeling of fullness perceived during a meal and satiety; the feeling of fullness perceived following cessation of an eating episode that, if it persists leads to eating less; and longer periods between meals, it is a combination of both satiation and satiety that determine the size and frequency of a meal consumed.

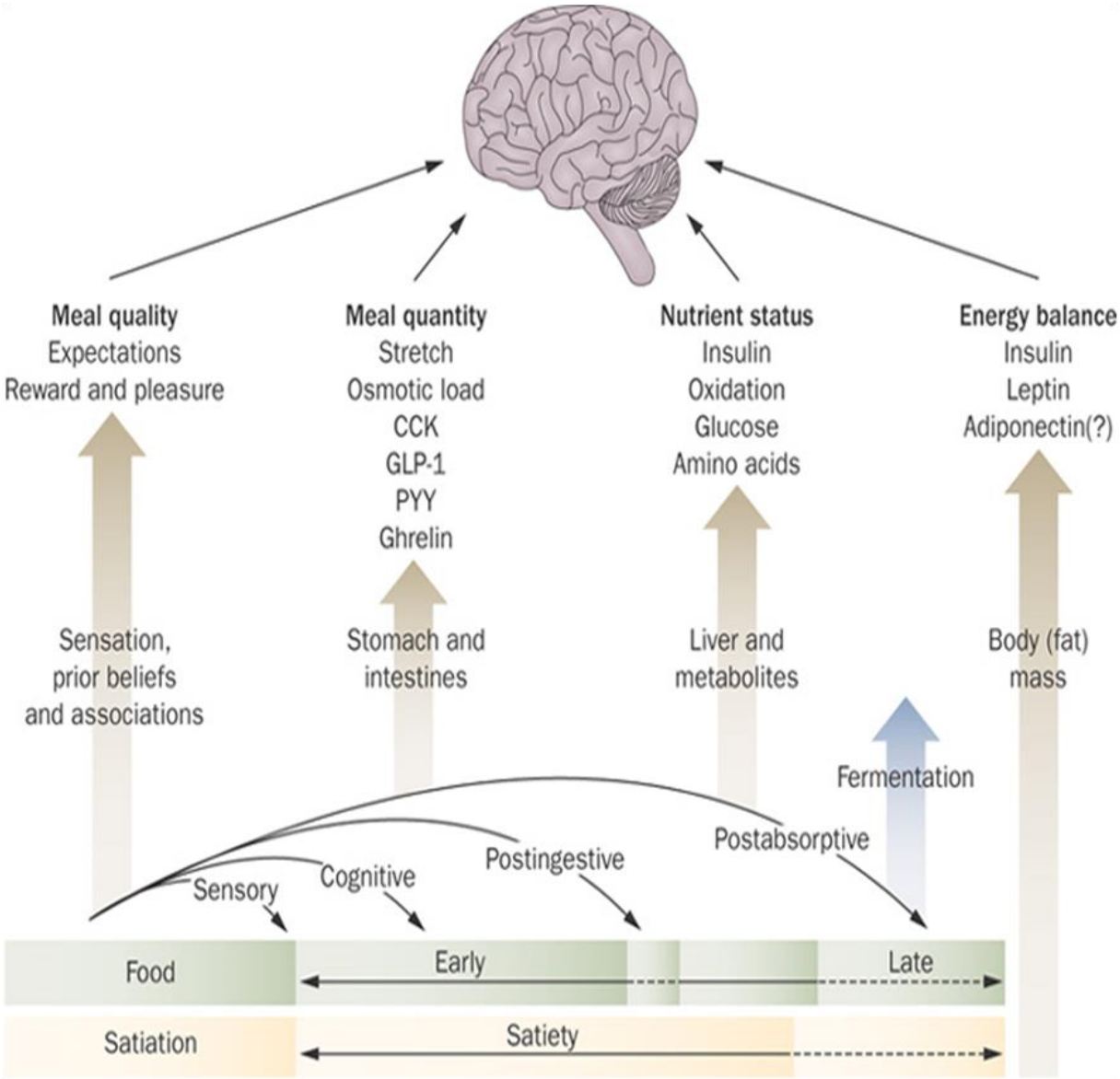

Figure 1. Satiety cascade showing the relationship between satiation and satiety, with mediating psychological and physiological processes. Illustrating how psychological and physiological stimuli arising from the consumption of a food modulate the effects of that food on appetite sensations and the pattern of eating (Blundell, 2010).

The cascade is characterised by three phases, initially the cephalic phase is influenced by neural mechanisms that stimulate the sensory system through liking or appeal of a food. Physiological aspects of this phase include production of the gut peptide ghrelin in response to hunger, stimulating orexigenic neuropeptide Y (NPY) to promote feeding by inhibiting anorexigenic pro-opiomelanocortin (POMC) neurons (**Figure 1).** Conversely the second pre-absorptive phase is dominated by the production of satiety hormones, glucagon peptide 1 (GLP-1) and peptide YY (PYY) that reduces NPY activity but stimulates POMC neurons. This inhibits food intake and slows gastric emptying, depending on the quantity and density of the food consumed. The third post absorptive phase includes satiety effects mediated by gut microbiota, through fermentation of non-digestible carbohydrates (NDCs) in the meal. This leads to the production of short chain fatty acids (SCFAs) acetate, propionate and butyrate. These metabolites have an affinity for G-protein coupled receptors (GPR43/42) on entero-endocrine cells located throughout the length of the colon, effectively stimulating production of GLP-1 and PYY. This in turn triggers anorexigenic pathways such as POMC neurons and reducing food intake (Blundell and Hill, 1987, Perry and Wang, 2012).

Prebiotics and appetite regulation

Prebiotics are often water binding and may be used as low calorie bulking agents in foods, in the stomach this bulk stimulates stretch receptors. This limits food intake through release of satiety signals (van Kleef *et al*., 2012). Prebiotics may also delay gastric emptying and, with low intestinal bioavailability, they decrease the glycaemic index of foods (Slavin and Green, 2007). This may smooth the post prandial insulin curve and suppress food seeking behaviour during the glucose slump. In the final post-absorptive phase of the satiety cascade, prebiotics are fermented in the colon yielding energy for the host in the form of SCFA. It is hypothesised that SCFA are satiety inducing metabolites produced during this process that may be increased to further positively impact on satiety mechanisms (Kaji *et al*., 2011, Karaki *et al*., 2006, Morrison *et al*., 2016). The role of fibre and prebiotics in appetite regulation is reviewed later.

Blending prebiotics

Candidate prebiotics exist as a range of different structures leading to varying functionality that can be used to target specific bacterial species to produce different ratios of SCFA (Brouns *et al*., 2002, Rivière *et al*., 2014) By investigating and characterising the fermentation profiles of complimentary blends it may be possible to attenuate colonic metabolism in different parts of the colon , thereby boosting health benefits, and this could be a route to manipulating metabolic processes such as appetite regulation.

Using a simulator of human intestinal microbial ecosystem, Marzorati *et al.* demonstrated that blending inulin and acacia gum allowed for a gradual fermentation along the length of a simulated colon compared to inulin alone (Marzorati *et al*., 2015) Similarly, during 24hr batch culture, inulin blended with gum acacia and pea fibre (0.5g per substrate in 140mL) induced a delay in the release of SCFA compared to fermentation of the individual substrates alone (Koecher *et al*., 2014).

Aim and outline of thesis

Individual prebiotics are known to selectively stimulate growth and activity of commensal bacterial groups to produce health benefits. Therefore, it is worthwhile investigating if additive prebiotic effects might occur through blending prebiotic oligosaccharides together ie inulin + GLOS or with polysaccharides ie inulin +RS, or inulin + arabinoxylan as this might improve appetite regulation and increase satiety. The aims of this project were;

1/ To screen 11 individual fibres through 48h *in vitro* batch culture to determine those that might complement inulin in a blended product. Increased acetate and propionate production, along with stimulation of *Bifidobacterium* and *Propionibacterium* growth at later time points were desirable.

2/ To test 3 novel prebiotic blends developed in aim 1 in a more realistic continuous culture fermentation model of the colon to identify an optimal prebiotic blend.

3/ To test the optimal blend as ascertained *in vitro* in a human dietary study with satiety as the primary endpoint.

The thesis begins with a literature review assessing the existing human intervention studies on prebiotics and satiety. In the general methodology chapter, I justify my experimental approaches. Chapter 4 describes the *in vitro* batch culture screening experiment in which the fermentation profiles of individual test substrates were characterised with a view to proposing candidate prebiotic blends. Chapter 5 describes fermentation profiles of 3 prebiotic blends in a three-stage continuous colonic system used to model changes to microbial ecology in anatomically distinct compartments, as well as SCFA production in these regions. In the final experimental chapter 6 the findings of a human, placebo-controlled crossover study used to test the satiety inducing effects of consumption of a prebiotic blend are discussed. Chapter 7 will cover the general discussion.

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Chapter 2

Prebiotics and Satiety - A Review of Randomised Controlled Feeding Studies.

Abstract

Prebiotics modify the composition of gut microbiota and this can lead to increases in the production of SCFAs. These metabolites are thought to be implicated in appetite regulation, and in particular: acetate and propionate are thought to act as ligands, stimulating production of satiety hormones such as GLP-1 and PYY and subsequently anorexigenic pathways to reduce food intake. To the extent in which prebiotics impact appetite regulation however, is yet to be elucidated. The purpose of this review is to identify those human dietary intervention studies that investigated the satiety inducing effects of prebiotics/candidate prebiotics, measuring subjective satiety as an endpoint, using visual analogue scale (VAS) but also changes in microbial ecology and satiety hormones following consumption of: resistant starch (RS), βeta glucan (β-GLU), oligofructose (OFS), polydextrose (PDX), and wheat bran (WB) Of the 26 studies identified, 17 elicited significant increases in subjective satiety, however as most of these studies were acute in design and chose a range of doses as well as administration methods, the disparity between results makes sense and instead it might be useful and beneficial to measure satiating effects of prebiotics in well designed, chronic human appetite studies, whereby satiety effects mediated by changes to gut microbiota might be considered.

Introduction

With 63% of the UK population either overweight or obese, obesity is a pressing public health issue. It is proposed that an imbalance or dysbiosis in the composition of gut microbiota may be implicated in the development of the obese phenotype (Ley *et al*., 2005) (Clarke *et al*., 2012). Emerging evidence suggests that this relationship can be mediated through gut metabolite influences on appetite regulation (Evans *et al*., 2013)**.** Dietary approaches that favourably manipulate microbial composition and metabolic processes to attenuate appetite include supplementation with prebiotics. The purpose of this review is to synthesise current evidence pertaining to the role of prebiotics in satiety.

Here we discuss human dietary intervention studies that have measured satiety as a primary endpoint, and which have also assessed satiety hormones in plasma, and characterised changes in gut microbial composition and/or activity following consumption of a prebiotic. We identified studies published between 2003-2017. Most of the human studies reviewed used the participant subjective visual analogue scale (VAS) as their main methodology, though subjective, VAS scores are considered reliable in terms of measuring satiety in human studies, using a within subject, repeated measures (Flint *et al*., 2000, Hobden *et al*., 2017). Bias is minimised by randomisation, single/double blinded placebo-controlled trials unless stated otherwise.

Resistant starch

Resistant starch (RS), refers to large polysaccharides that may be sub-classified as RS1-RS4 (Sajilata *et al.*, 2006) dependent on the relative amount of starch that reaches the colon (Englyst *et al*., 1982). High amylose resistant starch 2 (HAMRS-2) consists of 60% amylose, a helical polymer of α-D-glucose units and 40% amylopectin, a soluble polysaccharide comprised of highly branched polymers of glucose (Keenan *et al*., 2015).

Of the four human studies evaluating the satiating effects of RS were mainly of an acute crossover or parallel design using VAS to measure satiety and *ad libitum* meals to measure food intake (**Table 1)**.

Table 1: Fermentable & Soluble Fibre - Human Satiety Studies

Author, Yr Publication	Sample size	Fibre	Method	Dose (g)	Duration	Admin	Outcome
		Resistant Starch					
Willis *et al.*, 2009	20 healthy ♂ & ♀	Hi-Maize 260 high amylose corn starch	VAS (0-180mins)	8	1d	Muffins	Satiety ↑
Bodinham *et al.*, 2009	20 healthy ♂	Hi-Maize 260 high amylose corn starch	VAS (0-7hr) + glucose (Ria) & insulin	48	1d	Mousse	No diff in satiety, ↓ energy intake
Anderson *et al.*, 2010	n=17 ♂ & n=16 ♂ healthy	Hi-Maize 260 high amylose corn starch	VAS (0-120mins) + glucose (prick test)	23, 27	1d	Soup	Satiety ↑, ↓ energy intake
Harrold *et al.*, 2014	45 healthy ♂ & 46 ♀	Hi-Maize 260 high amylose corn starch	VAS	20, 30	1d	Fruit smoothie	Satiety ↑
Hoffman *et al.*, 2016	22 healthy ♂ & ♀	Unripe banana flour	VAS (0-180mins) + satiety peptides + insulin/glucose	5g	3x5g/wk for 6 wk	Soup	Satiety ↑, ↓ energy intake attenuated glucose
		Oligosaccharides					
Cani *et al.*, 2006	5 healthy ♂ & 5 ♀	Oligofructose	VAS (0-240mins)	16	2x8g/d dose for 2wk	Supplement	Satiety ↑, ↓ energy intake
Cani *et al.*, 2009	5 healthy ♂ & 5 ♀	Oligofructose	VAS (0-180mins) + finger prick – glucose: glucometer, breath hydrogen: MICROH2 breath test, Luminex: GLP-1, PYY, GIP, insulin	16	2x8g/d dose for 2 wk	Supplement	No diff in satiety, ↑ PYY & GLP-1.

Parnell *et al*., 2009	9 obese ♂ & 39 obese ♀	Oligofructose	VAS (0-360mins) + Lincoplex to detect Ghrelin, PYY, CKK, Leptin	21	21g/d dose for 12 wk	250ml drink	No diff in satiety, ↑ PYY, ↓ ghrelin
Hess *et al*., 2011	20 healthy ♂ & ♀	Oligofructose	VAS (0-240mins) + Breath hydrogen & energy intake	5, 8	1d	Cocoa drink & chocolate chews	No diff in satiety, ↓ energy intake only in ♀
Heap *et al*., 2016	19 healthy ♀	Inulin	VAS (0-180mins) & energy intake	0, 6	8d	Yoghurt	No diff in satiety or energy intake
		Polydextrose (PDX)					
King *et al.,* 2005	8 healthy ♂ & 8 ♀	PDX + xylitol	VAS	25g PDX &12.5g PDX + 12.5g xylitol	10d	Yoghurt	PDX - no difference, but PDX + xylitol ↑ Satiety
Ranawana *et al*., 2012	26 healthy ♂	PDX	VAS	12	1d	Smoothie	No diff in satiety, ↓ energy intake
Hull *et al*., 2012	24 healthy♀ & 10 ♂	PDX	VAS (0-450mins)	0, 6.25, 12.5	1d	Yoghurt	Satiety ↑, ↓ energy intake
Konings et al., 2013	9 obese ♂ & 9 ♀	PDX	VAS (0-24h) + breath hydrogen using Gastrolyser	30% of carb = PDX	1d	Orange or cocoa drink	Satiety ↑, no diff energy intake
Olli *et al.,* 2015	13 obese ♀ 5 obese ♂	PDX	VAS + gut hormones	15	1d	High fat meal	No diff in satiety, ↓ hunger & ↑ GLP-1
		βeta glucan					

Kim *et al*., 2006	19 obese ♂ & ♀	β-glucan	VAS + blood glucose	0,2g beta glucan	1 day	Yoghurt	No diff in satiety
Keogh *et al*., 2007	14 healthy ♀	β-glucan	VAS, insulin & glucose, respiratory quotient	11.9g	4wks	Muffin/cereal	no diff in energy intake
Vitaglione *et al*., 2009	14 healthy ♂ & ♀	β-glucan	VAS (0-180mins) + Glucose/PYY, ghrelin - Elisa plus insulin - Immulite one	0, 3	1 day	Bread	Satiety & PYY ↑, ghrelin ↓
Juvonen *et al*., 2009	4 healthy ♂ & 16 ♀	β-glucan	VAS – 180mins CCK, PYY, ghrelen	0, 30	1 day	Drink	HV ↑ satiety, ↑ CCK, PYY, ↓ ghrelin
Lyly *et al*., 2009	19 healthy ♂ & ♀	β-glucan	10 unit graphic intensity scale (0-120mins) + viscosity	0, 10.5	1 day	Drink	Satiety ↑
Beck *et al*., 2009	66 obese ♀	β-glucan	VAS (0-120mins) + Lincoplex to detect Ghrelin, CCK, PYY, leptin and glp-1	0, 6, 9	3 mth	Muesli bar	Satiety ↑No diff, except ↑ PYY
Pentikainen *et a*l., 2014	30 healthy ♀	β-glucan	VAS (0-210mins)	0, 4, 8	1 day	Biscuits & juice	Satiety ↑
Robello *et al.,* 2014	29 ♀ 19 ♂ healthy and overweight	β-glucan	VAS (0-240mins)	0, 2.68	1 day	Oatmeal	Satiety ↑ & ↓ energy intake
		Wheat bran					
Weickert *et al.,* 2006	14 healthy ♀	Wheat bran	equilateral seven-point rating scale (0-300mins) + gut hormones	0, 10.5	1 day	Bread	No diff in satiety

Lyly *et al*., 2009	19 ♂ ♀	Wheat bran	10 unit graphic intensity scale (0-120mins) + viscosity	0, 10.5	1 day	Drink	Satiety ↑
Freeland *et al*., 2009	19 ♂ ♀	Wheat bran (Fibre One)	VAS (0-120mins)	0, 41	1 day	Drink	No diff in satiety, ↓ energy intake

Willis *et al*. reported that corn bran and RS enriched muffins containing 9.6g and 8.0g fibre respectively, stimulated a significant reduction in the desire for food intake compared with the low fibre (LF) muffins (P=0.025) and (P=0.009) respectively) (Willis *et al*., 2009) RS muffins also kept subjects significantly less hungry compared to baseline at 120 minutes and fuller and more satisfied at 180 minutes. An effect of treatment was therefore observed with RS over time compared to control. In a similar acute crossover study, Anderson *et al* reported higher satiety scores at 30 minutes following placebo (maltodextrin), which corresponds to the greater increase in blood glucose concentration (P=<0.00) (Anderson *et al*., 2010). The high glycaemic load of maltodextrin is likely to have initially induced satiety according to the glucostatic theory (Mayer, 1996), however as maltodextrin is highly digestible, satiety was short lived. Glucose levels are tightly controlled as the brain relies on glucose as its main source of energy so multiple sites in the body are able to detect glucose and relay this to the brain through neuronal pathways affecting energy intake (Marty *et al*., 2007), therefore acute satiety signals are directly affected by glucose and subsequent insulin stimulation. In a review by Baggio *et al* satiety hormone GLP-1 was found to peak 20-30 minutes after eating (Baggio and Druker, 2007), therefore maltodextrin in this study may have induced gastric stimulation of GLP-1. However, at 120 minutes RS was reported to elicit a greater effect by reducing FI, indicating a different mechanism of satiety being exerted at this time point. In a small study in pigs (n=10) the satiety hormones GLP-1 and PYY did not rise following consumption of RS (Souza da *et al*., 2014), instead decreased postprandial glucose and insulin responses were reported and therefore the most likely mechanism of satiety is via delayed gastric emptying, which may account for the reduced food intake in both Willis *et al* and Anderson *et al.*

This hypothesis is supported by the observations of Hoffman *et al.* who fed 22 volunteers 15g of unripe banana flour (RS2) daily for six weeks and observed a significantly lower postprandial insulin response compared to maltodextrin placebo over the whole acute study period (P=0.029) (Hoffmann *et al*., 2016). Attenuated insulin release has been reported in other RCTs testing the effects of RS consumption (Behall *et al*., 2006, Klosterbuer *et al*., 2012). Hoffman *et al* additionally reported a post prandial increase in circulating peptide YY (PYY), which could explain reduced feelings of hunger following breakfast at 180 mins (P=0.026) and

increased satiety (P=0.044) at 180 mins. Significant reductions in food intake were also reported following consumption of a snack (P=0.033) and lunch (p=0.012).

Harrold *et al.,* 2014 also reported that both 20g and 30g doses of RS reduced subjective hunger significantly 3h post breakfast, however the 30g dose exerted further effects at 2 and 3hrs post lunch by reducing food intake, indicating a dose dependant effect. As reported by Anderson *et al*., 2010, Bodinham *et al.,* 2009*.* and Hoffman *et al*., 2016, the satiating effects of RS consumption may be due to improved glucose metabolism. Post absorptive effects of RS again were not measured in this study but may have been implicated in reduced food intake during dinner.

Contrary to the results of Anderson *et al*., 2010, Willis *et al.,* 2009, Hoffman *et al*., 2016 Harrold *et al.,*2014 whom all reported increased satiety effects in Bodinham *et al.,* 2010 study, following consumption of 24 g of RS at breakfast and again at lunch there was no increase in satiety, however food intake was significantly reduced during the study period (P=0.033) (Bodinham *et al*., 2010). Viscosity may have impacted satiety scores as the method of administration was a mousse which is fairly viscous, and in fact the intervention was liquid and placebo semi-solid, the gelling effect of which is likely masked any satiety effects of RS metabolism due to delayed gastric emptying associated with viscosity (Marciani *et al*., 2001).

Results from studies cited here indicate that 5g/day of RS is sufficient to test induced satiety effects and improved glucose homeostasis was observed; however further improvements may be feasible with greater levels of intake. All of the studies cited tested acute satiety with some chronic intervention, however the potential prebiotic effects of including RS in the diet and its effect on energy intake and satiety should be further evaluated using gut microbial analysis techniques such as fluorescent in situ hybridisation (FISH) in order to determine if a modification of gut microbiota over long term supplementation of RS can impact satiety mechanisms. However, it can be concluded that RS reduces perceptions of hunger, increased fullness, as a measure of satiety as well as reducing prospective food consumption when administered acutely. Prebiotic fermentation has been shown to induce increased circulating satiety hormones in animal, human and *in vitro* research **(Table 2)**.

Table 2: Animal and human studies demonstrating the stimulation of satiety hormones GLP-1 & PYY and reduction in food intake through prebiotic supplementation.

Author, Publication	Model	Fibre Dose/Treatment	Study Design	Food Intake	Outcome
Animal:					
Cani *et al*., 2005	Groups of adult male rats	OFS enriched diet (10%), high fat diet (HF) enriched or not with OFS (10%)	OFS 35d, then HF (15d): Satiety hormones measured (GLP-1 & PYY) at end (50d)	Energy intake ↓ with OFS enriched HF diet (p<0.05)	↑ GLP-1 in proximal region, portal vein GLP-1 doubled with HF OFS rats (p<0.05) no effect in distal region.
Delmee *et al*., 2006	Groups of adult mice	OFS enriched (10%) plus 2 HF diets (HF-1 50g/100g and HF-2 36g/100g)	OFS (21d), then HF-1 or HF-2 (28d): Plasma satiety hormones measured (GLP-1 & PYY) at end (50d)	Energy intake ↓ with 0.6g/d with OFS enriched HF-1 diet (P<0.05), no diff with HF-2	GLP-1 ↑ (66%), 100% ↑ in GLP-1 in proximal colon of mice on HF OFS
Reimer *et al*.,2012	Male rats	High prebiotic fibre (HF-21% wt-wt), or high protein (HP-40% wt-wt), high fat/sucrose (HFS-40%-45%).	HF weaning, then HFS (6wks) then C, HF or HP (4 wks): plasma satiety hormones measured at end (28d)	Energy intake ↓ (P=0.03). Final 3wks ↑ energy intake on HP and control compared to HF (P=0.05)	HF diet: ↑GLP-1 & PYY (P=0.05) compared to C & HP
Da Souza *et al*., 2014	Weaning pigs	35% pregelatinized starch (PS) and 34% retrograded starch (RS)	Crossover - PS or RS (14d): Satiety hormones measured at the end of each treatment	Energy intake ↓ by 3% with RS (P<0.00) compared with PS diet	GLP-1 ↓in RS compared with PS (P<0.001)
Zhou *et al*., 2015	Wild type (C57BL/) & GLP-1R knock out (KO) mice	Control or 30% RS diet	Control or RS(10wks): single intraperitoneal injection of saline or PYY receptor antagonist at end	Energy intake ↑ with RS (P < 0.05) compared with control	RS ↑ GLP-1 & PYY above detection unlike C. Proglucagon and PYY ↑ RS-fed mice (p < 0.01)

Kaji *et al*., 2010	Rats	Control: NDC free diet Treatment: 5% FOS diet for 28 days	28d followed by FFA2-, GLP-1-, enteroendocrine cells quantified immuno-histochemically in the colon, cecum, and terminal ileum.		↑ density FFA2+ enteroendocrine cells in rat proximal colon doubled compared to control. FOS supplementation ↑ number of FFA2+ L cells in proximal colon
Human:					
Cani *et al*., 2006	Five males and five females (21–39)	16g/d OFS or 16g/d control maltodextrin	Crossover - OFS & C (2wks) or C with 2wk washout: FI measured *ad libitum* meals	Energy intake ↓at breakfast (P=0.01) & lunch (P=0.03). Total energy intake ↓ 5%/d during OFS treatment	Satiety ↑, ↓ energy intake
Greenway *et al*., 2007	7 obese, 7 healthy weight	4g/d Viscofibre (oats/barley)	Weight loss (16wks): PYY & GLP-1 measured 1hr before & after standard meal test (Wk14)		Fasting PYY ↑ (P<0.05), fasting GLP-1 ↑ (P<0.01) (wk14). Weight reduced 3.07 +/- 3.13 kg (P <0.05) over 16 weeks.

Oligosaccharides

Oligofructose is a linear mix of fructose oligomers linked by β (2—1) bond and at the end of each fructose chain is a glucose molecule linked by α (1—2) bond, with an average chain length, or degree of polymerization (DP) of 10 (Niness, 1999). Four out of the five studies reviewed here were a crossover or parallel design to investigate the satiety inducing effects of chronic consumption of oligosaccharides using VAS additionally satiety hormones were measured in two of the studies **(Table 1).**

No significant increase in satiety scores were reported in the 360 minutes following consumption of OFS at breakfast and lunch at the end of a 12-week weight loss study Parnell *et al*., however circulating plasma PYY did increase by 13% (P=0.03) whereas the hunger hormone, ghrelin, decreased by 23% (P<0.00) by day 14. The subjects, though healthy, were overweight at baseline; low grade inflammation associated with increased adiposity has been shown to dysregulate appetite by blunting the response of satiety signals, which may have occurred here. Adam *et al.,* reported blunted GLP-1 response in 28 obese subjects compared to 30 lean subjects (P=0.03) following supplementation of 50g galactose or guar gum based test meal (Adam and Westerterp-Plantenga, 2005).

Parnell *et al.,* additionally reported a significant 29% reduction in food intake by week 6 (P< 0.00) via 3-day food records completed by the volunteers, which corresponds with the 1.03 ±0.43 kg weight loss in the OFS group at end of the study period. There is accumulating evidence of the fermentation of prebiotic fibres by commensal bacterial groups such as bifidobacteria, leading to production of SCFAs, integral to the mechanisms of appetite regulation, through stimulation of satiety hormones, particularly GLP-1 and PYY, implicit in reducing food intake and increasing satiety **(Table 3)**.

Table 3: Animal, human and *in vitro* research demonstrating stimulation of satiety hormones: GLP-1 & PYY by SCFAs.

Author, Publication	Model	Treatment	Study Design	Outcome
Animal:				
Lin *et al.,* 2012	Wild type & FFAR3 KO mice.	Acetate, propionate, and butyrate - 400mg/kg	Plasma gut hormone GLP-1 & PYY levels measured following oral SCFA challenge at 10mins - 1hr	Butyrate ↑ GLP-1 & PYY at 10 mins post prandial, however acetate did not ↑ GLP-1 & PYY. Propionate ↑ GIP, insulin, and amylin, but not GLP-1 or PYY.
Psichas *et al*., 2015	Wistar rats, male wild type (WT) mice & FFA2 knockout (KO)mice	A single intra-colonic propionate (0.45mmol) or Saline control	Jugular vein blood samples were collected at 60 mins post injection & single blood sample taken from the portal vein at 15 min.	Propionate ↑ GLP-1 (P=0.02) & PYY (P=0.02) in rats (GLP-1 peaked at 30mins, PYY at 60mins). In WT mice GLP-1 & PYY ↑ (P<0.05). No ↑ in KO mice
Human:				
Chambers *et al*., 2014	60 overweight adults	10g/d inulin (I) or 10g/d inulin-proiponate ester (IP)	Parallel, 24wks. Energy intake and plasma PYY and GLP-1 concentrations measured at baseline and post treatment	IP ↑ plasma GLP-1 & PYY (p<0.05)
in vitro:				

Brown *et al*., 2003	Mammalian cells (calf)	Control cells: transiently transfected with hGPR40 or the μ-opioid receptor. Or HEK293 cells transiently transfected with hGPR43	HEK293 cells transiently transfected with hGPR43 & loaded with Ca+ sensitive fluorescent dye Fluo-4 & intracellular [Ca+] measured using FLIPR.	Acetate ↑ in Ca+ whereas control cells did not.
Tolhurst *et al*., 2010	3 mth WT mice (C57B/6) & FFA2/3 KO mice	Control: 10 mmol/L glucose only or addition of acetate, propionate or butyrate (1 mmol/L)	Bowels of WT & KO mice to produce mixed cultures to detect GLP-1 stimulation in 2hr incubation	GLP-1 secretion in WT cultures but not KO cultures, propionate stimulated GLP-1 reduced by 70% (P= 0.001). Response to acetate abolished (P<0.00).
Chambers *et al*., 2014	Cultured gut model	Inulin-propionate (IP) ester and inulin	Gut model used to test inulin-propionate (IP) ester compared with inulin alone	Propionate ↑ PYY threefold above basal (P>0.00) & GLP fourfold (P<0.00)
Psichas *et al*.,2015	Adult male C57BL6 & FFA2 KO mice	Supplemented with 0.1% free fatty acid (bovine)	Colonic crypt cultures used to assess effects of propionate on satiety hormones release in vitro, followed by gut hormone secretion experiments	(1–50 mmol l−1) Propionate ↑ production of PYY & GLP-1 In wild mice and was significantly attenuated in cultures from FFA2-/- mice

In a pilot study carried out by Cani *et al.,* it was reported that following chronic consumption of 16g/d OFS for 2 weeks, satiety was increased at breakfast (P<0.00) and dinner. Whereas food intake was reduced at breakfast (P=0.01) and lunch (P=0.03) but not dinner (Cani *et al*., 2006). Conflicting with results Cani *et al* reported in his 2009 study, in which no increase in satiety was reported following 16g/d for 2wk OFS consumption. Both of these studies were small (n=5 per gender) and are likely to have been underpowered; according to Flint *et al* between 12 and 18 single gender subjects are necessary to detect a 5mm difference in self-reported satiety scores at 4.5h (Flint *et al*., 2000). Furthermore, the control breakfast appears to be 150Kcals greater than the OFS breakfast. Increased energy density can improve satiety scores, by increasing the transit time and therefore the rate at which nutrients reach receptors involved in satiety, and this impacts the amount of food consumed directly. This was demonstrated by Kissileff *et al* in food loading experiments, conducted in which eating rate corresponded with gastric distention (Kissileff *et al*., 1984) Though chronic consumption of OFS did not increase in subjective satiety scores in Cani *et al* 2009 study, GLP-1 and PYY production was significantly increased following (P=0.007). Disparity between subjective satiety scores and objective measurements in appetite studies is prevalent and an issue when it comes to designing studies in the future (Lesdema *et al*., 2016).

Similarly, Hess *et al* observed no difference in satiety scores following the consumption of 0, 10 or 16g OFS, in in 10 males and 10 females, in an acute setting. The female group did report reduced food intake with 8g/d dose, however the males did not. A study of 22 men and women investigated whether the brain responds differently to satiety and is gender specific, and using blood flow as a cognitive marker for neuronal activity, reported that possible differences in cognitive and emotional processing of hunger and satiation can occur (Del Parigi *et al*., 2002). A later study by Wang *et al* of 13 women and 10 men observed that only male participants could suppress metabolic activation in the amygdala when exposed to an appealing food and asked to suppress the desire to eat (Wang *et al*., 2009) the amygdala is an area of the brain that is thought to be involved in satiety regulation and motivation to eat(Farr *et al*., 2016). Heap *et al.,* may have missed the relevant temporal satiety response as VAS scores were only measured for 180mins following consumption of 6g/d OFS for 8 days.

Though lower desire to eat, prospective food consumption and increased satiety were reported, none were significant. Longer term studies appear to have better impact on satiety scores than acute studies following consumption of OFS (Heap *et al*., 2016).

Unlike more complex polysaccharides that elicit physiological mechanisms of satiety, OFS is a short chain oligosaccharide and is thought to be more rapidly fermented, in the proximal region of the colon, thereby exerting a prebiotic effect on fibre degrading bacterial groups such as bifidobacteria which produce SCFAs. The mechanism OFS impacts satiety may be through exerting prebiotic modification on bacterial groups to increase SCFA production and stimulate satiety hormones. Previous research suggests that up to 3 weeks supplementation is required to observe significant prebiotic induced microbial changes therefore longer term, properly powered studies would be required (Macfarlane *et al*., 2006). Cani e*t al.,* points out that acute prebiotic supplementation does not necessarily impact appetite regulation and that longer term adaptive effects need to take place to observe more pronounced and significant effect of prebiotics with satiety. The very small sample size in both of Cani *et al.,* studies (n=5 single gender subjects) and Hess *et al*., (n=10 single gender subjects) could have mitigated any acute satiating impact of OFS as well as short duration of supplementation, which would not have considered any prebiotic induced increase in satiety mechanisms.

Polydextrose

Polydextrose is a non-digestible oligosaccharide comprising glucose monomers randomly linked, whilst containing small amounts of sorbitol and citric acid. As a low molecular weight compound, it is also low in energy density (1kcal/g) (do Carmo *et al.*, 2016). Five randomized, controlled human studies investigating the satiety inducing effects of polydextrose (PDX) were identified under acute conditions with VAS as the primary method of measuring subjective satiety scores (**Table 1).**

King *et al.,* reported no significant increase in satiety scores in 8 healthy weight men using VAS, following chronic consumption of 12g PDX in the form of yoghurt. It may be that the study was underpowered (Flint *et al*., 2000), and highlights the importance for properly powered intervention studies to observe desired effects. However, it may also be that the viscosity of the yoghurt potentially masked any additionally satiety. Viscosity has been

reported to increase subjective satiety through reduced gastric emptying, leading to increased stimulation of gastric receptors that can trigger production of satiety hormones. Ranawana *et al.,* study was larger, consisting of a population of 26 males, so unlikely to have been underpowered, however Ranawana *et al.,* also observed no significant increase in satiety following consumption of 12g PDX in 26 healthy males. As the method of administration was a viscous 400g smoothie, this may have skewed results, as well as the short duration of the study of one day, which may have been insufficient. Conversely, Hull *et al.,* study did report increases in subjective satiety when yoghurt enriched with 0, 6.25, 12.5g PDX was consumed by 24 healthy weight men and 10 women over the course of one day. Consumption of 12.5g PDX induced a significant reduction in FI during the *ad libitum* lunch (P=0.022), whereas consumption of 6.25g PDX additionally significantly increased subjective satiety following the *ad libitum* lunch (P=0.02). Hull *et al.,* concedes the viscosity of the 6.25g PDX enriched yoghurt was greater than control or 12.5g PDX enriched yoghurt. Therefore, the different rates of transit could account for the altered response to subjective satiety and FI, however unlike King *et al.,* and Ranawana *et al.,* significant increases in satiety were reported.

Ranawana *et al.,* study reported no significant increase in satiety scores in 26 healthy weight males, only significant reduction of food intake following an *ad libitum* lunch (102Kcal). Hull *et al.,* study however reported both food intake was significantly reduced (52.14Kcal) during the *ad libitum* lunch, as well as a significant increase in satiety scores in 24 healthy females and 10 males. As both studies included a similar population size, dose and administration of PDX, it may that gender differences might be the reason for the disparity in results.

Konings *et al.,* and Olli *et al.,* tested the satiety inducing effects of administering 15g PDX to obese participants in an acute setting (Konings *et al*., 2014, Olli *et al*., 2015). However, whereas Konings *et al.,* reported significant increases in satiety scores following intervention (P<0.05) in women, Olli *et al* did not. Increased adiposity could account for blunting the response to satiety hormones, which can skew results and has been reported in previous studies (Delgado-aros *et al*., 2004). Gender differences in the effect of satiation have been reported ((Del Parigi *et al*., 2002), which could account for the disparity in results between men and women within Koning *et al*., study. The study duration may have also impacted results here, as 4/5 studies cited, only tested acute satiety over the period of 1 day, and

therefore any post absorptive satiety mechanisms would not have been considered or reported.

The evidence from the dietary intervention studies indicates that at higher doses (12.5g-25g) PDX triggers a reduction in food intake and increased satiety. The growth stimulatory effect of dietary PDX on colonic bifidobacteria (Probert *et al.,* 2004) might induce satiety signals mediated through an increase in SCFA production during fermentation. Of the dietary interventions with PDX reviewed here, all used VAS to measure satiety following intervention in the absence of other measures some show an increase in feelings of satiety relative to control and there is weak evidence linking satiety response to colonic fermentation.

β-glucan

Beta-Glucan (β-GLU) is partially soluble depending on linkages, branching and molecular weight. β-GLU from oat and barley comprise of linear polysaccharides joined by 1,3 and 1,4 carbon linkages. Most cereal β-glucan bonds consist of 3 or 4 β-1,4 glycosidic bonds. Eight randomised, controlled, blinded human satiety studies with β-GLU were identified: (Kim *et al*., 2006, Vitaglione *et al*., 2009, Lyly *et al*., 2009, Beck *et al*., 2009, Juvonen *et al*., 2009, Pentikainen *et al*., 2014, Rebello *et al*., 2014) and of these studies, four reported significant increases in perceived feelings of satiety following consumption of β-Glu in doses 3-30g, relative to control. All except Beck *et al.,* were acute in design and VAS was used to measure subjective satiety in all six studies between 120-560min, two studies additionally measured circulating satiety hormones PYY and GLP-1 as well as hunger hormone ghrelin.

Satiety scores were significantly increased ($P<0.05$) following consumption of β-GLU at 3-30g/d in healthy weight males and females (Vitaglione *et al*., 2009, Rebello *et a*l., 2014, Pentikainen S *et al*., 2014). Lyly *et al* however, did not report an increase in satiety scores in 19 healthy weight males following consumption of 10.5g/d β-GLU. VAS was completed for just 120min following consumption, the shortest duration of the studies cited, and therefore may not have been long enough to observe any increase in satiety. Conversely, Vitaglione *et al* did report significant increases in satiety as well as increased fullness at 120mins ($P>0.05$), so it may be that Lyly *et al.,* study suffered from "carry over effects." An effect from one treatment that "carries over" to another and can occur if the washout is insufficient between treatments. Lyly *et al*., reported "at least one day between sessions" however, given the 10.5g

dose of β-GLU, it is possible that one day was not long enough for washout in this crossover design.

Using a crossover design, Pentikainen *et al.,* 2014also investigated the satiety inducing effects of differently administered oat β-GLU in 30 healthy weight women (Pentikainen *et al*.,2014): β-GLU enriched biscuits and enriched juice (EBEJ) and biscuits and enriched juice (BEJ) elicited the greatest and significant increase in satiety in 180mins compared with enriched biscuits and juice (EBJ), and biscuits and juice (BJ) and it may be that adding β-GLU is more efficacious at inducing satiety in a liquid form than solid, in this study. Food composition impacts on viscosity, and during an *In vitro* investigation of the 4 different meals, increased viscosity was reported following the transit from stomach to large intestine. EBEJ had a starting viscosity of 19.89pas in stomach increasing to 24.9pas in the intestine. EBJ and BEJ had a much lower viscosity at 2.92pas and 2.11pas respectively and increased to 8.16pas and 6.29pas in the intestine. The viscosity is far greater in EBEJ than the other interventions. Therefore viscosity or mode of administration can impact satiety as observed by Juvonen *et al.,* who explored the satiety inducing effects of modified oat bran consumption on 20 healthy weight participants with two iso-calorific drinks (300ml) of low viscosity (LV) β-GLU and high viscosity β-GLU (Lin *et al*., 2012). The LV β-GLU drink induced a greater postprandial satiety compared to HV β-GLU drink ($P<0.05$). Additionally, the satiety hormone Cholecystokinin (CCK) significantly increased ($P=0.03$), whilst ghrelin was significantly reduced ($P<0.00$) but only with LV β-GLU drink. The authors hypothesise this effect could be related to differences in the rate of gastric emptying, with LV eliciting greater acute effects due to a faster absorption rate. Satiety and plasma measurements may need to be taken for longer than 180 mins after consumption in to determine if the HV had a longer-term impact on satiety. A lower viscosity may liberate nutrients faster which could enhance short term satiety, however longer-term satiety may have been experienced with HV with nutrients being released more slowly which may have impacted prospective food intake at subsequent meals, as has been observed by Pentikainen *et al*.

Kim *et al.,*2006 administered 2g/d β-GLU to 19 obese women in an acute study over the period of 1 day, to investigate the satiety inducing effects, however there was no significant effect. The dose was quite low at 2g/d; additionally, the results could have been confounded

due to the increased adiposity of the study population, which can blunt satiety signals (Hellstrom, 2013). Beck *et al*., 2009 also reported no significant increase in subjective satiety following 3 months chronic consumption of 0, 6 or 9g/d β-GLU in 66 obese women. Circulating plasma PYY (P=0.018) and CCK plasma levels (P<0.00) were significantly increased however. Vitaglione *et al*., however, reported both increased satiety scores and circulating satiety hormones (PYY) after supplementing healthy weight participants with β-GLU at 3g/d. Again, this highlights how disparity between results can occur, and should be a consideration when choosing a study population within the study design.

β-GLU becomes gel like in the GI tract and may therefore increase distension and stretch receptors, in addition it is a fermentable substrate for saccharolytic flora. Variation in the physiological properties of β-Glucans may contribute to the varying outcomes of the satiety studies reported (El Khoury *et al*., 2012). However, as β-GLU is complex viscous polysaccharide exhibiting a slower fermentation rate, these acute studies could only investigate pre-absorptive and some post absorptive satiety mechanisms. Any increase in satiety mediated by gut microbial fermentation of the β-GLU, through prebiotic activity has not been measured. Prebiotic activity of β-GLU could account for increases in satiety hormones howeverβ-GLU has been reported to be a prebiotic candidate in human/animal studies carried out (**Table 2),** therefore carrying out longer term, chronic studies in which changes to microbial ecology and SCFA production are measured as well as gut hormones might be worthwhile. Increased SCFA is thought to impact appetite regulation by acting as a signalling molecule on endocrine cells in the colon, thereby stimulating satiety hormones (Baggio and Drucker, 2007).

Wheat bran

Wheat bran is a complex structure, composed of about 53% dietary fibre (xylans, lignin, cellulose, galactan and fructans (Onipe *et al*., 2015). Of the three randomised, blinded, placebo controlled human intervention studies included in this review, all were acute studies (Lyly *et al*.,2009, Freeland *et al*., 2009 & Weickert *et al*., 2006).

Only Lyly *et al.,* 2009 reported an increase in satiety following consumption of 10.5g/d wheat bran in an acute setting in 19 healthy women. Both Weickert *et al.,* 2006 and Freeland *et al.,*

2009 reported no significant increases in satiety following 10.5 and 41g/d consumption respectively. Freeland et al however did report a reduction in energy intake following consumption of 41g wheat bran, and Weickert *et al.,* 2006 reported an increase in AUC PYY *(*P=0.016), and a significant decrease in ghrelin (P<0.00). Again, a difference has been reported in results from measurement of subjective satiety and the objective measurement of both energy intake and satiety hormones. The duration could be a confounding factor as these are very short studies; however, using a subjective measurement such as VAS is limited and prone to error (Lesdema *et al*., 2016), such as individual variation in use of the line scale, however, using a crossover design can reduce this type of variability. It could be that the duration of the studies is too short. Initial satiety from wheat bran is likely to occur from gastric distention due the physical bulking and structure of wheat bran, any post absorptive effect on satiety has not been evaluated. Wheat bran has been shown have prebiotic effects, due to the xylan and inulin within the structure.

A summary of the prebiotic effects **(Table 4)** is reported below for each fibre reviewed based on the weight of evidence collated from the study results, highlighting those fibres that had the greatest impact on satiety mechanisms.

Table 4: Summary of prebiotic effects exerted during human appetite studies of: resistant starch (RS), oligofructose (OFS), polydextrose (PDX), β-glucan (β-GLU), wheat bran (WB). 10-20% of studies = ↑, 50% of studies = ↑↑, 90% of studies = ↑↑↑

Substrate	Influence on subjective satiety	Satiety hormones corrborate subjective satiety	Energy intake reduced (24h)	Glucose attenuated/insulin blunted
RS	↑↑↑	↑	↑↑↑	↑↑
OFS	↑	↑	↑↑	↑
PDX	↑↑	↑	↑↑	↑
β-GLU	↑↑↑	↑↑	↑↑	↑↑
WB	↑↑		↑	

The focus of all three studies was on testing the short-term satiety effects of wheat bran, and the results are conflicting. Potential confounders may have affected the results reported. Costible *et al.,* 2016 carried out a placebo-controlled, crossover, randomised study in which 31 healthy weight volunteers consumed 48g wholegrain cereal or wheat bran every day for 3 weeks. The results showed that faecal levels of bifidobacteria and lactobacilli were significantly increased, indicating a prebiotic effect. It could be that over time adaptive effects occur to positively impact satiety, such as increasing commensal bacteria to produce SCFAs that have been reported to impact on and impact satiety signals (**Table 3).** Therefore, more human studies are required with a chronic, long term aspect to more fully understand the satiety inducing effects and prebiotic effects of wheat bran. It might also be preferable to isolate some of the potential satiety inducing components of wheat bran such as lignin and xylans which have shown promise as a prebiotic candidate.

Conclusion

There is some evidence to suggest that intervention with fibre and prebiotic oligosaccharides may positively impact satiety through multi-mechanistic pathways; the results however, are conflicting, mainly because of the differences in study design, some of which are likely to be underpowered. Most of these studies are acute studies, focusing on short term benefits of prebiotic consumption, whereas properly powered chronic RCTS, would enable measurement of the prebiotic and adaptive effects of prebiotic consumption. It is difficult to measure subjective satiety, VAS is really the gold standard, but it has its limitations, however the validity of VAS is only as good as the study design it is used in and combining with objective measurements can increase it validity within a design. There is a complexity in measuring satiety as there are multiple mechanisms at work here. Perceived satiety scores are subjective, and do not necessarily corroborates actual energy intake, satiety or hormone production.

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Chapter 3:

General Methodology

Introduction

This thesis describes experimental investigations which may be divided into 2 parts: In the first phase of the study *in vitro* batch culture fermentations were used to investigate the metabolic fate and microbial effects of 11 individual fibres. The results from this preliminary screening exercise were used to develop 3 novel blends of prebiotics tested in a more realistic continuous colonic gut model system, again to ascertain metabolic and microbial responses. The second phase of the project involved a human appetite RCT, to test whether markers of prebiotic activity and satiety are improved in healthy weight men consuming a novel prebiotic blend. In this chapter we discuss the methodologies used in the project.

Prebiotics

Table 1: Physico-chemical properties and uses of Individual substrates tested in 48hr batch culture fermentations.

Substrates	Physio-chemical properties	Approach/process	Soluble or not?	Taste/Uses	Evidence of prebiotic potential - human studies
Long Chain fructo-oligosaccharides (LC-FOS) (Fine Foods)	DP 2-65 - β (2-1)-Fructans (Niness, 1999)	Direct extraction - from raw plant material (chicory root)	Yes	Slightly sweet - baked goods, drinks	Gibson *et al.,*1995, Kolida *et al.,*2007, Costabile *et al.,*2010
Oligofructose, (OFS) (Orafti P95)	DP 2-9 - β (2-1) Fructans (Niness, 1999)	Controlled hydrolysis - using enzymatic hydrolysis if inulin from chicory	Yes	Slightly sweet - baked goods, drinks	Gibson *et al*., 1995, Bouhnik *et al.,*2006, Knol *et al.,* 2005
Resistant starch, (RS) (Himaize 260 – Sigma potato starch)	DP 35-60 - 60% amylose, a helical polymer of α-D-glucose units and 40% amylopectin, a soluble polysaccharide comprised of highly branched polymers of glucose (Leszczyñski, 2004)	Enzymatic hydrolysis of potato starch	No	Bland, neutral - flour replacer baked goods, cereal, batter, cheese	Hald *et al.,*2016, Maier *et al.,* 2017

Resistant maltodextrin, R-MLX (Promitor T&L)	DP 5-20 - polysaccharide – α (1-4), α (1-6), a/b (1-2), and a/b (1-3) glucosidic bonds (Brouns *et al*., 2007)	Chemical modification of maltodextrin	Yes	Neutral to slightly sweet - sugar replacement in drinks and baked goods	Fastinger et al.,2008, Miyazato et al., 2016
Gluco-oligosaccharides, GLOS (Biocolians)	DP 3-6 - alternate α-(1,3)/α-(1,6)-linked glucosyl residues (Meyer *et al*., 2014)	Controlled enzymatic hydrolysis of D-glucose	Yes	Slightly sweet - baked goods, drinks	Limited human studies: Djouzi *et al.,* 1995 (rat study), Sarbini *et al.,* 2013 (*in vitro* study)
β-glucan, β-Glu (Glucagel)	DP-5-28 - glucose residues which are linked by β-(1–4) and β-(1–3) glycosidic bondsβ-glucans, are linear homopolymers of d-glucopyranosyl (Glcp) residues	Controlled enzymatic hydrolysis of barley grains, (1-3) & (1-4) β-glucan	Partially	Neutral flavour - frozen desserts, sour cream, and cheese spreads.	Mitsou *et al*., 2010, Wang *et al*., 2016

Oat Fibre, OAT (Fine foods)	DP-5-28 - glucose residues β-(1–4) and β-(1–3) glycosidic bonds β-glucans, are linear homopolymers of d-glucopyranosyl (Glcp) residues linked mostly via two or three consecutive β-(1 → 4) linkages that are separated by a single β-(1 → 3) linkage (Izydorczyk & Dexter, 2008)	Controlled enzymatic, hydrolysis from barley grains	Partially	Neutral - frozen desserts, sour cream, and cheese spreads.	Mitsou *et al*., 2010, Wang *et al*., 2016
Low viscosity arabinoxylan LV-ABX (Megazyme	linear chain backbone of ß-d-xylopyranosyl (Xylp) residues linked through (1 → 4) glycosidic linkages. α-l-Arabinofuranosyl	Controlled enzymatic, hydrolysed from wheat and rye	Yes	Neutral - animal feed, distilling	Costabile *et al*., 2008
Medium viscosity arabinoxylan, MV-ABX (Megazyme)	linear chain backbone of ß-d-xylopyranosyl (Xylp) residues linked through (1 → 4) glycosidic linkages. α-l-Arabinofuranosyl	Controlled enzymatic, hydrolysed from wheat and rye	Yes	neutral - animal feed, distilling	Costabile *et al*., 2008

Xylo-oligosaccharides, (XOS_ Santori	DP 2-6 - β (1–4)-Linked xylose (Zhou *et al.*, 2008)	Controlled enzymatic hydrolysed of polysaccharides; arabinoxylan	Yes	Slightly sweet - increases functionality in baked goods, uses varied	Okazaki *et al*., 1990, Na & Kim, 2007, Chung *et al*., 2007
Polydextrose, PDX (Danisco Sweeteners)	DP 3-60 - branched, randomly bonded glucose polymer combinations of α- and β-linked 1→2, 1→3, 1→4, and 1→6 glycosidic linkages, though the 1→6 (both α and β), (do Carmo *et al*., 2016)	Synthesised from dextrose, with 10% percent sorbitol, and 1% citric acid added	Yes	Slightly tart, replacement for sugar, starch, and fat in commercial beverages, cakes, candies, dessert mixes, breakfast cereals, gelatins, frozen desserts, puddings, and salad dressings	Jie *et al*., 2000, Boler *et al*., 2011

Arabinoxylan

Arabinoxylan (ABX) is a hemi-cellulose polysaccharide; it is found in the cell walls of plants such a cereal grains and is structured as a linear xylan backbone with α-L- arabinofuranose units attached as side chains by β (1 → 4) linkages. These can be substituted with arabinose residues on the C(O)-2 and/or C(O)-3 position. Ferulic acid is esterified on the C(O)-5 position of arabinose (Dornez *et al*., 2009) **(Table 1).** Colonic bacteria such as *Bifidobacterium* can utilise arabinofuranosidases to cleave these side chains (Saha, 2000), however the complete degradation of arabinoxylan requires the synergistic activity of several hemicellulolytic enzymes, such as β-1,4-endoxylanase, β-xylosidase, α-glucuronidase, α-L-arabinosidase, and acetylxylan esterase (Dodd and Cann, 2009, Rogowski *et al*., 2015).

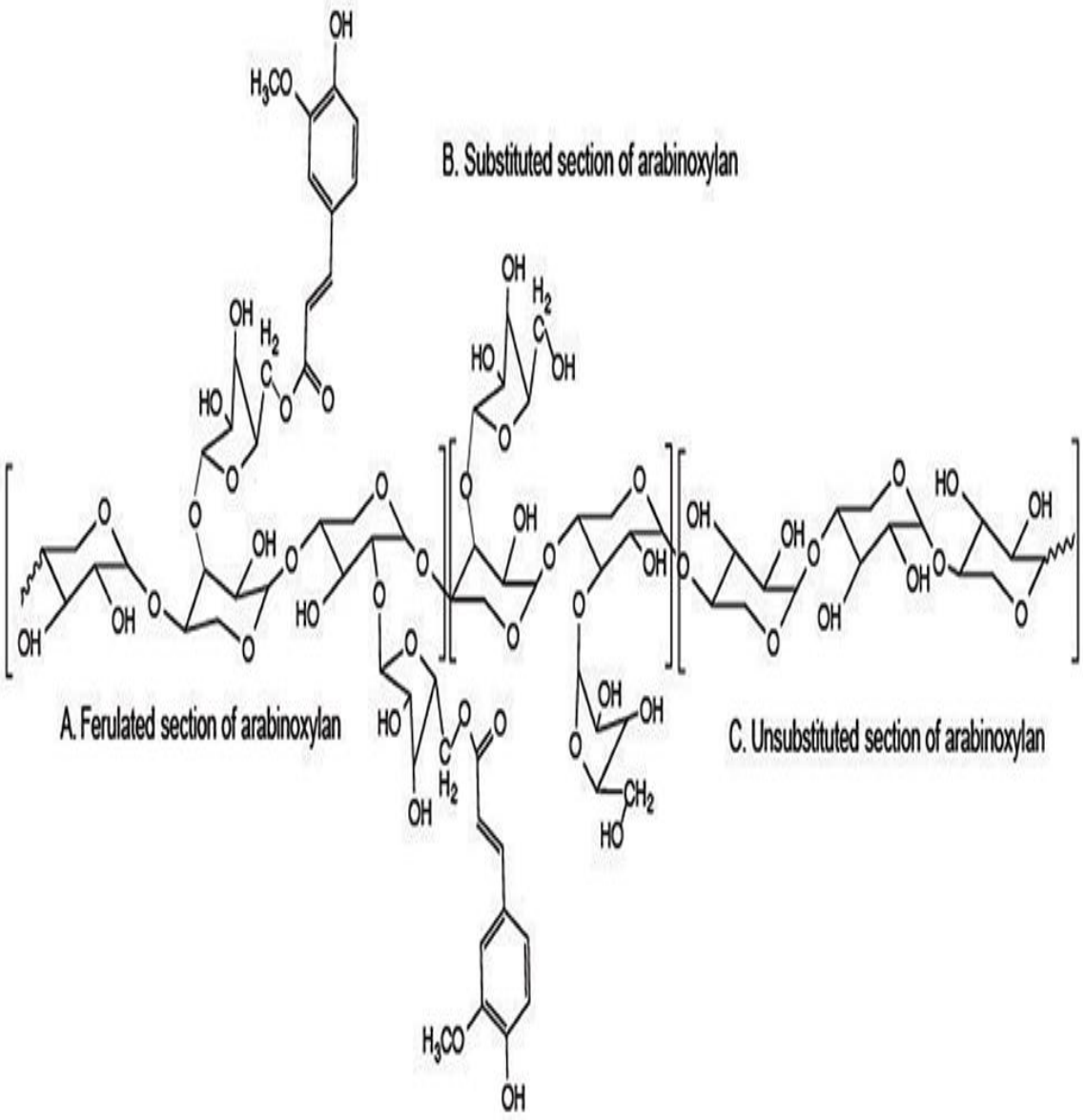

Figure 1: Chemical structure of arabinoxylan, a polysaccharide (Hatfield, 2017)

Inulin

Fructans are oligo- and polysaccharides composed of β-D-fructosyl residues (n = 2–60) connected with β-(2→1) linkages, often including a glucosyl moiety. Oligofructose is the shortest fructan with a chain length of <10 monomeric fructose units, whereas fructans >10 units (DP_{ave}= >20 units) are called inulin. Fructans occur naturally in chicory, banana and onion in small quantities (Mitmesser & Combs, 2017).

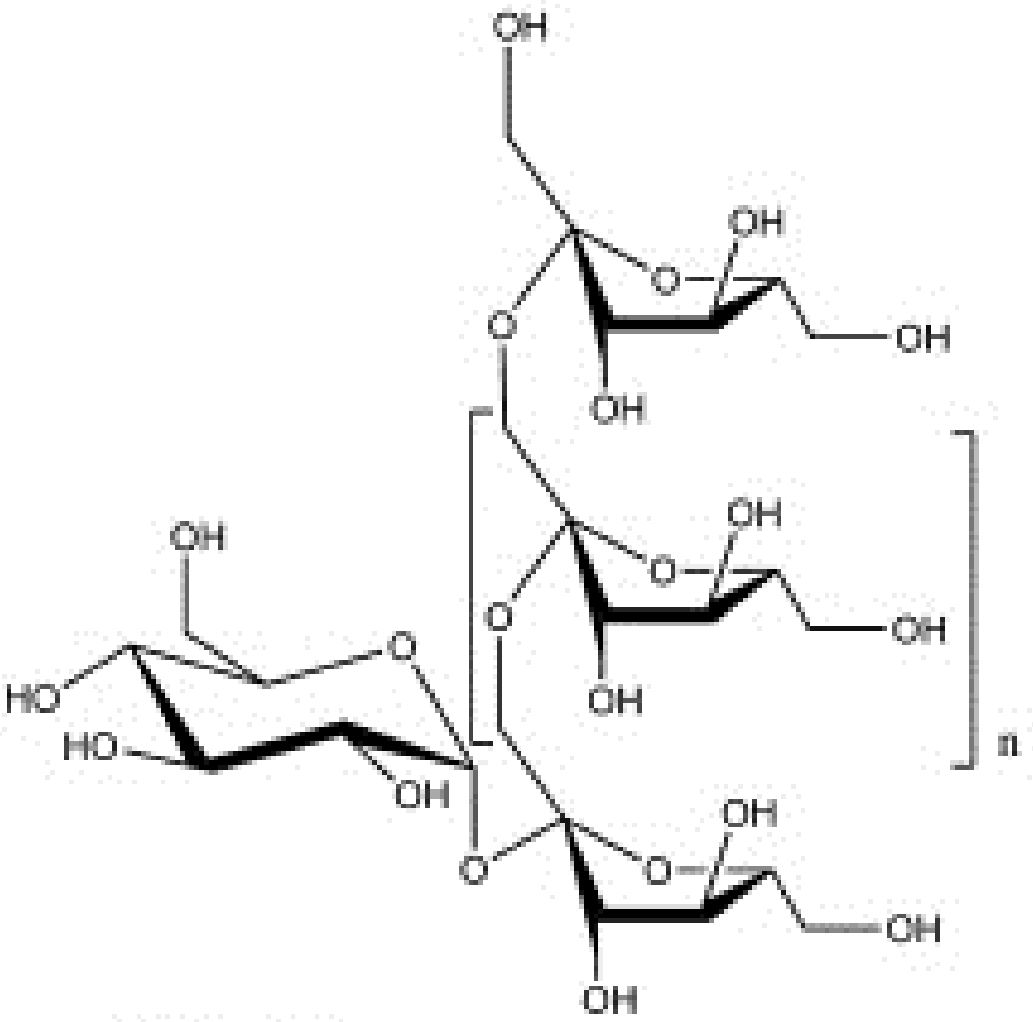

Figure 2: Chemical strcuture of inulin (Mensink, 2015)

Producing prebiotics

Extraction of prebiotics from plant sources such as chicory (*Cichorium intybus)* can yield inulin and FOS. Whilst modification by transglycosylation reactions can produce GOS. (Patel, 2012). Inulin is mainly isolated from chicory using hot water diffusion, purification, then spray drying. Inulin can also be enzymatically synthesised from sucrose via fructosyltransferase, which catalyses the transglycosylation reactions whereby sugar residues are transferred form one glycoside to another (Mitmesser and Combs, 2017). This process can also be used to obtain fructo-oligosaccharide (FOS), except there is an additional step, in which Inulin is hydrolysed by an inulase enzyme to reduce the chain length to <10. The resulting oligofructose product has ∼30% of the sweetness of sucrose and contains ∼5% glucose, fructose and sucrose (Niness, 1999). Galacto-oligosacchairdes (GOS) are produced by hydrolysing lactose using glycoside hydrolases, via transglycolation reactions. An enzyme commonly used in this process is β-galactosidase from *Aspergillus oryzae* (Torres *et al*., 2010).

Desirable characteristics of prebiotics in this research: <10% digestibility, resists hydrolyzation in upper GI tract, selectively stimulates commensal bacterial groups: *Bifidobacterium* and *Propionibacterium,* does not stimulate potentially pathogenic bacteria such as *Desulfovibrio* and *Clostridium histolyticum,* and stable at high temperatures such as cooking, as this increases the possible uses and increases commercially viability.

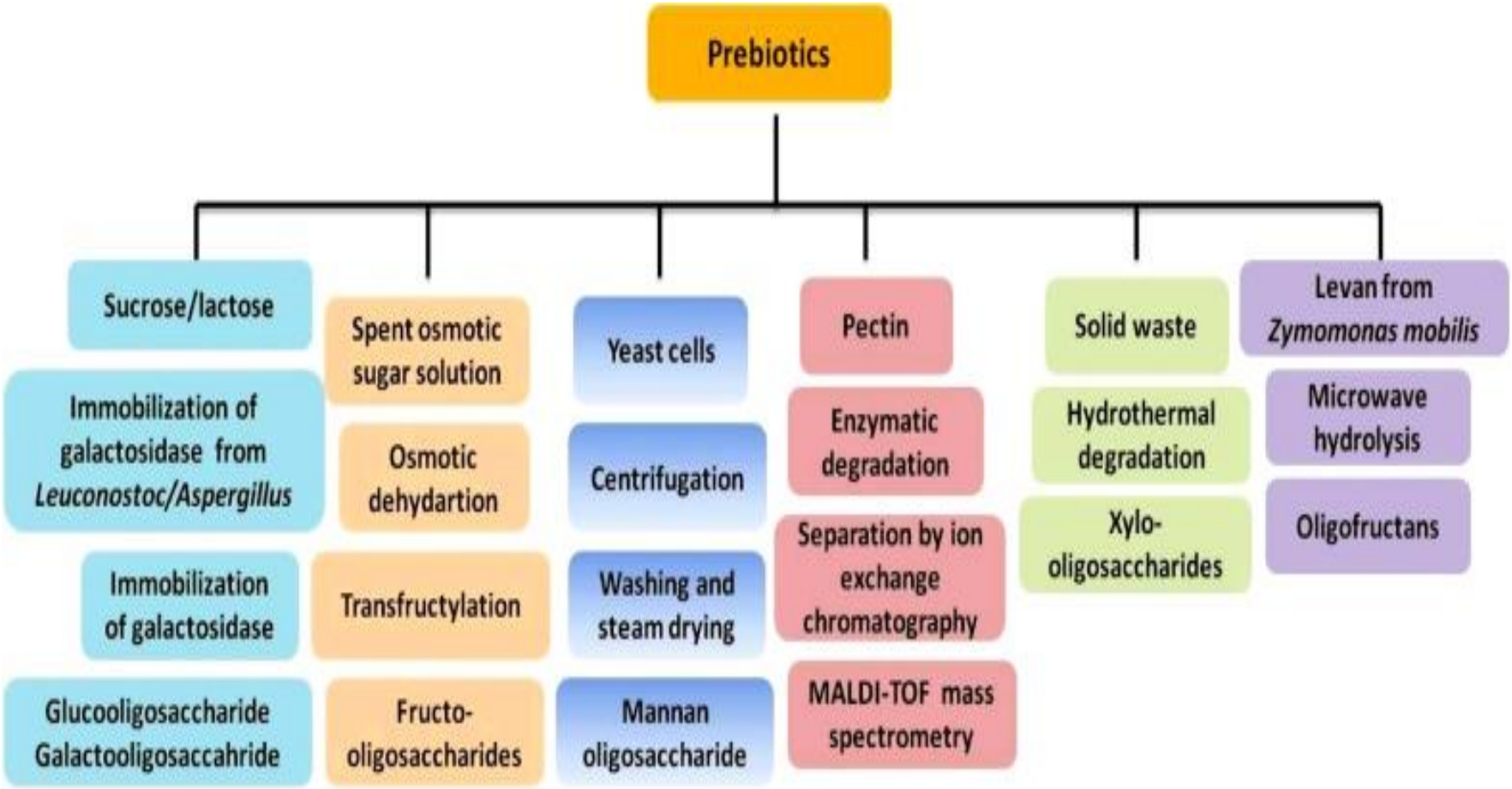

Figure 2: Different methods used to isolate or synthesise prebiotic (Patel, 2012)

Batch culture fermentation

The human colon is not easily accessible; obtaining samples from this region for the purpose of investigating fermentation processes of foods and ingredients is considered invasive, expensive and unethical (Booijink *et al*., 2007). Not only this, the colon is home to a large and complex ecosystem with multiple metabolic reactions occurring simultaneously and any attempt to isolate effects and identify diet-microbial interactions can be problematic.

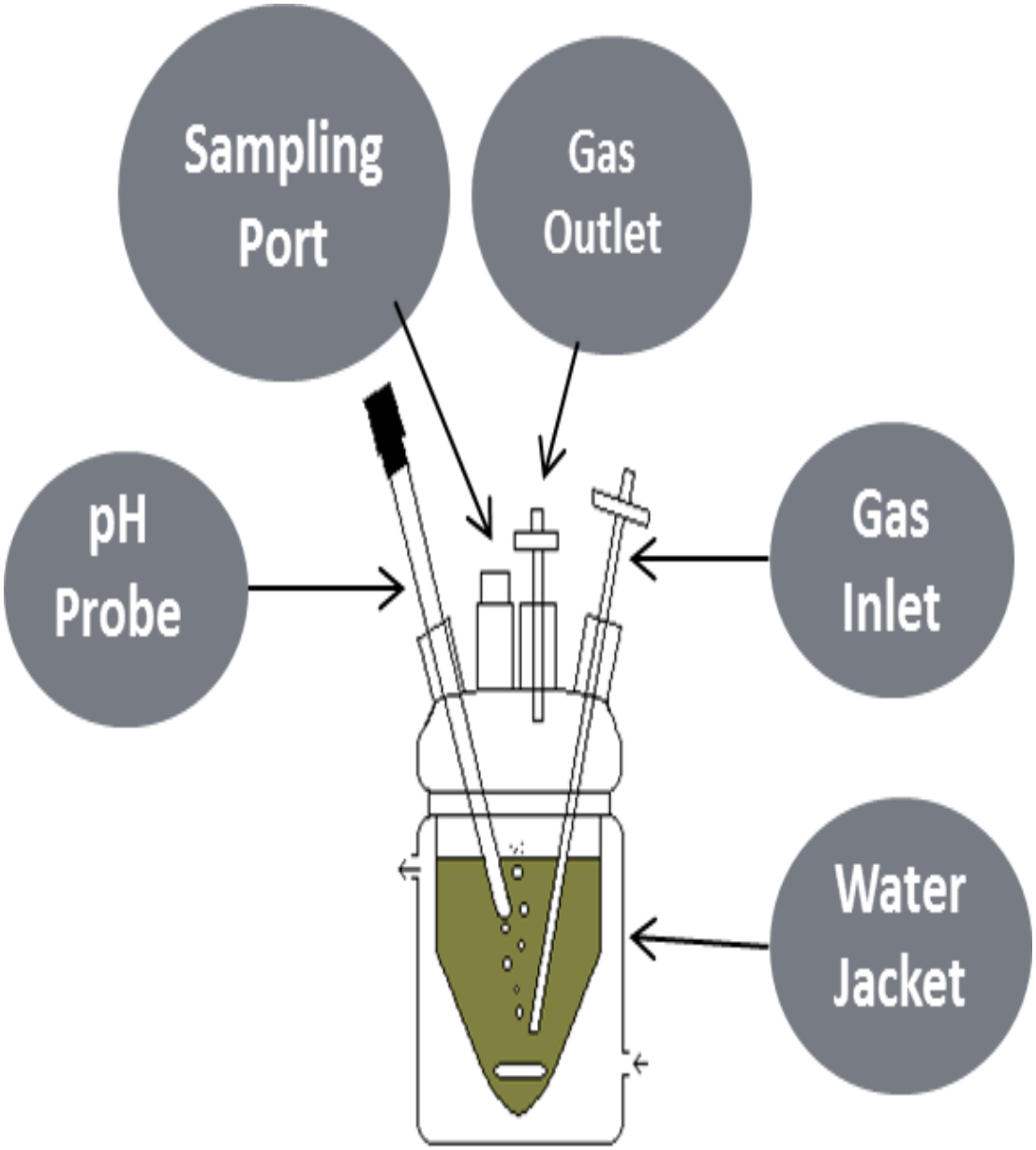

Figure 3: Schematic anaerobic batch culture vessel, with sampling port, pH controller to maintain a constant pH 6.8, N_2 outlet and inlet, and water jacket to control temperature to that of the human colon at 37˚C

In vitro batch culture fermentation with faecal inoculate is an inexpensive and well documented method used to gauge the metabolic fate of different foods and pharmaceuticals by identifying changes in gut microbiota composition and subsequent metabolic output (Khalil *et al*., 2014, Rycroft *et al*., 2001, Salazar *et al*., 2009, Vardakou *et al*., 2008). Conditions in batch culture are controlled, by maintaining a pH of 6.8, temperature at 37^{o}C, nutrients and oxygen, the conditions of the distal region of the colon can be simulated. The distal region of the colon is characterised by the relative dominance of proteolytic metabolism, compared to the caecum where saccharolytic fermentation dominates. The distal colon may also be more prone to disease due to an accumulation of the by-products of proteolytic fermentation (Smith and Macfarlane, 1996), increasing saccharolytic metabolism in the distal colon therefore may be beneficial to health, as it could help negate the negative effects of proteolysis. Though carbohydrate metabolism mainly occurs in the proximal region of the colon, some residual parts of larger chain fibres polysaccharides such as arabinoxylan and resistant starch may persist to be metabolised further along the colon. Similarly, shorter chain oligo-saccharides metabolised by bacterial groups such as bifidobacteria in the proximal region of the colon to produce lactate, the lactate can be metabolised by bacterial groups further along the colon, such as *Bacteroides* producing butyrate from lactate (Dodd *et al*., 2011).

As a closed system with no absorptive epithelial surface to account for absorption, amplification effects can occur in batch culture, particularly at the later stages of the culture. Thus an accumulation of intermediary, and end products of fermentation, can potentially overshadow the detection of metabolic products with health effects (Macfarlane and Macfarlane, 2007). We used this model to screen 11 individual substrates to ascertain any prebiotic potential over the course of 48 hours (**Table 1**). By sampling at timepoints 0, 24 and 48hr, the production of SCFAs were quantitatively measured, and bacterial growth assessed, thus allowing an approximation of the rate of fermentation to be reported.

Table 2: Major elements, their sources and function required for bacterial growth in batch culture.

Basal medium ingredient/source	Element	Function
Glucose, yeast extract	Carbon	Main component of bacterial cell, backbone of biological molecules
L-cystine, yeast extract, peptone water	Nitrogen	Synthesis of proteins, amino acids, DNA, and RNA
Water	Hydrogen	Main component of organic compounds and cell water
Yeast extract	Phosphorus	Nucleic acid synthesis
Magnesium sulfate ($MgSO_4$)	Sulfur	Component of coenzymes and amino acids: cysteine, methionine
Di-potassium phosphate (K_2HPO_4) & Monopotassium phosphate (KH_2PO_4)	Potassium	Component of enzymes, (inorganic cellular cation and co-factor)
Magnesium sulfate ($MgSO_4$)	Magnesium	Co-factor for enzymatic reactions (inorganic cellular cation)
Calcium chloride ($CaCl_2$)	Calcium	Component of enzymes, (inorganic cellular cation and co-factor)
Hemin	Iron	Co-factor for enzymatic reactions

Three faecal donors were chosen to inoculate the batch cultures; they were selected based on phenotype and age to reduce inter-individual variation between batches. Age-related changes in microbial composition, such as reduced bifidobacterial counts in older people, are common (Bartosch *et al*., 2004), and differences have also been observed when comparing the microbiota of lean with obese individuals (Andoh *et al*., 2016). Whilst we did control for age and body mass when selecting volunteer faecal donors, individual differences in the fermentation performance were likely due to variance in the composition of the initial microbiota population of the volunteer, leading to variable responses throughout the *in vitro* batch culture experiments. Further, an individual's gut microbiota does not necessarily respond in a consistent manner to a particular dietary intervention (Healey *et al*., 2016). Those with very low bifidobacterial counts at baseline, can be shown to have greater increases following prebiotic intervention than those with high baseline bifidobacterial counts (De Preter *et al*., 2008). Additionally, habitual fibre intake influences baseline gut microbiota composition (Cotillard *et al*., 2013), and this varying degree of consumption of dietary fibre may influence how the gut microbiota responds to the prebiotic intervention. In a human placebo-controlled study, healthy individuals with habitual high (HDF) and low fibre (LDF) intake consumed 16g Beneo Orafti Synergy for 3 weeks, or 16g maltodextrin. In both groups, bifidobacteria was increased (P=0.001), however the HDF group also showed increased *Faecalibacterium* (P=0.010), and decreased numbers of *Coprococcus* (P=0.010), *Dorea* (P=0.043) and *Ruminococcus* (P=0.032). Thus the HDF group exhibited a greater response to the intervention than those with LDF intakes (Healey *et al*., 2018).

It was considered desirable to observe an increase in acetate and propionate producing bacterial groups: *Bifidobacterium* and *Propionibacterium*, as well as increased concentration of their respective metabolites: acetate and propionate in the supernatant. This was based on hypothesised anorexigenic effects of these SCFAs, mediated via the stimulation of satiety hormones (Psichas *et al*., 2015, Karaki *et al*., 2006). Therefore, those substrates that elicited the greatest increase in acetate and propionate production through *in vitro* batch culture were deemed the most suitable candidate prebiotics. These substrates were then blended together and tested further in a three-stage continuous culture model of the gut.

Three stage continuous gut model

Capturing real time colonic microbial metabolism *in vivo* is challenging, however a 3-stage colonic system continuous culture fermentation system models changes to microbial ecology through the functional compartments of the colon.

Our three stage colonic fermentation model was previously validated against the luminal contents of the proximal and distal regions of 4 healthy sudden death victims *(*Macfarlane *et al.,* 1998*),* studies based on this model to screen for prebiotic activity have been widely published in the academic literature (Gibson and Wang, 1994, Gibson *et al*., 1995, Hobden *et al*., 2013, Liu *et al*., 2016*)*. This model controls environment (temperature, pH, transit time and substrate) allowing us to replicate the influence of substrate on the mixed culture system of a given faecal donor.

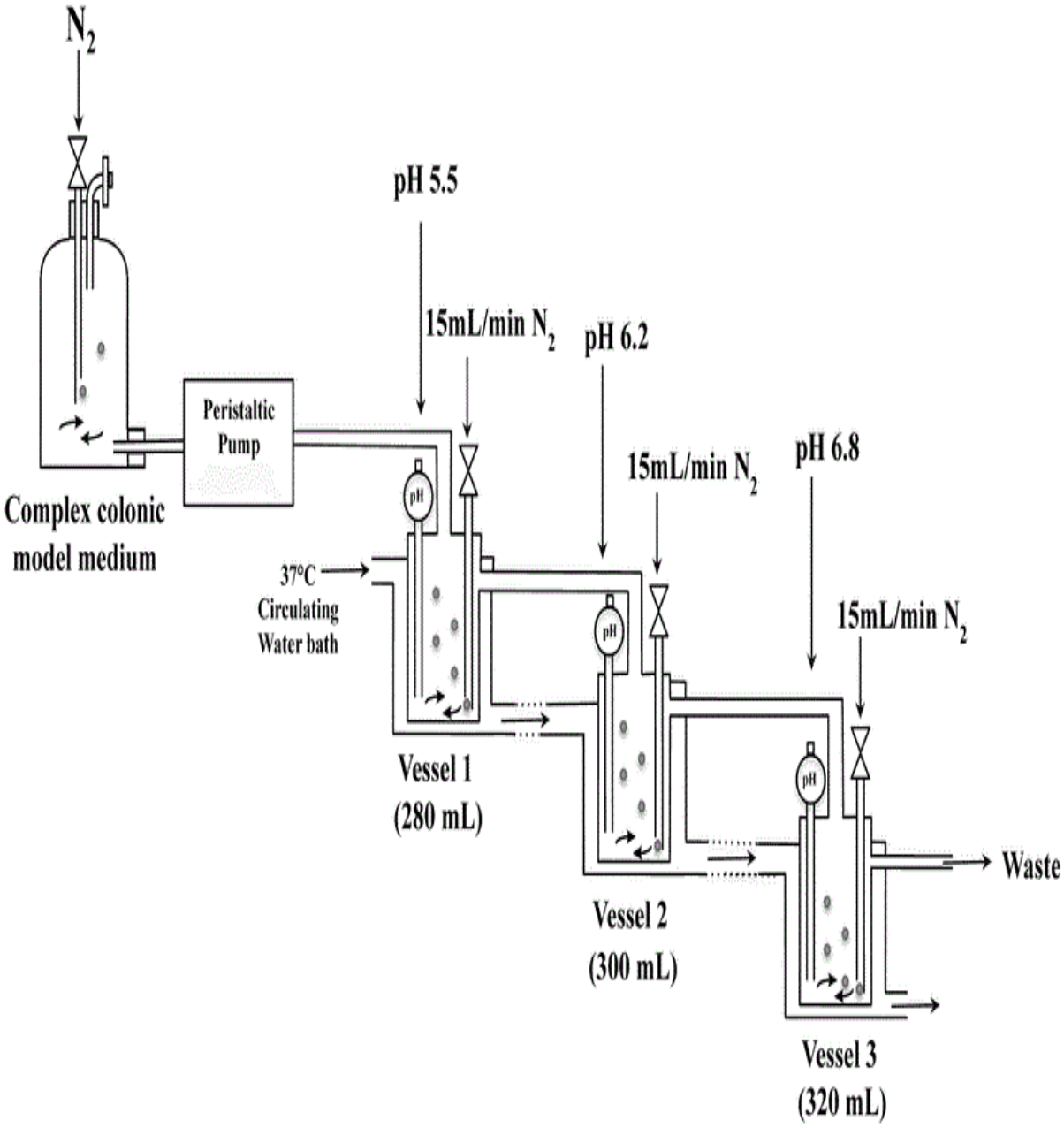

Figure 4: schematic representation of a 3-stage continuous colonic model inoculated with human faeces. The system comprises of a trio of connected vessel controlled to simulate conditions in each region by adding 1M HCl or NaOH as required, temperature maintained at 37°C using a water jacket and contents continually stirred using a magnetic flea.

Sampling

It was necessary to monitor SCFA production over 3 consecutive days to ensure the colonic model had reached equilibrium, a steady state in which bacteria are producing as much as they are consuming. Steady state must be reached before intervention occurs, this ensures that observed changes in activity are due to the intervention and are not just fluctuations in an equilibrating system. The sampling to establish the steady state began at day 16 of routine continuous culture. Once the intervention began, daily for another 15 days, samples were again taken over 3 consecutive days for SCFA analysis, to ensure the second steady state had been reached.

The aim was to identify a fermentation profile of all three blends (inulin + gluco-oligosaccharides (I+GLOS), inulin + long chain fructo-oligosaccharides, (I+LC-FOS), inulin + arabinoxylan (I+ABX), thereby determining the optimal blend to further test in a human RCT. It was desirable to observe an increase in SCFA production, *Propionibacterium* and *Bifidobacterium* growth towards V3, as this could indicate increased fermentation and possible additive prebiotic effects over inulin alone. It was hypothesised that blending fibres together would increase and prolong production of SCFA through *in vitro* fermentation compared with prebiotic oligosaccharides alone, thus also potentially sustaining anorexigenic pathways of appetite control, by acting as ligands onto the receptors of L-cells, located along the length of the colon, to stimulate a more sustained production of gut hormones such as GLP-1 and PYY, for a longer period, thereby improving satiety and satiation.

Bacterial Characterisation

A combination of fluorescent in situ hybridisation (FISH) and flow cytometry (FLOW) can help detect changes in microbial ecology from *in vitro* and *in vivo* samples (FISH-FLOW).

Fluorescent in situ hybridization (FISH) was chosen to assess microbial ecology as it is a quantitative approach. Fluorescent oligonucleotide probes are targeted at 16S rRNA **(Table 3)** and oligonucleotide probes are used during FISH for enumeration of target bacterial groups. 16S rRNA is a component of the 30S small subunit of prokaryotic ribosome (Kim and Chun, 2014) present in all bacteria in high copy numbers. It is a highly conserved genetic region (Chakravorty *et al*., 2007), and is a reliable method for identification of prokaryotic cells at the phyla level, independent of cultivability (Zwirglmaier, 2005). Unfortunately, within phyla level characterisation is not possible due to conservation of the 16SrRNA sequence. For this research genus and species level interrogation of the mixed culture was considered sufficient as the overall aim was to detect functional changes in microbial composition. RTqPCR is also robust, reproducible and sensitive method to quantitatively track functional gene changes in experimental conditions (Smith & Osborn, 2009). However, given the additional cost of setting up and using the equipment, it was decided the FISH-FLOW would be robust enough for this research.

Table 3. Oligonucleotide probes used during FISH for enumeration of target bacterial groups.

Probe Name	Target species	Sequence 5' to 3'	Reference
Non Eub	Non bacteria	ACTCCTACGGGAGGCAGC	Wallner *et al.,* 1993
Eub338‡	Most bacteria	GCTGCCTCCCGTAGGAGT	Daims *et al*., 1995
Eub338II‡	Most bacteria	GCAGCCACCCGTAGGTGT	Daims *et al*., 1995
Eub338III‡	Most bacteria	GCTGCCACCCGTAGGTGT	Daims *et al*., 1995
Bif164	*Bifidobacterium* spp.	CATCCGGCATTACCACCC	Langendijk *et al*., 1995
Lab158	*Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis;* all *Enterococcus, Vagococcus, Melisococcus, Catellicoccus, Tetragenococcus, Pediococcus* and *Paralactobacillus* spp.	GGTATTAGCAYCTGTTTCCA	Harmsen *et al*., 2002
Bac303	Most *Bacteroidaceae* and P*revotellaceae*	CCAATGTGGGGGACCTT	Manz *et al.,* 1996

Erec482	Most *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster *XIVa and XIVb)*	GCTTCTTAGTCARGTACCG	Franks *et al*., 1998
Rrec584	*Roseburia - Eubacterium rectale*	TCAGACTTGCCGYACCGC	Walker *et al.*, 2005
Ato291	*Atopobium, Colinsella, Olsenella* and *Eggerthella spp.; Cryptobacterium curtum; Mycoplasma equigenitalium* and *Mycoplasma elephantis*	GGTCGGTCTCTCAACCC	Harmsen *et al*., 2002
Prop853	*Propionibacterium* (Clostridial Cluster IX)	ATTGCGTTAACTCCGGCAC	Walker *et al.*, 2005
Fprau655	*Faecalibacterium prausnitzii*	CGCCTACCTCTGCACTAC	Devereux *et al.*, 1992
DSV687	Most *Desulfovibrionales* and *Desulfuromonales*	TACGGATTTCACTCCT	Hold *et al*., 2003
Chis150	*Clostridium histolyticum* (*Clostridium* cluster I and II)	TTATGCGGTATTAATCTYCCTTT	Franks *et al*., 1998

Metagenomic gene sequencing was considered as an alternate means of microbial characterisation; we were however concerned about its propensity towards giving false negative results on Bifidobacteria (Lee & O'Sullivan, 2010). In addition, we were interested in quantifying the functional bacterial groups, we felt that sequencing would have been unsuitable as a quantitative measure.

Flow cytometry

Flow cytomtery (FCM) (Accuri C6) is a high throughput technique in which fluorescently labelled probes attached to bacterial cells are excited and pass through a laser, thereby emitting light at varying wavelengths, allowing enumeration of individual bacterial groups). FCM is thought to be less subjective and less time consuming than microscopy techniques (Namsolleck *et al*., 2004).

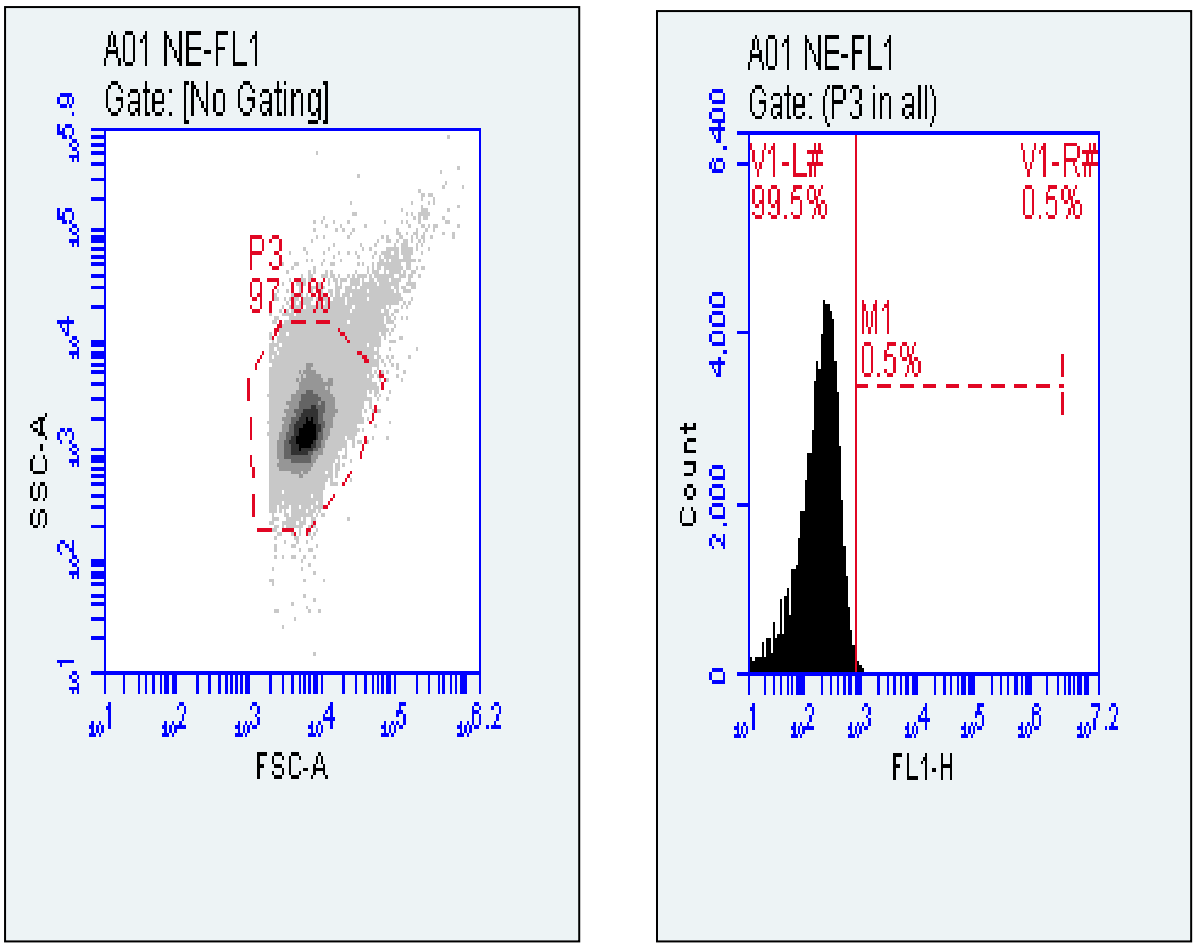

Figure 5: The 1st plot shows EUB I II III cell counts following flow cytometry analysis. The interest should be gated around the main dense dot, with at least 90% total events. On the 2nd plot, the left side represents total bacteria, and the right indicates non-bacteria (NE-FL1). Here we are aiming for <0.5% to reduce false positive events.

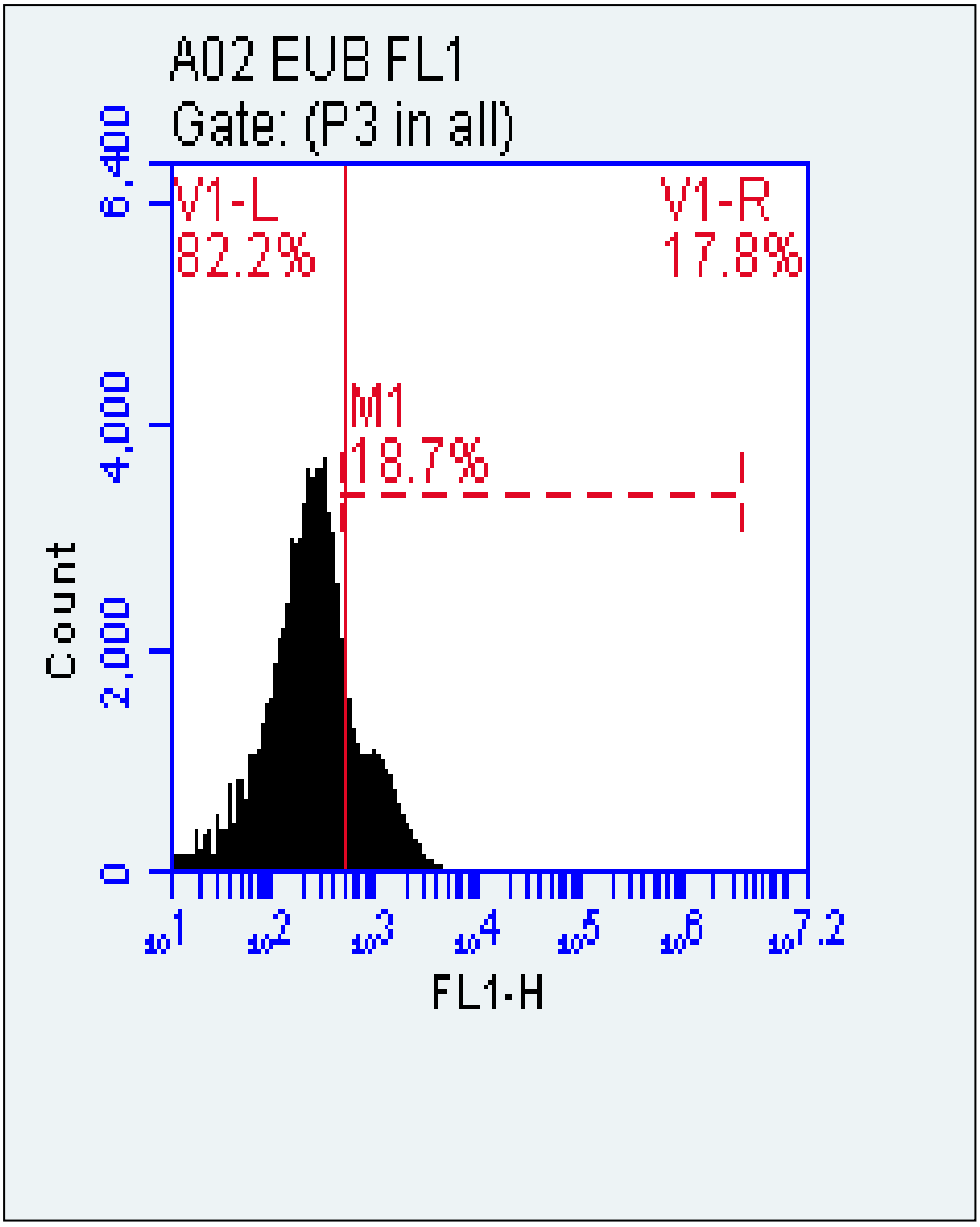

Figure 6: Total bacteria represented here on the right side of the plot (18.7%) – total bacterial counts will depend on the limit set previously in the negative control (NE-FL1)

SCFA analysis

Short-chain fatty acids (SCFAs) are metabolic end products of bacterial fermentation, produced at an approximate ratio of 60:20:20 acetate, propionate and butyrate in the human colon. Lactate is also produced by *Bifidobacterium* and *Lactobacillus*, this is usually then converted to acetate through cross-feeding mechanisms by groups such as *Bifidobacterium.* Gut microbiota metabolise fibre via two main metabolic routes, for glycolysis of 6 carbon sugars the Embden-Meyerhof-Parnas pathway is utilised, or the pentose-phosphate pathway for 5 carbon sugars converting monosaccharides into phosphoenolpyruvate that are subsequently fermented to organic acids (Stincone *et al*., 2015). Acetate is formed by hydrolysis of acetyl-coA via the Wood-Ljungdahl pathway which is then taken up by organs via the hepatic portal as a source of energy, however the majority – 70% is taken up by the liver as an energy source and as a pre-cursor for cholesterol synthesis. Butyrate conversely is formed by a condensation reaction in which two molecules of acetyl-to CoA and reduction to butyryl-CoA. Butyrate is the preferred energy source for colonocytes and has been implicated as an anti-carcinogenic compound due to its effects on cancer cell growth (Gonçalves & Martel, 2013). The predominant formation of propionate occurs via the succinate pathway from hexose sugars by decarboxylation of methylmalonyl-CoA to propionyl-CoA and coupled with sodium transport across the membrane, generating ATP through sodium-translocating ATPase (Miller & Wolin, 1996, Reichardt *et al*.,2014). Propionate has been implicated in maintaining cholesterol homeostasis, reducing lipogenesis in the liver as well as being implicated in positively impacting appetite regulation. This is due to the strong affinity propionate has as a ligand on the free fatty acid receptors (FFAR2/3) located on L-endocrine cells along the length of the colon, thus stimulating production of satiety hormones, in particular GLP-1 and PYY, which via the vagus nerve and therefore gut brain axis to promote anorexigenic pathways (Brown, 2003, Canfora *et al*., 2015, Chambers *et al*., 2014, Hosseini *et al*., 2011, Lin *et al.*, 2012)

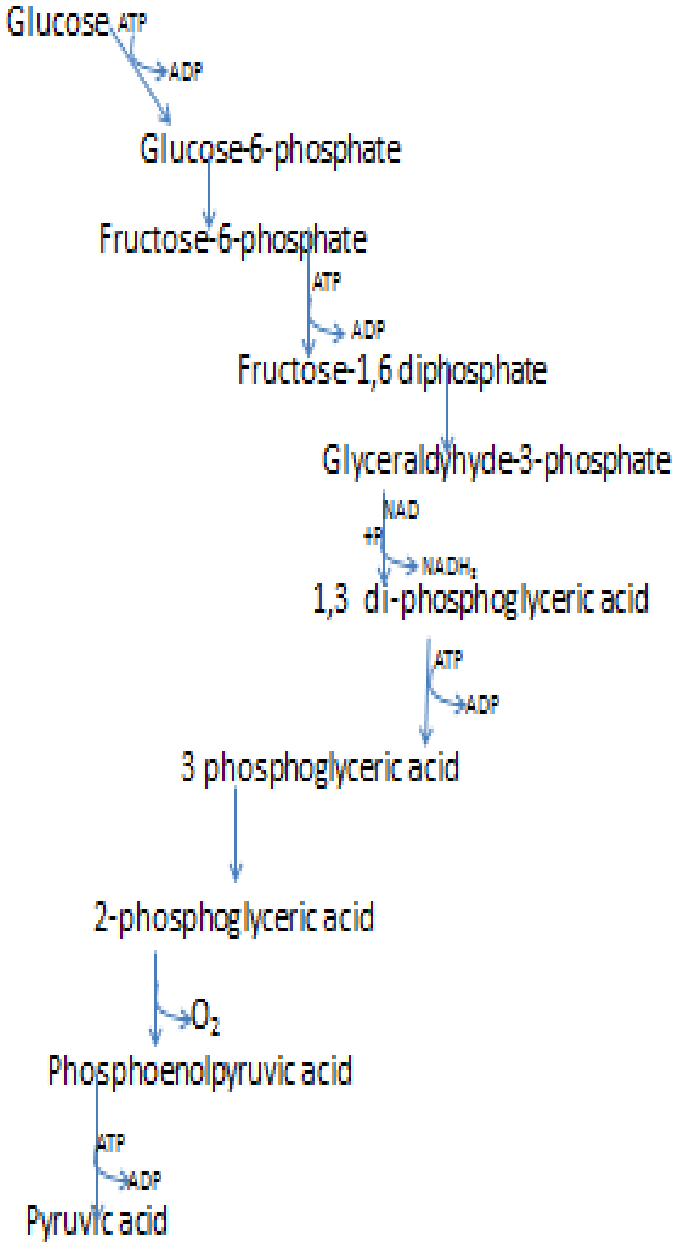

Figure 7: The Embden-Meyerhof-Parnas pathway. The metabolic pathway favoured by gut bacterial groups in glycolosis of 6 carbon sugars. (Adapted from Miller *et al*., 1996).

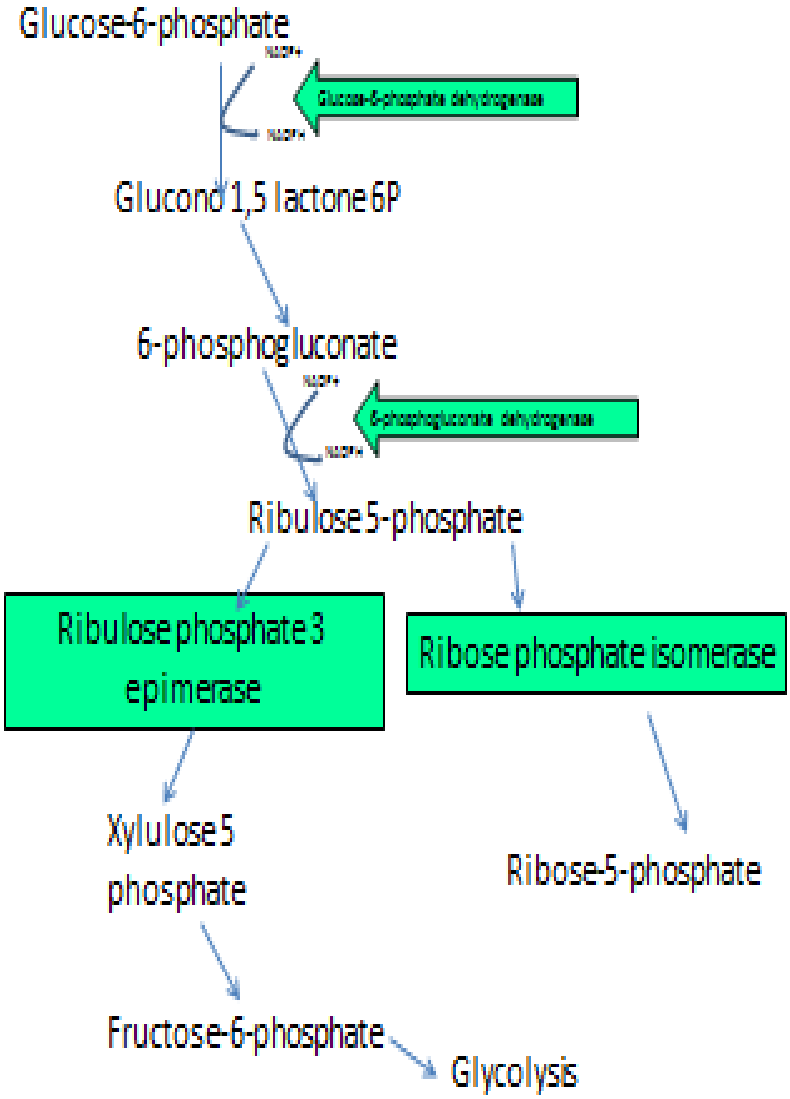

Figure 8: Schematic of the Pentose Phosphate Pathway, the metabolic pathway favoured by colonic bacterial groups to metabolise of 5 carbon sugars to organic acids. (Adapted from Miller *et al*., 1996).

HPLC

High performance liquid chromatography (HPLC) allows for quick detection of most organic acids because it is a highly sensitive method. By running samples through an organic acid column (phenomenex) then through a refractive index detector, analysis of SCFAs produces rapid equilibration, stable baselines, and reproducible results (Fernández *et al*., 2016). The main advantage of this method over gas chromatography is that it negates the need for derivatization step, thereby reducing the potential for prepping errors to occur during analysis.

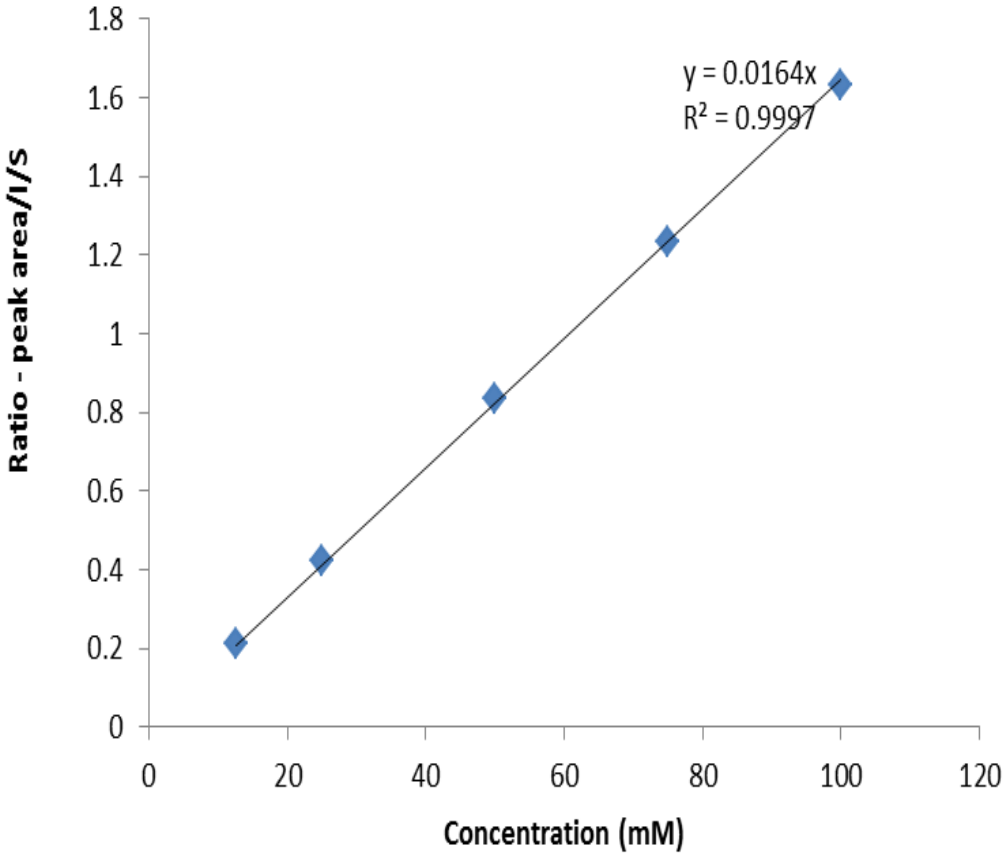

Figure 9: Calibration curve used to quantitatively measure acetate in samples collected.

Human double blinded, crossover appetite study

Methods

Testing the optimal blend (I+ABX) in a controlled human intervention study was necessary to confirm *in vivo* prebiotic activity. A within-subjects, acute within chronic, double-blinded placebo-controlled satiety study was chosen to assess any increase in satiety mechanisms in healthy weight men following consumption of I+ABX for 3 weeks. The study employed a preload design, allowing for assessments of satiety mechanisms by use of *ad libitum* food intake at lunch meal in response to preloads in the morning. Maltodextrin, commonly used in appetite studies was chosen as a placebo, providing the dose is under the threshold to provoke a glucose spike in humans and therefore less likely to mask any effects of the I+ABX intervention, it was considered a suitable placebo.

Strict exclusion criteria were implemented during volunteer screening to limit non-controlled external influences on the study outcomes; volunteers were excluded if their BMI was over 26, because as BMI increases a blunting of satiety signals can occur (Camilleri, 2015). Differences have also been observed in the microbiotas of lean and obese which can increase the possibility of inter-individual variation (Long *et al.,* 2015) and wanted to minimise this. Healthy males were chosen as the study population as gender differences have been reported in hedonic responses to food intake (Chao *et al*., 2017).

Those with illness, and gastro-intestinal conditions were excluded this study, as well as those on medication such as anti-biotics which can cause dysbiosis of the microbiome, modifying the baseline composition. This is undesirable because those with reduced bifidobacterial numbers are likely to respond with greater increases in growth than they would with balance microbial composition and this can lead to false positive results (Francino, 2015). Those on medications, particularly mood stabilisers were excluded as these can impact appetite regulation (Wysokinski & Kloszewska, 2014). Poor sleep habits have been associated with dysregulation of appetite (Öztürk, 2018), therefore those that regularly experience disrupted sleep or sleep <5hrs per night were also excluded, as determined by the Pittsburgh questionnaire. Those with disinhibited eating patterns,

determined by the 3 factor eating questionnaire were excluded as disinhibition (Anglé *et al*., 2009). A novel fibre blend was being tested here, it was important to recruit volunteers that consumed an average amount of fibre, therefore vegetarians were excluded, as well as those that consumed >22g fibre/d, determined by 2 x 24 recall dietary analysis in the screening process.

Faecal samples were collected at the beginning and the end of both treatment periods, giving a total of 4 sample time points to compare through SCFA analysis and determine if the treatment influenced SCFA production.

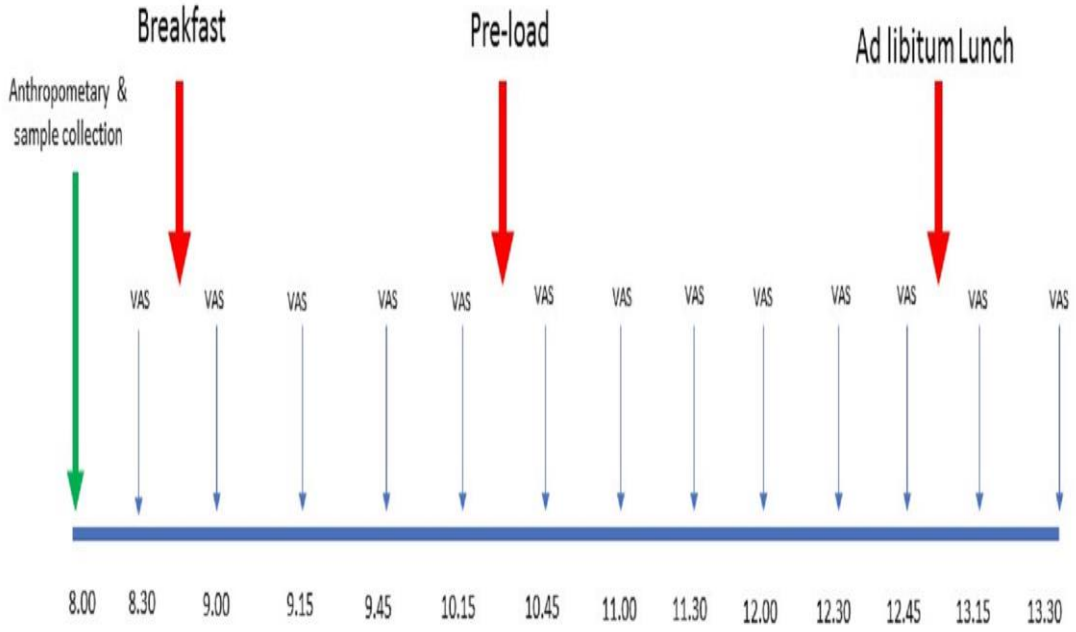

Figure 10: Time line of study day. Time points of visual analogue scale (VAS) for satiety scores anthropometrics & sample collection and meal time points.

VAS

Several methods may be used to evaluate the satiety inducing effect of a food or ingredient. Objective measurements include directly observing total energy consumed during in an *ad libitum* meal following pre-load, (Griffioen-Roose *et al*., 2012, Adam *et al.*, 2005) or measuring circulating colonic satiety hormones such GLP-1 and PYY. Self-reporting food intake or measuring subjective satiety scores using weighed food diaries and recalls or visual analogue scale (VAS) respectively (Blundell *et al*., 2010) are subjective methods.

VAS is a validated method of recording subjective satiety in appetite studies as it is highly reproducible (*Flint et al*., 2000, Stubbs *et al*., 2000). It is more efficacious to use VAS satiety scores in conjunction with other measurable parameters of satiety processes, such as SCFAs, gut hormones and energy intake as this helps objectively corroborate the findings of the subjective VAS measurements (Flint *et al*., 2000, Blundell *et al*., 2010, Hobden *et al*., 2017). Inter-individual variability can occur with self-reporting satiety scores, due to differences in using the line scale; however, as a crossover design was chosen, variability in individual response was minimised as each volunteer acts as their own control (Lesdema *et al*., 2016). Satiety questionnaires by visual analogue scale (VAS) were used in conjunction with energy intake measured during the *ad libitum* lunch at the end of each study day to determine if any increase to subjective satiety had occurred.

Appetite sensations are difficult to assess as their appearance depends on many external and internal factors (Blundell *et al*., 2010). Using VAS scores to determine true satiety sensations is dependent on the study population and design. Healthy weight males (mean BMI: 23.24) were chosen, with 20 completing. Between 12-18 single sex subjects are thought to be sufficient to observe a 5mm difference in VAS line scale (Flint *et al*., 2000). Conditions were controlled as much as possible so that the evening meal prior to the study day was standardised, as was breakfast on the study day itself, and timings were strict. Volunteers were partitioned off in a specific quiet area to minimise external stimuli whilst completing VAS satiety scores and at mealtimes. The timing in which VAS were completed were strictly regulated throughout the study day.

Gut hormones and VAS

Measuring circulating plasma satiety hormones such as GLP-1, PYY and insulin at specific time point throughout the study day would have been an effective method of corroborating any significant increases in subjective satiety using VAS as other satiety studies have used this combination of methods with good results (Parnell *et al*., 2009, Bodinham *et al*., 2010, Hoffmann *et al*., 2016). However, plasma analysis is time consuming and costly, for the purposes of this research it was deemed viable instead to choose measuring subjective satiety scores using VAS in conjunction with measuring energy intake during a blinded *ad libitum* lunch following the pre-load. Additionally, SCFA analysis was carried out as these metabolites are thought to be implicated in appetite regulation, an increase of acetate and propionate could indicate increased satiety in VAS scores.

Nuclear magnetic resonance (NMR)

The study of small molecule metabolites in an organism is referred to as metabolomics (Dettmer & Hammock, 2004). By analysing at a metabolomic level, the response and effect of dietary components can be observed and is an effective way of analysing systemic effects produced from a specific food or ingredient (Markley *et al*., 2017).

An untargeted approach was chosen based on the hypothesis that variation in metabolites will occur between treatment and control and I wanted to detect as many metabolites as possible, as opposed to exploring a defined set as is the case with targeted NMR. The main advantage of NMR is that a wide range of molecules can be detected from non-destructible samples very quickly, with no need for separation or derivatization that are highly reproducible, which is necessary when using mass spectrometry and unlike MS, NMR is quantitative. Historically, however MS has been shown to be more sensitive than NMR, thereby increasing the number of metabolites that may be detected, although sensitivity has improved with NMR in recent years. MS is a useful approach for use if sensitivity and selectivity combined is necessary in metabolomics research (Emwas, 2015). The spectral data collected following analysis not only allows quantification of metabolite concentration, but also offers data on chemical structure. Each molecule generates a peak gives information on the amount of metabolite whereas the pattern of peaks gives us information to help identify

the type of metabolite being measured and can be viewed in both 2-D, based on two frequency axis, allowing for overlapping peaks to be highlighted, the chemical shift, that would otherwise be hidden in 1-D axis (Alonso *et al*., 2015).

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Chapter 4: An Investigation into the Fermentation Characteristics of Novel Prebiotics in a 48 Hour *in vitro* Batch Culture Model of the Gut

Abstract

Prebiotics can induce changes in the relative composition of the microbiota, conferring a health benefit. It is advantageous to investigate the prebiotic ability of other fibres, as these too may be favourable to health. The objective of this study was to compare the fermentation characteristics of eleven commonly consumed fibres in an *in vitro* batch culture model of the colon (n=3). Faecal inoculate from three healthy volunteers was incubated in a basal media containing the candidate prebiotic substrates at 1% w/v under anaerobic conditions simulating the distal colon. The putative prebiotic substrates evaluated were: long chain fructo-oligosaccharides (LC-FOS), oligofructose (OFS), xylo-oligosaccharides (XOS), α-gluco-oligosaccharides (GLOS), polydextrose (PDX), resistant maltodextrin (R-MLX), resistant starch (RS), medium viscosity arabinoxylan (MV-ABX), low viscosity arabinoxylan (LV-ABX), oat fibre (OAT) and β-glucan (β-GLU). Outcome measures were, changes from baseline in the composition of the mixed culture microbiota, and changes in the concentration of short chain fatty acids at 0, 24 and 48 hours. LC-FOS was the only substrate to induce bifidogenic growth between both 0-24h (P=0.02) and 24-48h (P=0.01). OFS (1.65 $\log_{10}$) XOS (1.54 $\log_{10}$), R-MLX (1.37 $\log_{10}$), LV-ABX (1.35 $\log_{10}$) exerted the greatest bifidogenic effects, corresponding with the significant increases in acetate concentration between 0-24h. OFS elicited the greatest increase in acetate 106mM (P=0.01) and GLOS was associated with high propionate concentrations of 43mM (P=0.01). It appears the oligosaccharides and polysaccharides induce structure specific compositional changes in the microbiota and metabolite production. A *priori* consideration of favourable prebiotic endpoints should guide a purposeful selection of optimal products and or the construction of blends of products to achievable favourable outcomes in the consumer.

Introduction

In adults, the composition of human gut microbiota remains relatively stable (Rodríguez *et al.*, 2015) however illness or low-grade inflammation from increased adiposity, can cause disruption to this ecosystem (Ley *et al.*, 2005, Ley *et al.*, 2006). This can increase the risk of the host developing ailments such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), and can also dysregulate metabolic processes such as appetite regulation (Flint *et al.*, 2012, Jostins *et al.*, 2012).

Prebiotics are defined as 'a substrate that is selectively utilised by host microorganisms conferring a health benefit" (Gibson *et al.*, 2017). Supplementing the diet with prebiotics can improve bacterial composition, by increasing beneficial commensal colonic bacterial groups, such as bifidobacteria (Gibson *et al.*, 1995, Kolida and Gibson 2007, Kolida *et al.*, 2007). Fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are established prebiotics (Gibson *et al.*, 1995, Ramirez-Farias *et al.*, 2009), however research continues to establish other substrates as prebiotics, including candidates such as resistant starch (RS) and arabinoxylan (ABX) (Birt *et al*., 2013, Hald *et al.*, 2016). Well-designed *in vitro* and human dietary-intervention studies are required to understand the underlying mechanisms and to justify classifying these substrates as prebiotics.

An initial assessment of the prebiotic potential of a substrate can be made by measuring changes to functional bacterial populations in models of colonic fermentation (Czechowska *et al.*, 2008). Selective growth of non-pathogenic commensal bacterial groups, and enhancement of short chain fatty acid (SCFA) production, in mixed culture, in response to candidate prebiotic substrate, can be used to justify subsequent human randomised control trials to evaluate prebiotics effects (Gibson *et al.*, 2004).

Metabolites associated with prebiotic fermentation include butyrate, the primary energy source for colonocytes and a possible anti-carcinogenic metabolite (Peluzio *et al.*, 2009), and acetate and propionate which are implicated in lipogenesis, cholesterol synthesis and appetite regulation (Canfora *et al.*, 2015). Further, microbial cross-feeding may result in the production of other metabolites that could not be predicted from the culture of isolated

bacteria and which may have significance for health, which highlights the importance of mixed cultures in the evaluation of candidate prebiotics (Sarbini and Rastall, 2011).

This study aimed to screen 11 candidate substrates in 48-hour batch culture fermentations with human faecal inoculate. These cultures were controlled to simulate an environment approximate to the distal region of the colon, an area of interest due to increased disease occurrence therein (Yao *et al.*, 2016). Static batch cultures have previously been used to screen potential candidate prebiotics intended for human use (Rycroft *et al.*, 2001, Liu *et al.*, 2016). Quantification of changes in bacterial ecology were assessed through fluorescent *in situ* hybridisation and flow cytometry (FISH-FLOW), and with quantification of the SCFAs in the supernatant assessed through HPLC analysis.

Methods

Substrates

Eleven commercially available fibres were selected for testing in gut simulated 48 hr batch culture fermentation long chain fructo-oligosaccharides (LC-FOS) (Fine Foods), oligofructose, (OFS) (Orafti P95), resistant starch (RS) (Himaize 260 – Sigma potato starch), resistant maltodextrin (R-MLX) (Promitor T&L) α-gluco-oligosaccharides, (GLOS) (Biocolians), β-glucan (β-Glu) (Glucagel), oat fibre (OAT) (Fine foods), low viscosity arabinoxylan (LV-ABX) (Megazyme), medium viscosity arabinoxylan (MV-ABX) (Megazyme), Xylo-oligosacchairde, (XOS) (Santori), Polydextrose (PDX) (Danisco Sweeteners).

Faecal Donors

Faecal samples were collected from three human volunteers (BMI 19–27kg/m^2), aged 25-39, 2 females, one male. One faecal donor was used for each batch culture run. There was no recent history of gastrointestinal disorders and no anti-biotic use in the last 6 months. The volunteers had not consumed prebiotic/probiotic supplements within 3 weeks of donating. Samples were collected on the day of inoculation and kept in an anaerobic container (AnaeroJar™ 2.5L; Oxoid Ltd) for no more than 1h with a gas generating kit (AnaeroGen™; Oxoid), in which the atmospheric O_2 is absorbed by the with the simultaneous generation of CO_2 so within 30 minutes O_2 levels reach approximately 1% and CO_2 between 9% and 13%.

Samples were diluted 1:10 in anaerobically stored PBS (PBS; 0.1 M; pH 7.4), then homogenised in a stomacher (Steward 400) for 2 min at 240 paddle beats per minute.

Basal medium

Basal medium was prepared by heating and stirring the following substrates: peptone water (2 g/L), yeast extract (2g/L), NaCl (0.1 g/L), K_2HPO_4 (0.04 g/L), KH_2PO_4 (0.04 g/L), $MgSO_4.7H_2O$ (0.01 g/L), $CaCl_2.{}_6H_2O$ (0.01 g/L), $NaHCO_3$ (2 g/L), Tween 80 (2ml/L), Hemin (0.05 g/L), Vitamin K (10 ml/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L) and resazurin (0.25 g/L). The medium was then autoclaved at 121˚C for 15 mins to sterilise and prevent contamination and aseptically added to individual batch culture vessels at 135mL volume, then adjusted to pH 6.8. The fermenters were purged with O_2 free N_2 and they were magnetically stirred to maintain pH and temperature.

The method for running the batch cultures has been previously described (Liu *et al.*, 2016). Twelve sterile batch culture fermenters (300mL volume) were aseptically filled with 135mL autoclaved basal medium. All vessels were then gassed overnight with O_2 free N_2 (15ml/min). Each vessel was inoculated with 15mM faecal slurry, then fermentation was initiated with the addition of 1.35g (1% w/v) individual substrate into the fermenters. Inulin was chosen as a positive control and there was an additional 12^{th} vessel containing no substrate included as a control vessel. The pH was maintained between 6.7–6.9 using pH controllers (Fermac 260; Electrolab) and automatically adjusted by adding 0.5 mM NaOH or HCl to the vessels when required. The pH of 6.8 and temperature of 37˚C was controlled to simulate conditions of the distal region of the human large intestine. Batch culture fermentations were run for 48 hours, and samples were collected at 0, 24 and 48 hours (5ml from each vessel) for analysis of bacterial populations and metabolite production. Fermentation of each substrate was performed in triplicate for each volunteer.

Enumeration of specific bacterial groups with fluorescent *in-situ* hybridization (FISH)

Enumeration of faecal bacterial groups was via fluorescent *in-situ* hybridisation (FISH) and flow cytometry in combination (FLOW-FISH). Samples were collected from V1-V12 at 0, 24 and 48hr and from this 375 µl was centrifuged for 5mins at 1300 x g, the supernatant removed, and pellet re-suspended with 375 µl filtered PBS (0.22µm filter Millipore, Bedford,

MA) and 1125 µL of 4% (v/v) paraformaldehyde. Following incubation at 4˚C for 4 hours, samples were then centrifuged at 13000 x g for 5 min and washed in 1ml filtered PBS. This was repeated twice and the resultant washed cells were then re-suspended in 150 µl filtered PBS and 150 µl ethanol (99%) and stored at -20˚C as previously described (Grimaldi *et al.*, 2016).

For permeabilisation, 75 µL of the fixed sample was mixed with 500µl of cold PBS at 4°C and centrifuged at 11360 × *g* for 3 min, the supernatant removed and pellet resuspended with 100 µL of filtered TE-FISH (Tris/HCl 1M pH 8, EDTA 0.5M pH 8, distilled H_2O) containing lysozyme (1 mg/mL of 50 000 U/mg protein) and filtered with a 0.22µm filter (Millipore, Bedford). This was followed by hybridisation steps in which pellets were re-suspended in 150 µL of hybridisation buffer (5M NaCl, 1 M Tris/HCl pH 8, 30% formamide, ddH_2O, 10% SDS, then vortexed and centrifuged at 11360 × *g* for 3 min). Pellets were then re-suspended in 1 mL of HB and 50 µL aliquoted into Eppendorf tubes, for individual bacterial groups to be enumerated using specific probes as detailed in (**Table 2)** (Devereux *et al.*, 1992, Wallner *et al.*, 1993., Langendijk *et al.*, 1995, Manz *et al.*, 1996, Franks *et al.*, 1998, Daims *et al.*, 1999, Harmsen *et al.*, 2002, Hold *et al.*, 2003, Walker *et al.*, 2005). For the control tube, no probes were added to the 50µl sample. NON EUB338 probe was used to control for non-specific binding to EUB338. Simultaneously EUB338 I, II & III linked at their 5' end either to Alexa488 and Alexa647 was used to target total bacterial species, as EUB338 I alone is insufficient for the detection of all bacterial groups and also using EUB I, II, III together allows for more accurate quantification (Daims *et al.*, 1999). In each eppendorf, 4 µL of Eub338 I-II-III linked to Alexa488 was added for total bacteria and 4µL of the specific probe linked with Alexa647 at the 5' end, then incubated in a heating block at 35°C for a minimum of 12 hours to enable hybridisation.

Following hybridisation, samples were centrifuged for 3 min at 13000 x g and supernatant removed. The control tube (no probe) was re-suspended with cold PBS (4˚C) and analysed using the flow using the BD Accuri™ C6 flow cytometer according to the manufacturer's instructions to determine background noise and subtract this from the analysis. Samples containing probes were then re-suspended with the same volume as negative control. Specific

and total bacterial groups were then enumerated using a dilution factor (DF) which was calculated from different volumes used during preparation of the samples and events/µl determined from NON EUB338 and EUB I-II-III probes that were analysed by flow cytometry.

Table 2. Oligonucleotide probes used during FISH for enumeration of bacterial groups.

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

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

SCFA analysis

The concentration of total and individual SCFAs was measured using HPLC (Agilent 1260 Infinity) as previously described by Grimaldi *et al.,* and Salazar *et al*., 2009. A, 320 µl filtered sample was mixed with 120 µl internal standard (I/S) which was 2-ethylbutiric acid (10mM) giving a ratio of 1:4, and this was then added to HPLC vials. By adding the same amount of I/S to each sample and standard and using the ratio of the area of the peak analyte and I/S, the peaks can be corrected, and quantitative analysis is improved instead of keeping track of absolute peak area which may have volumetric losses in preparation and injection. A 20 µl volume was then injected into the HPLC system with a run time of 45 minutes to allow all peaks required to be measured. Agilent (Chemstation) software was then used to integrate the peaks, using calibration curves of individual SCFAS: lactic, acetic, propionic and butyric acid of increasing concentration (12.5, 25, 50, 75, 100mM).

Statistical analyses

Statistical analysis was performed using SPSS for windows (version 16.0; SPSS, Inc). Repeated measures, one-way ANOVA and post hoc tests (Bonferri) and t-tests were used to ascertain if there was any significant effect of treatment on SCFA production and bacterial growth during fermentation between 0-24h and 24-48h at ($P<0.05$). Bacteriology results were not normally distributed, therefore non-parametric Friedmans tests were carried out and this was followed by paired t-tests to determine significance ($P<0.05$), between 0-24h and 24-48h.

Results

Bacterial enumeration

The results of changes to microbial ecology throughout the 48h batch culture are detailed in **Table 3.**

Table 3. Bacterial ecology during batch culture fermentation of various substrates: long chain fructo-oligosaccharides (LC-FOS), oligofructose (OFS), resistant starch (RS), resistant maltodextrin (R-MLX), α-gluco-oligosaccharides (GLOS), β-glucan (β-GLU), oat fibre (OAT), low viscosity arabinoxylan (LV-ABX), medium viscosity arabinoxylan (MV-ABX), xylo-oligosaccharide, (XOS), polydextrose (PDX). Values are means ± SD for three separate cultures, statistically significant differences between population levels at 0-24h and 24-48h as determined by one way ANOVA repeated measures and paired t-tests, indicated by an asterisk (*). Significance level P<0.05.

Substrate & sampling time (h)	Log10(cells/ml)									
	EUB I II II	**Bif164**	**Lab158**	**Bac303**	**Erec482**	**RRec584**	**Prop853**	**Fprau655**	**DSV687**	**Chis150**
LC-FOS										
0	8.59±0.06	6.7±0.14	6.51±0.23	7.63±0.50	7.64±0.30	6.79±0.20	7.20±0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	**9.86±0.09***	**7.64±0.37***	6.22±0.64	7.50±0.11	8.74±0.60	7.026±0.14	7.92±086	7.67±0.48	6.33±0.62	6.9±0.26
48	**10.02±0.06***	**8.50±0.38***	6.56±0.67	**8.00±0.05***	8± 0.42	7.72±0.22	8.24±0.50	8.10±0.032	6.30±0.75	7.01±0.15
OFS										
0	8.59±0.06	6.7±0.14	6.51±0.23	7.63±0.50	7.64± 0.30	6.79±0.20	7.20±0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	9.6±0.26	**8.35±0.63***	7.07±0.16	7.67±0.50	8.24±0.88	6.90±1.12	**8.07±0.78***	7.51±0.56	6.41±0.22	6.5±0.25
48	9.66±0.23	8.61±0.16	**7.68±0.07***	8.05±0.11	8.39±0.44	7.30±0.99	8.13±0.51	7.69±0.52	6.94±0.73	6.54±0.11
XOS										
0	8.59±0.06	6.7±0.14	6.51±0.23	7.63±0.50	7.64±0.30	6.79±0.20	7.20±0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	**9.37±0.22***	**8.24±0.37***	6.98±0.14	7.36±0.48	7.68±0.92	6.92±0.32	7.33±0.95	7.62±0.59	6.22±0.54	6.21±0.47
48	9.69±0.3	8.58±0.46	**7.35±0.06***	7.78±0.61	**8.17±0.81***	7.57±0.33	8.40±0.34	8.12±0.08	6.39±0.53	6.99±0.60
GLOS										
0	8.59±0.06	6.7 ± 0.14	6.51±0.23	7.63±0.50	7.64± 0.30	6.79±0.20	7.20±0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	**9.59±0.07***	**8.02 ± 0.42***	7.06±0.14	7.52±0.30	9.05 ± 0.37	7.14±0.45	8.19±0.36	7.97±0.36	6.51±0.56	6.94±0.28
48	9.62±0.1	8.59 ± 0.33	7.54±0.04	7.8±0.56	8.70±0.21	**7.84±0.60***	8.67±0.32	8.33±0.31	6.31±0.25	6.55±0.54
PDX										

0	8.59±0.06	6.7±0.14	6.51±0.23	7.63±0.50	7.64± 0.30	6.79±0.20	7.20±0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	**9.51±0.43***	6.8±0.35	6.92±0.12	7.8±0.10	7.56±0.59	6.95±0.86	7.71±0.83	7.66±0.52	6.44±0.49	6.32±0.16
48	9.66±0.2	**7.69±0.61***	7.32±0.07	7.78±0.32	7.59±0.50	7.23±1.40	8.05±0.76	7.55±0.54	6.7±0.44	6.14±0.33
R-MLX										
0	8.59±0.06	6.7±0.14	6.51±0.23	7.63±0.50	7.64±0.30	6.79±0.20	7.20±0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	**9.67±0.09***	**8.07±0.62***	7.13±0.08	7.84±0.16	**9.03±0.37***	7.42±0.45	7.92±0.61	8.19±0.66	6.77±0.39	6.33±0.50
48	9.7±0.2	8.55±0.48	7.46±0.00	7.64±0.51	8.76±0.70	7.44±0.76	8.21±0.38	7.82±0.51	6.59±0.45	6.82±0.17
RS										
0	8.59±0.6	6.7±0.14	6.51±0.23	7.63±0.50	7.64±0.30	6.79±0.20	7.20± 0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	9.4±0.43	7.77±0.28	6.93±0.28	7.55±0.67	8.05±0.57	7.17±0.37	7.60±0.53	6.94±1.94	6.49±0.72	6.61±0.44
48	9.65±0.03	8.22±1.01	7.33±0.23	7.76±0.22	8.81±0.83	7.51±0.24	8.09±0.33	7.44±2.30	6.43±0.72	5.94±0.47
MV-ABX										
0	8.59±0.06	6.7±0.14	6.51±0.23	7.63±0.50	7.64±3.0	6.79±0.20	7.20±0.77	7.55±0.30	6.03±0.35	5.45±0.46
24	**9.73±0.01***	**7.78±0.01***	**7.08±0.20***	7.6±0.34	7.87±0.60	7.24±0.28	7.72±0.13	8.23±0.43	6.84±0.44	6.16±0.20
48	9.86±0.27	8.41±0.46	7.56±0.09	**8.2±0.30***	8.35±0.08	7.48±0.86	8.27±0.25	7.94±0.36	7.01±0.29	6.08±0.10
LV-ABX										
0	8.59±0.06	6.7±0.14	6.51±0.23	7.63±0.50	7.64±0.30	6.79±0.20	7.20±0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	**9.39±0.27***	**8.05±0.39***	6.92±0.03	7.75±0.52	7.63±0.47	7.26±0.18	7.65±0.97	7.73±0.49	6.53±0.52	6.64±0.54
48	9.61±0.7	8.19±0.09	6.73±0.44	8.17±0.72	7.48±0.51	7.75±0.53	7.90±0.53	7.85±0.51	6.15±0.39	6.62±0.53
OAT										
0	8.59±0.06	6.7±0.14	6.51±0.23	7.63±0.50	7.64±0.30	6.79±0.20	7.20±0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	8.68±0.36	7.34±0.46	6.45±0.05	7.32±0.32	7.45±0.30	6.80±0.46	7.48±0.88	7.32±0.35	6.5±0.14	6.34±1.1
48	8.73±0.26	7.15±0.18	6.73±0.11	7.15±0.33	7.25±0.50	6.87±0.97	8.00±0.01	7.68±0.01	**5.86±0.02***	6.05±0.69
Negative										
0	8.59±0.06	6.7±0.14	6.51±0.23	7.63±0.50	7.64±0.30	6.79±0.20	7.20±0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	8.03±0.53	6.97±0.42	6.66±0.08	7.02±0.32	7.26±0.36	6.78±0.17	7.24±0.82	7.14±0.65	6.68±0.01	6.78±0.08
48	8.56±0.23	7.11±0.22	6.79±0.11	7.27±0.23	7.34±0.42	7.55±0.38	7.57±0.37	7.21±0.41	6.61±0.2	6.66±0.03

β-GLU										
0	8.59±0.06	6.7±0.14	6.51±0.23	7.6±0.30	7.64±0.30	6.79±0.20	7.21±0.77	7.55±0.30	6.03±0.35	6.45±0.46
24	8.89±0.39	**7.52±0.41***	6.94±0.13	7.6±0.28	**8.31±0.47***	7.50±0.13	7.53±0.82	7.81±0.27	**6.77±0.5***	6.29±0.74
48	9.26±0.14	7.8±1.52	6.74±0.14	7.73±0.07	8.39±0.46b	7.63±0.36	8.39±0.76	7.78±0.56	6.38±0.3	6.21±0.62

SCFA analysis

Table 4 below details the production of SCFAs produced during the *in vitro* fermentation of all eleven substrates between 0-24 and 24-48h.

Table 4: SCFA concentration during batch culture fermentation of various substrates: long chain fructo-oligosaccharides (LC-FOS), oligofructose (OFS), resistant starch (RS), resistant maltodextrin (R-MLX), α-gluco-oligosaccharides (GLOS), β-glucan (β-GLU), oat fibre (OAT), low viscosity arabinoxylan (LV-ABX), medium viscosity arabinoxylan (MV-ABX), xylo-oligosaccharide, (XOS), polydextrose (PDX). Values are means ± SD for three separate cultures, with statistically significant differences between population levels at 0-24h and 24-48h determined by one way ANOVA repeated measures and paired t-tests, indicated by an asterisk (*). Significance level P<0.05.

Substrate & Timepoint (h)		Acetate	propionate	butyrate	total	Substrate & Timepoint (h)		acetate	propionate	butyrate	total
LC-FOS	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32	**RS**	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32
	24	**53.13±16.27***	21.72±11.29	12.99±6.52	**97.84±11.19***		24	**59.63±18.96***	**22.26±4.42***	13.39±5.31	**92.61±13.22***
	48	85.13±36.86	30.73±2.19	20.86±1.27	146.74±37.84		48	92.89±19.11	33.55±21.46	18.12±2.11	144.57±16.19
OFS	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32	**MV-ABX**	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32
	24	**79.38±25.2***	34.04±18.94	**17.91±2.34***	**131.35±39.01a**		24	**41.03±36.03***	22.6±7.26	10.85±10.14	**85.82±22.86***
	48	82.36±37.38	36.8±22.4	19.16±2.96	131.66±26.98		48	56.63±33.81	33.26±15.89	10.07±8.8	79.1±28.86
XOS	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32	**LV-ABX**	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32
	24	**77.4±7.71***	**22.94±8.47***	**20.67±9.26***	**121.03±16.36***		24	**74.99±9.52***	22.84±16.86	**12.53±1.56***	**102.37±20.72***
	48	92.88±12.88	43.55±12.93	20.51±8.01	156.94±7.28		48	80.41±17.54	33.84±29.6	15.81±2.39	120.07±19.19
GLOS	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32	**OAT**	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32
	24	**53.78±22.95***	**48.17±14.55***	17.05±2.14	**119.01±38.17***		24	18.15±5.11	19.89±3.03	10.06±2.06	**48.1±5.39***
	48	57.52±12.99	51.21±11.4	20.08±5.73	128.82±27.07		48	20.58±5.5	19.69±5.16	11.32±1.28	51.6±11.77
PDX	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32	**NEG**	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32
	24	**35.5±11.57***	**30.82±4.47***	11.8±4.51	**78.13±13.26***		24	22.52±12.78	15.33±9.54	8.54±1.57	46.4±21.24
	48	39.59±13.91	42.65±10.08	16.44±7.67	107.03±30.51		48	27.25±20.57	16.37±9.46	10.95±4.41	54.58±31.56

R-MLX	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32	**β-GLU**	**0**	7.36±4.38	5.33±1.57	6.85±1.54	19.54±4.32
	24	**67.07±8.79***	21.73±7.59	**17.34±3.42***	**126.15±15.24***		**24**	**26.7±7.11***	24.54±13.26	13.87±2.66	65.11±17.72
	48	85.93±9.76	27.4±7.69	21.44±5.13	154.77±6.77		48	25.33±9.69	37.86±6.4	13.54±2.83	**76.74±13.25***

LC-FOS

LC-FOS was the only substrate to elicit significant bifidogenic effects in both earlier and later stages of the culture, with a significant 1.27, and 0.16 log_{10} increase in total microbial EUB I II III cells (P<0.00) between 0-24h, and 24-48h respectively. This corresponds with the 0.94 and 0.86 log_{10} increase in bifidobacterial cells between 0-24 (P=0.02) and 24-48h (P=0.01) respectively. The significant 46mM increase in acetate concentrations (0-24h) is reflective of this growth. Though the initial fermentation of LC-FOS was dominated by a bifidobacterial growth between 0-24h, reflected in the decline of *Bacteroides* numbers (0.13 log_{10} cells) at this time, there was a shift between 24-48h towards *Bacteroides* growth with a 0.50 log_{10} increase their numbers (P=0.03).

OFS

During the initial fermentation of OFS, bacterial groups: *Bifidobacterium* and *Propionibacterium* dominated growth with 1.65 log_{10} (P<0.00) and 0.87 log_{10} (P=0.02) increase in cell numbers, that was reflected in the significant increase of 1.01 log_{10} in EUB I II III cells. However, between 24-48h there was a shift towards *Lactobacillus* and *Bacteroides* growth with a 0.61 (P=0.03) and 0.38 (P=0.05) log_{10} increase in cell numbers respectively. The production of SCFA during this time is reflective of the increased bacterial activity with significant increases in acetate and butyrate production of 72mM (P=0.04) and 11mM (P=0.03) respectively. Though there was a large 29mM increase in propionate production it was not significant (P=0.13).

XOS

Initially, the fermentation of XOS led to bifidogenic effects, with a cell growth of 1.54 log_{10} between 0-24h (P<0.00), which accounts for the significant 101mM increase in total SCFA (P<0.00), reflected in the 70mM rise in acetate production (P=0.01) at this time. Butyrate levels increased by 14mM (P=0.05) as did propionate with a 18mM increase (P=0.01). Between 24-48h however, bacterial growth shifted towards R*oseburia* (P<0.00) and *Lactobacillus* growth (P=0.03) with a 0.65 and 0.37 log_{10} cell increase respectively. Propionate production increased by 21mM, which was close to significance (P=0.06).

GLOS

A 1.00 log_{10} increased in total EUB I II III cell numbers occurred between 0-24h (P<0.01), and can account for the 1.32 log_{10} cell increase in bifidobacterial numbers during the initial stages of GLOS fermentation (P=0.02) and their increased is reflected in the 46mM increase in acetate at this time. Growth shifted towards *Roseburia* cell numbers between 24-48h with a 0.70 log_{10} cell increase (P=0.03). Increases of 46mM, 10mM and 43mM in acetate (P=0.01), butyrate (P=0.05) and propionate (P=0.01) production respectively is indicative of greater bacterial activity.

RS

There was a large 1.52 log_{10} increase in cell growth of bifidobacterial between 0-24h, however it was not significant (P=0.14). However, it is this elevation in bifidobacterial numbers that led to a significant 53mM increase in acetate production between 0-24h (P=0.04). Also, propionate production was elevated during this time with a significant 17mM increase. There was a shift towards *Propionibacterium* growth between 24-48h with a 0.49 log_{10} cell increase, and this was close to significance (P=0.06).

LV-ABX

Bifidogenic effects were observed during the initial stages of LV-ABX fermentation, with a 1.35 log_{10} increase in cell numbers between 0-24h (P=0.01), and 0.47 log_{10} growth in *Roseburia* cell numbers which was close to significance (P=0.06). The 0.80 log_{10} growth of total microbial EUB I II III cells is reflective of this, as is the acetate production between 0-24h which significantly increased by 68mM (P<0.00), as did butyrate production by 6mM (P=0.04). *Roseburia* cell numbers increased by 0.46 log_{10} between 24-48h but was not significant (P=0.35)

MV-ABX

During the earlier stages of MV-ABX fermentation there was a significant increase in the cell growth of *Bifidobacterium* and *Lactobacillus* respectively 1.08 log_{10} (P<0.00) and 0.80 log_{10} (P=0.01), and correlates with significant 1.14 log_{10} growth of total microbial EUB I II III cells (P<0.00), and corresponds to the significant and elevated acetate concentration of 34mM

(P<0.00) at this time. Bacterial growth then shifted towards *Bacteroides* between 24-48h with a 0.6 log_{10} increase in cell numbers (P=0.03).

R-MLX

R-MLX fermentation appears to have occurred mainly between 0-24h, with a significant 1.08 log_{10} rise in total microbial EUB I II III cell numbers, and is indicative of the 1.37 and 1.39 log_{10} cell growth in *Bifidobacterium* (P=0.03) and *Eubacterium rectale* (P=0.03) numbers respectively. Elevation in SCFA production corresponds with this change in microbial activity with a 67mM (P<0.00) and 10mM (P=0.04) increase in both acetate and butyrate concentration respectively.

PDX

Between 0-24h there was a significant 1.08 log_{10} cell increase in total microbial EUB I II III cell numbers (P<0.00), however there was no significant increase in individual bacterial groups. Bifidobacterial cell numbers were significantly increased between 24-48h though, with a 0.89 log_{10} cell increase. A significant 28mM (P=0.02) and 25mM (P<0.00) rise in acetate and propionate respectively, indicates increased microbial activity at this time.

β-GLU

Fermentation of β–GLU induced a 0.82 log_{10} cell increase of *Bifidobacterium* between 0-24h, which corresponds with the significant 19mM rise in acetate levels (P=0.02). *Eubacterium rectale* cell numbers also increased by 0.67 log_{10}, during 0-24h, whereas *Desulfulvibrio* cell numbers significantly decreased by 0.74 log_{10} (P=0.03).

OAT

Bifidobacterial cell numbers increased by 0.54 log_{10} during OAT fermentation between 0-24h, however this result was not significant, *Desulfulvibrio* cell numbers decreased by 0.64 log_{10} which was significant (P=0.01). Increased bifidobacterial activity is indicated by the significant increase of 28mM in total SCFA production at this time (P<0.00).

There was no significant increase in *Faecalibacterium prausnitzii* during 48h batch culture of all 11 substrates.

Between 24-48h there were significant increases in *Bacteroides* following fermentation of LC-FOS and MV-ABX. *Roseburia* was also increased through XOS, GLOS, R-MLX and R-MLX fermentation between 24-48h, most likely a result of cross-feeding mechanisms. Fermentation of 11 substrates led to a growth in bacterial groups and increased SCFA between 0-24h and 24-48h, except for R-MLX, OAT, LV-ABX and negative control.

A summary of prebiotic effects observed during the 48h *in vitro* batch culture (n=3) are summarised in **Table: 5**, with prebiotic effects symbolised by ↑ = low prebiotic effects, ↑↑ = moderate prebiotic effects and ↑↑↑ = high prebiotic effects. Acetate (mM): low; ↑ (0-25), medium; ↑↑ (25-45), high; ↑↑↑ (50+). Propionate (mM): low; ↑ (0-15), medium; ↑↑ (15-30), high; ↑↑↑ (30+). Butyrate (mM): low; ↑ (0-5), medium; ↑↑ (5-10), high; ↑↑↑ (10+). Bifidogenic effects between 0-24 & 24-48h (log_{10} cells/mL): low; ↑ (0.0-0.5), medium; ↑↑ (0.5-1.0), high; ↑↑↑ (1.0+). Stimulates acetate/propionate/butyrate producing bacterial groups between 0-24h OR 24-48h (log_{10} cells/mL): low; ↑ (0.0-0.4), medium; ↑↑ (0.4-1.0), high; ↑↑↑ (0-24h + 24-48h OR 1.00+). Bacterial growth between 0-24h & 24-48h indicating a slower fermentation (log_{10} cells/mL): low; ↑ (0.0-0.3), medium; ↑↑ (0.3-0.7), high; ↑↑↑ (0.7+).

Table 5: Summary of the 11 prebiotics/prebiotic candidates fermentation characteristics observed during 48h *in vitro* batch culture (n=3). Prebiotic effects are denoted by ↑ = low, ↑↑ = moderate and ↑↑↑ = high.

Substrates	Acetate	Propionate	Butyrate	Bifidogenic 0-24h	Bifidogenic 24-48h	Stimulates propionate producing bacteria (*Bacteroides*, *Propionibacterium*)	Stimulates butyrate producing bacteria (*Roseburia, Eubacterium rectale*)	Stimulates acetate producing bacteria (*Bacteroides*, *Bifidobacterium, Lactobacillus)*	Influence on potentially pathogenic bacterial groups (*Desulfovibrio, Clostridium histolyticum)*	Slower fermentation (↑ bacterial growth 24-48h)
LC-FOS	↑↑			↑↑↑	↑↑	↑↑		↑↑↑		↑↑↑
OFS	↑↑↑		↑↑↑	↑↑↑	↑	↑	↑↑	↑↑↑		↑↑
XOS	↑↑↑	↑↑	↑↑↑	↑↑↑	↑		↑↑	↑↑↑		↑↑
GLOS	↑↑	↑↑↑	↑↑	↑↑↑	↑↑		↑↑	↑↑↑		↑↑↑
RS	↑↑	↑↑		↑↑↑	↑	↑↑				↑↑
LV-ABX	↑↑↑		↑↑	↑↑↑	↑		↑↑	↑↑↑		↑↑
MV-ABX	↑↑			↑↑↑	↑↑	↑↑		↑↑↑		↑↑
R-MLX	↑↑↑		↑↑↑	↑↑↑	↑		↑↑↑	↑↑↑		↑↑↑
PDX	↑↑	↑↑		↑	↑↑			↑↑		↑↑↑
B-glu	↑			↑↑	↑		↑↑	↑↑	↑↑	↑
OAT	↑↑↑	↑↑		↑↑	↑				↑↑	↑

Discussion

Bifidogenic effects of substrate fermentation.

Fermentation of all 11 substrates led to an increase in cell numbers of *Bifidobacterium*, above growth observed in the negative control vessel, between 0-24h (0.27 log_{10}) with the exception of PDX, which was the only substrate in which a decrease in bifidobacterial cell numbers occurred. Of the results that were significant: OFS (1.65 log_{10}) XOS (1.54 log_{10}), R-MLX (1.37 log_{10}), LV-ABX (1.35 log_{10}), GLOS (1.32 log_{10}) were the most bifidogenic, characterised by significant increases in acetate observed at this time. The least bifidogenic were: β-GLU ~ (0.82 log_{10}), OAT (0.54 log_{10}), and PDX (0.1 log_{10}) between 0-24h, however LC-FOS and PDX were the only substrates to induce significant growth in bifidobacterial cell numbers in the latter stages of the culture (24-48h).

The fermentation of OFS, an oligosaccharide comprising β (2-1)-fructans, (DP_{ave}=8), induced the greatest bifidogenic effect between 0-24h with a 1.65 log_{10} cell increase in bifidobacteria. Shorter chain oligofructose is a well-documented prebiotic, with many strains of bifidobacteria selectively and preferentially metabolising OFS (Bouhnik *et al*., 1996, Gibson *et al*., 1995). The only substrate to induce significant bifidogenic effects between both 0-24h (P=0.02) and 24-48h (P=0.01) was LC-FOS, a fructan-type polysaccharide comprising of (2→1) linked β-D-fructosyl residues (n=2-60) and a α-D-glucose moiety (Mensink *et al*.,2015) Though *Bifidobacterium* prefer the shorter chain OFS, some species such as *Bifidobacterium adolescentis* can metabolise inulin by utilising β-fructofuranosidases to hydrolyse the β(2,1) bonds (Rossi M *et al*.,2005). XOS, an oligosaccharide with β (1–4)-Linked xylose, (DP_{ave}=2-6) induced the 2nd greatest bifidogenic effects between 0-24h (P=0.01). A large increase in bifidobacterial numbers (P-<0.00) along with acetate and butyrate levels was also reported during an *in vitro* 48 batch culture of XOS (Makelainen et al 2010). Furthermore, in Amaretti *et al*, *in vitro* investigation of XOS, reported that *Bifidobacterium adolescentis* was most likely to utilise β-xylosidase to hydrolyse XOS (Amaretti *et al*., 2013). Fermentation of GLOS, an oligosaccharide with alternating α-(1,3)/α-(1,6)-linked glucosyl residues (DP_{ave} =3-6) also resulted in significant bifidogenic effects between 0-24h (P=0.02). Increased bifidobacterial activity from is reflected in the significant and elevated concentration of acetate.

Fermentation of R-MLX, comprised of α(1-4), α(1-6), α/β(1-2), and α/β (1-3) linkages (DP_{ave}=12) led to an increase in bifidobacterial numbers as is reflected in the significant increases in acetate production, however this was not significant, most likely due to variation across the batches in terms of starting inoculum. Previous batch culture experiments by Rosch *et al*, reported that R-MLX fermentation leads to increased *Bifidobacterium* and that following analysis of the culture afterwards, reported that glucosidases were present. This is an enzyme commonly utilised by bifidobacterial strains (Rosch *et al*., 2015). The significant growth in *Lactobacillus* numbers could also account for the significant rise in propionate as this bacterial group produces lactate, which via the acrylate pathway may be converted to propionate (Louis & Flint, 2017).

MV-ABX is a polysaccharide comprised of a linear chain backbone with ß-d-xylopyranosyl (Xylp) residues linked through (1 → 4) glycosidic linkages. α-l-Arabinofuranosyl (Araf) residues. There was significant growth of *Bifidobacterium* and *Lactobacillus* between 0-24h, correlating with significant acetate production at this time point. As with LV-ABX, it is possible that *Bifidobacterium adolescentis* utilised arabinofuranohydrolases to cleave the outer arabinofuranosyl residues of the wheat LV-ABX (Rivere *et al*.,2014). Regarding the shift towards Bacteroides growth later in the culture could be due to *Bacteroides thetaiotaomicron,* a bacterial group that have been shown to utilise many glycosidases for polysaccharide degradation (Flint et al, 2012). Furthermore, *Bacteroides ovatus* contain two PUL, integral that are activated when grown on wheat arabinoxylan (Rogowski *et al*, 2015).

β -GLU (DP-5-28) is a polysaccharide formed of glucose residues, and linked by β (1–4) and β (1–3) glycosidic bonds, and appears as though fermentation of this complex structure is initiated by *Bifidobacterium*, based on significant increases in cell numbers earlier on in the culture, and characterised by the elevated acetate concentration and a decrease in *Desulfulvibrio* cells numbers (P=0.03), possibly from competitive inhibition. In this study RS, exhibited a slower fermentation, with increases in bifidobacterial cell growth of 1.52log between 0-24h, however this was not significant, most likely due to variation in starting inoculum of the batches. During *in vitro* fermentation of RS, Wang *et al* reported that bifidobacterial groups can effectively metabolise high-amylose starch granules, (Wang *et al.,*

1999). Further to this, in a metagenomics study, it was reported that genes coding for glycoside hydrolases (GH) required for degrading starch and starch hydrolysate were higher in most bifidobacterial strains than in strains of other genus, and that starch hydrolysates were favourable carbon sources for *Bifidobacterium* (Liu *et al*., 2016).

Only fermentation of PDX resulted in a decrease in *Bifidobacterium* cell numbers between 0-24h followed by significant increases between 24-48h was PDX (structural description), indicating a more sustained fermentation, as was the observation by Probert et al, in which a continuous colonic model was used to assess the effects of fermentation on bacterial growth. Bifidobacterial levels were increased in vessels 2 and 3, simulating transverse and distal regions, and thereby demonstrating that PDX exerted a more sustained prebiotic effect throughout the model (Probert *et al*., 2004).

Fermentation characteristics

It appears that the fermentation of longer chain polysaccharides: LC-FOS, MV-ABX, RS, XOS was dominated by *Bifidobacterium*, with a shift towards *Bacteroides* growth between 24-48h, characterised by a significant elevation in acetate and propionate concentration at this time. Furthermore, substrate fermentation initially dominated by *Bifidobacterium,* but then shifting towards butyrate producing bacteria in the latter stages of the culture was: R-MLX, LV-ABX, GLOS, XOS and OFS, most of these substrates being oligosaccharide in structure. The difference in fermentation characteristics between oligosaccharides and polysaccharides is due to the structural differences between these two types of substrates. The ability of bacterial groups to metabolise different substrates is dependent on their capacity to utilise enzymes, and those with larger enzymatic abilities at their disposal can metabolise larger, more complex substrates. (Sarbini and Rastall, 2011).

Bacteroides have a diverse array of enzymes at their disposal, and are more generally associated with glycan degradation, whereas bacterial groups such as *Bifidobacterium*, though they have glycan degrading capabilities, exhibit a selective preference for starch and fructans (Martens *et al*., 2008).

Effect of fermentation on acetate and propionate producing bacterial groups

A genomic study of *Bacteroides,* revealed that *Bacteroides thetaiotaomicron* strains possess complete polysaccharide utilising locus (PUL), which corresponds with a range of fructan metabolising capabilities (joglekar *et al*., 2018). This could account for the significant growth of *Bacteroides* between 24-48h during fermentation of both OFS and LC-FOS. There was a stimulation of bacterial growth in both earlier and later stages of the culture, indicating a slower fermentation profile, most likely through cross-feeding mechanisms, in which *Bifidobacterium* appears to have initiated fermentation, and then shifted towards *Bacteroides* growth. Lactate produced by bifidobacterial groups can be utilised by Bacteroides to produce acetate, which was significantly increased here, alternatively specific bifidobacterial groups can metabolism LC-FOS, but not all, leaving the majority to be fermented by *Bacteroides.* OFS fermentation stimulated the growth in *Propionibacterium* cells (P=0.02) in the initial 24h, but then shifted towards *Lactobacillus*. *Propionibacterium* are thought to metabolise FOS to produce propionate, and although this was increased, it was not significant. Butyrate is likely to have appeared through cross-feeding mechanisms most likely from the conversion of lactate, which is produced during *Lactobacillus* growth (Kolida S & Gibson GR 2007). Similarly, to LC-FOS and OFS, MV-ABX fermentation shifted towards *Bacteroides* growth between 24-48h (P<0.00), reflected in the elevated acetate reported between 0-24h. Again, it is likely that cross-feeding occurred, but possibly through a different mechanism than LC-FOS and OFS. Some bifidobacterial groups can cleave the LV-ABX side groups, using arabinofurinosidases (Wang *et al*., 2014), whereas groups such as *Bacteroides,* are able to metabolise the whole polysaccharide: ~20% of *Bacteroides* genome is associated with the transport and breakdown of a wide variety of polysaccharides, such as ABX (Schwalm III and Groisman, 2017).

RS is a soluble polysaccharide that comprises 60% amylose, a helical polymer of α-D-glucose units and 40% amylopectin with highly branched polymers of glucose, Bacterial growth shifted towards *Propionibacterium* growth between 24-48h (P=0.06). This slower fermentation could be attributed to the complex structure of RS, as a full set of glycosidases is required to fully metabolise polysaccharides, or a number of bacterial groups are required for full metabolism (Grondin et al., 2017) In Wang *et al in vitro* investigation of RS, it was reported that *Propionibacterium* could degrade the amylopectin fraction of RS (Wang et al

1999) and this could potentially account for increases in this bacterial group, as well as elevated propionate levels at this time.

Effect of fermentation on butryate producing bacterial groups

The fermentation of XOS appears to have shifted from *Bifidobacterium* towards R*oseburia* ($P<0.00$) and *Lactobacillus* ($P=0.03$) between 24-48h. This indicates a slower fermentation, potentially through cross-feeding mechanisms. XOS may be degraded by *Roseburia intestinalis*, through their ability to ferment xylan (Mirande *et al*, 2010), and though *Roseburia* are generally butyrate producing bacteria, which was significantly increased here, Qing *et al* study observed that *Roseburia intestinalis*, were able to utilise the deoxy sugars rhamnose and fucose fractions, present in small quantities in XOS (Qing *et al*., 2013). Furthermore, a transcriptomics study by Scott *et al*, observed that through transcription profiling, *Roseburia inulinivorans* was able to convert the fucose fractions of XOS to propionate via the propanediol pathway (Scott *et al*, 2006). This could account for the significant rise in propionate production between both 0-24 and 24-48h. The fermentation of GLOS led to similar fermentation characteristics, whereby Bifidobacterial growth shifted towards *Roseburia* ($P=0.03$) later in the culture, characterised by elevated butyrate levels. LV-ABX is a polysaccharide that comprises a linear backbone with ß-d-xylopyranosyl (Xylp) residues linked through (1 → 4) glycosidic linkages, and α-l-Arabinofuranosyl (Araf) residues. In this experiment, LV-ABX exhibited a fast fermentation rate, only significantly increasing growth of bacterial groups between 0-24h. Bifidogenic effects were induced during this time ($P=0.01$) as did growth in *Roseburia* cell numbers, close to significance ($P=0.06$). The elevated acetate and butyrate production between 0-24h is evidence of increased activity by these bacterial groups. An *in vitro* investigation of arabinoxylan by *Bifidobacterium adolescentis*, reported that arabinofuranohydrolases were used to cleave the outer arabinofuranosyl residues of arabinoxylan (Rivere *et al*., 2014) and could account for their increase in numbers.

R-MLX did not exhibit a slower fermentation profile as expected, instead *Bifidobacterium* and *Eubacterium rectale* were significantly increased between 0-24h, accounting for the elevated levels of both acetate and butyrate, but no significant results in the latter half of the culture.

Limitations

There was a large SE across the batches, most likely from inter-individual differences in starting composition in faecal inoculate. Differences have been reported in microbial composition between lower and higher BMI (Andoh *et al*., 2016) and the BMI of the three donors ranged from normal to overweight (19.5-28 kg/m^2) which may have contributed to the large SE.

All three donors were from different parts of Europe, which may have caused different responses to the same prebiotic. Geographical/racial variation in the composition of gut microbiota is mainly attributed to varying environmental exposure such as diet (Gupta *et al.*, 2017). Individual responses to the same prebiotic were investigated *in vitro*, and when large differences in response were reported it was concluded that investigating potential prebiotic activity through *in vitro* substrate fermentation requires both low level nutrients and nutrient dense cultures to be carried out (Long *et al.*, 2015). In future work, donors will be chosen from a very similar in phenotype, particularly BMI, as well as increasing experimental runs to reduce this type of variation, taking care to ensure there is still sufficient variation for a significant effect to be observed.

Conclusion

In conclusion, all substrates, on fermentation in batch culture conditions increased SCFA above control, and it appears that oligosaccharides and polysaccharides exhibit different pathways of metabolism, that can be potentially manipulated in future work, in order to obtain specific ratios of SCFAs, through stimulation of specific bacterial groups.

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Chapter 5 - An Assessment of the Fermentation Characteristics of 3 Fibre Blends Using an *In Vitro* Continuous Colonic Model

Abstract

Human colonic microbiota can metabolise prebiotic carbohydrates to produce short chain fatty acids (SCFA) that may be associated with a myriad of health benefits. Our aim was to investigate the potential of blending prebiotic fibres, i.e. inulin + gluco-oligosaccharides (I+GLOS), inulin + arabinoxylan (I+ABX) and inulin + resistant starch (I+RS) to achieve sustained SCFA production through the large bowel. Here we assessed this possibility *in vitro* by using a continuous culture fermentation model of the colon simulating anatomically distinct regions of the large intestine. Blends of prebiotics were administered as a powder, at 1.33g twice daily for 18 days and samples collected over 3 days for analyses of SCFAs via HPLC as well as characterisation of changes in the microbial composition, using 16S rRNA based fluorescence *in situ* hybridisation. I+ABX induced a significant rise in propionate production during *in vitro* fermentation (P=0.04) as well as a significant 0.9 $\log_{10}$ cells/mL increase (P=0.02) in cell numbers of bifidobacterial species in V1, simulating the caecum. Acetate was significantly increased following fermentation of all 3 blends in V2: I+RS (P=0.028), I+ABX (P=0.44), I+GLOS (P=0.50). Acetate was also increased in V3, though not significantly, suggesting sustained saccharolytic activity in the transverse and distal regions of the colon. Combining complimentary blends, may increase SCFA production in areas of the colon they are less abundant i.e. towards the distal region, an area more associated with disease risk, due to an increase in proteolytic metabolites (H_2S, ammonia), implicated in inflammatory disease aetiology. Creating a more saccharolytic environment could also be beneficial, for metabolic functions such as appetite regulation and take part in anti-carcinogenic, and anti-pathogenic mechanisms, however further *in vivo* testing is required.

Introduction

The human microbiome comprises gut microbiota that integrate with organs such as the brain and liver to mediate metabolic functions (Evans *et al.*, 2013). The majority of bacterial groups belong to the *Bacteroidaceae* and *Bacillaceae* families (Ley *et al.*, 2008), however composition at genus and species levels is more individualistic and modifiable through dietary intervention with prebiotics, namely "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson *et al*., 2017). These include the production of short chain fatty acids (SCFA), that can act signalling molecules, possibly stimulating satiety hormones via free fatty acid receptors (FFA2) (Tolhurst *et al.*, 2012, Psichas *et al.*, 2015). Furthermore, acetate and propionate have been implicated in lipogenesis and reduced cholesterol synthesis in the liver (Canfora *et al.*, 2015). Additional health benefits associated with SCFAs include inhibiting potentially pathogenic bacterial groups such as certain *Clostridium* spp. *and Desulfibrio* spp., by reducing colonic pH (Macfarlane *et al.,* 1992), and lowering the risk of carcinogenic development by regulating cell growth through butyrate production (Peluzio *et al.*, 2009; Vanhoutvin *et al.*, 2009).

There are some naturally rich food sources of prebiotic fibre (Moshfegh e*t al.*, 1999) however enriched functional foods generally contain higher concentrations, and in a review of *in vivo* studies, consumption of 5g/d oligosaccharides for 11 days elicits bifidogenic effects (Delzenne, 2002). Well studied prebiotics such as fructo-oligosaccharides (FOS) are short chain in length and preferentially metabolised by *Bifidobacterium* (Gibson *et al.,* 1995), One goal is to target prebiotic function towards the distal region of the colon, an area associated with higher disease risk, related to the production of metabolites of proteolytic metabolism, such as phenols and indoles compounds (Macfarlane *et al.*, 1992; Hijova & Chmelarova 2007). It is thought that increasing saccharolytic metabolism towards this region may be beneficial to health.

The physio-chemical structure of prebiotic substrates denotes functionality (Sarbini & Rastal, 2011). By blending novel prebiotic fibres together, and in particular, longer chain fibres such as RS and ABX, the aim was to extend carbohydrate fermentation towards the distal region. Blends were chosen based on their ability to stimulate bacterial groups such as *Bifidobacterium, Propionibacterium* and *Bacteroides* that produce propionate and acetate, as

these metabolites been reported to take part in anorexigenic pathways of metabolism, which was desirable (Psichas , 2015). RS is a polysaccharide classified as RS1-RS4 (Sajilata *et al.*, 2006), defined by the amount of starch that reaches the colon (Englyst *et al.*, 1982). High amylose resistant starch 2 (HAMRS-2), a prebiotic candidate that consists of ~60% amylose, a helical polymer of α-D-glucose units and 40% amylopectin, a soluble polysaccharide of highly branched polymers of glucose (Keenan *et al.*, 2015). Arabinoxylan (ABX) is a hemicellulose with a linear xylan backbone with α-L-arabinofuranose units attached as side chains by β-1 → 4 linkages (Izydorczyk & Biliaderis, 1995, Vardakou *et al.,* 2008, Dodd *et al.,* et al., 2011, Van den Abbeele *et al.*, 2013) as well as gluco-oligosacharides (GLOS), oligosaccharide with alternating α-(1,3)/α-(1,6)-linked glucosyl residues (DP_{ave} =3-6), (Wichienchot *et al,.* 2006). Through blending RS, ABX and GLOS with inulin (LC-FOS), a fructan-type polysaccharide is comprised of (2→1) linked β-D-fructosyl residues (n=2-60) and a (1↔2) α-D-glucose moiety (Mensink *et al*., 2015), we aimed to assess any additive potential prebiotic effect *in vitro*.

Here we utilise a 3 stage continuous colonic culture system (Macfarlane *et al.*, 1998) to assess the impact of *in vitro* fermentation of carbohydrate blends: inulin + resistant starch (I+RS), inulin + α-gluco-oligosaccharides (I+GLOS), inulin + wheat arabinoxylan I+ABX on microbial ecology and SCFA production. This method enables analysis of fermentation in each anatomically distinct region, which was an important consideration as the aim was to sustain SCFA release through the colon.

Methods

Faecal Donors

Feacal samples were collected on the day of inoculation and kept in an anaerobic container 10% H_2, CO_2 and 80% N_2 for no more than 1.5hrs prior to inoculation. Donors were three healthy females BMI 19–23 kg/m^2, non-smokers, aged 25-40 with no history of gastro-intestinal disorders, no anti-biotic use in the previous 6 months and had not consumed prebiotic/probiotic enriched functional foods within 3 weeks prior to participating. Samples were diluted 1:10 w/w in anaerobic PBS 0.1 mol/L at pH 7.4 and homogenised in a stomacher Stomacher 400; Seward for 2 min at 240 paddle beats per minute.

Fermentation media g/L

The gut model medium consisted per Litre of: starch (5g), peptone water (5g), tryptone (5g), yeast extract (4.5g), NaCl (4.5g), KCl (4.5g), mucin (4g), casein (3g), pectin (2g), Xylan (2g), arabinogalactan (2g), NaHCO3 (1.5g), $MgSO_4$ (1.25g), guar gum (1g), inulin (1g), cysteine (0.8g), HCl (0.8g), KH_2PO_4 (0.5g), K_2HPO_4 (0.5g), bile salts (0.4g), $CaCl_2.6H_2$ (0.15g), $FeSO_4.7H_2O$ 0.005g), hemin (0.5g), tween 80 (1mL), vitamin K (10µL).

A three-stage continuous gut model system that simulates the physio-chemical conditions of anatomically distinct areas in the large intestine was used to investigate the fermentation characteristics of the fibre blends. A trio of cascading connected glass fermentation vessels in descending order of smallest to largest volume corresponding with the caecum V1, 80ml at pH=5.5, transverse V2, 100ml at pH=6.2, and distal colon V3, 120ml at pH=6.8. The models were inoculated with 20% wt:v faeces from healthy weight donors aged 25-38 (Macfarlane *et al.*, 1998).

The system was operated at a retention time of 48hr, approximating the transition rate of dietary fibre through the adult human colon. Following inoculation, the gut model was left for 24h to equilibrate at which time the medium pump switched on, which fed medium into V1 and via gravity, through to the connected vessels V2 and V3. The system was run for 8 full turnovers which took 15 days. A turnover representing the rate at which the full volume of the vessel was replenished by medium. At this point steady state 1 (SS1) was achieved, determined by carrying out HPLC analysis of SCFA production over 3 consecutive days and intervention commenced with two 1.33g doses of the blend administered to V1 at 10.30am and again at 3pm. This continued until day 31 when the second steady state (SS2) was determined, again by HPLC analysis of SCFAs over 3 consecutive days. Samples were additionally collected during SS1 and SS2 over 3 consecutive days and centrifuged at 13,000 x g for 10min for analysis, with the supernatant used for SCFA analysis and the resultant pellet for 16S RNA bacterial enumeration.

Novel prebiotic blends

Three different blends of commercially available fibres were chosen: inulin (Fruitafit) sourced from Chimab + resistant starch (RS) (HI-MAIZE® 260) supplied by Ingredion, inulin + α-gluco-oligosacchairdes (I+GLOS) (Bioecolia) suppled byAston Chemicals and inulin + arabinoxylan (Megazyme) from Megazyme (**Table 1).** Blends were tested in triplicate in the continuous culture colonic model**.** Each blend was administered to the gut model system twice a day, at 1.33g, representing a third of the 8g/d to be tested in a human trial later. The aim of scaling down the volume was to reduce the amount of media required to conserve resources.

Table 1. The physio-chemical composition of individual fibres blended together, and fermentation properties tested in a continuous gut model system

Substrate	Source	DP	insoluble fibre %	soluble fibre %	total fibre/100g	kcal/g	Brand name	Supplier
Inulin	Chicory root	10-60	0	90	90	2.00	Fruitafit IQ	Chimab
Resistant starch	High amylose corn	40-600	56	0	56	2.36	Hi-Maize	Ingredion
Gluco-oligosaccahride	Sucrose/maltose	6-17	0	87	87	2.00	Bioecolia	Aston Chemicals
Arabinoxylan	Wheat bran	500	0	70	70	2.3	Megazyme	Megazyme

Enumeration of specific bacterial groups with fluorescent *in-situ* hybridization FISH

Enumeration of faecal bacterial groups was carried out by fluorescent *in-situ* hybridisation (FISH) and flow cytometry (FISH-FLOW). Samples were taken from V1-V3 over 3 consecutive days at SS1 & SS2 and from each sample 750 µl was centrifuged for 5mins at 13000 x g for 5 min, the supernatant then removed and pellet re-suspended with 375 µl filtered PBS 0.22µm filter Millipore, Bedford, MA and 1125 µL of 4% v/v paraformaldehyde. Following incubation at 4˚C for 4 hours, samples were centrifuged at 13 000 x g for 5 min and washed twice in 1ml filtered PBS. Washed cells were then re-suspended in 150 µl filtered PBS and 150 µl ethanol 99% and stored at -20˚C as described by Grimaldi *et al,* (Grimaldi *et al*., 2016).

For permeabilisation, 75ul of the fixed sample was mixed with 500µl of cold PBS at 4°C and centrifuged at 11300 × g for 3 min, the supernatant removed and pellet resuspended with 100 µL of filtered TE-FISH (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8, distilled H_2O) filtered with 0.22µm filter Millipore, Bedford, MA. This was followed by hybridisation steps in which pellets were resuspended in 150 µL of hybridisation buffer (HB) (5 M NaCl, 1 M Tris/HCl pH 8, 30% formamide, ddH_2O, 10% SDS), then vortexed and centrifuged at 1136 × g for 3 min). Pellets were then resuspended in 1 mL of HB and 50 µL aliquoted into Eppendorf tubes, for individual bacterial groups to be enumerated using specific probes (**Table 2**) (Devereux *et al.*, 1992; Wallner *et al.*, 1993; Langendijk *et al.*, 1995; Manz *et al.*, 1996; Franks *et al.*, 1998; Daims *et al.*, 1999; Harmsen *et al.*, 2002; Hold *et al.*, 2003; Walker *et al.*, 2005). For the control, no probes were added to the 50µl sample. NON EUB338 probe was used to control for non-specific binding to EUB338. Simultaneously EUB338 I, II & III linked at their 5' end either to Alexa488 and Alexa647 was used to target total bacterial species, as EUB338 I alone is insufficient for the detection of all bacterial groups and also using EUB I, II, III together allows for more accurate quantification (Daims *et al.*, 1999). In each eppendorf, 4 µL of Eub338 I-II-III linked to Alexa488 was added for total bacteria and 4µL of the specific probe linked with Alexa647 at the 5' end, then incubated in a heating block at 35°C for a minimum of 12 hours to enable hybridisation.

Table 2. Oligonucleotide probes used during FISH for enumeration of bacterial groups.

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

Rrec584	*Roseburia - Eubacterium rectale*	TCAGACTTGCCGYACCGC	Walker *et al.*, 2005
Ato291	*Atopobium, Colinsella, Olsenella* and *Eggerthella* spp.; *Cryptobacterium curtum; Mycoplasma equigenitalium* and *Mycoplasma elephantis*	GGTCGGTCTCTCAACCC	Harmsen *et al*., 2002
Prop853	*Propionibacterium* (Clostridial Cluster IX)	ATTGCGTTAACTCCGGCAC	Walker *et al.*, 2005
Fprau655	*Faecalibacterium prausnitzii*	CGCCTACCTCTGCACTAC	Devereux *et al.*, 1992
DSV687	Most *Desulfovibrionales* and *Desulfuromonales*	TACGGATTTCACTCCT	Hold *et al*. 2003
Chis150	*Clostridium histolyticum* (*Clostridium* cluster I and II)	TTATGCGGTATTAATCTYCCTTT	Franks *et al*., 1998

Following hybridisation, samples were centrifuged for 3min at 13000 x g and supernatant removed. The negative control no probe was re-suspended with cold PBS (4˚C) and analysed using the flow using the BD Accuri™ C6 flow cytometer according to the manufacturer's instructions to determine background noise and subtract this from the analysis. Samples containing probes were then re-suspended with the same volume as negative control. Specific and total bacterial groups were then enumerated taking into account a dilution factor (DF) which was calculated from different volumes used during preparation of the samples and events/µl determined from NON EUB338 and EUB I-II-III probes that were analysed by flow cytometry.

SCFA measured by HPLC

The concentration of total and individual SCFAS was measured using HPLC (Agilent-2) as previously described (Grimaldi *et al*., 2016). A 20µL volume from each sample was injected into the HPLC system with a run time of 45 minutes to allow all peaks required to be measured, including internal standard (I/S) of 10mM 2-ethylbutiric acid. I/S is used to correct peaks and improve accuracy of quantitative analysis. Software (Agilent Chemstation, Hewlett Packard) was used to integrate peaks, and quantification was obtained using calibration curves of individual SCFAS: lactic, acetic, propionic and butyric acid of increasing concentration (12.5, 25, 50, 75, 100mM).

Statistical analysis

Statistical analysis was performed using SPSS for windows version 21.0. Tests for normality were carried out using Shapiro-Wilk. Most of the data were normally distributed. If not, distributions were compared using the Wilcoxon signed ranking tests. Data were analysed by two-way ANOVA and Tukeys post-hoc testing. Additional paired t-tests were applied to assess the significance of single pairs of data. Statistical significance was accepted at $P < 0.05$ for all analyses.

Results

Bacterial analysis by fluorescent *in situ* hybridisation (FISH)

Average bacterial counts of all groups enumerated by FISH detailed in **Table 3** are expressed as $\log_{10}$cells/mL ± standard deviations.

Total bacterial growth occurred following fermentation in all three treatments but was only significant following I+ABX fermentation with a 0.29 $\log_{10}$ cells/mL increase reported in V1 (P=0.011). This increase was largely due to the significant 0.9 $\log_{10}$ cells/mL growth of *Bifidobacterium* observed with I+ABX (P=0.021) in V1. Fermentation of I+RS elicited a significant 0.54 $\log_{10}$ cells/mL growth of *Bifidobacterium* numbers in V1 (P=0.027). Growth of *Bifidobacterium* was observed during fermentation across all treatments in all 3 vessels but not significantly. There was a trend for *Propionibacterium*. increases (P=0.084) during I+ABX fermentation in V1.

Table 3: Bacterial populations in log_{10} cells/mL V1, V2 and V3 of the gut models before SS1 and after SS2 treatment with I+RS, I+GLOS and I+ABX. Data presented as means of the three gut models ± standard deviations SS1 and SS2 and calculated as mean values over three consecutive days. *P < 0.05 significantly different from SS1.

I+RS

	Total bacteria		***Bifidobacterium***		***Bacteroides***		***Propionibacterium***	
	SS1	SS2	SS1	SS2	SS1	SS2	SS1	SS2
V1	9.51 ± 0.06	9.52 ± 0.17	7.92 ± 0.78	**8.46 ± 0.43***	8.35 ± 0.26	8.82 ± 0.08	7.9 ± 0.65	7.92 ± 0.89
V2	9.21 ± 0.01	9.45 ± 0.16	7.97 ± 0.64	8.59 ± 0.43	8.19 ± 0.13	8.08 ± 0.73	7.87 ± 0.64	8.04 ± 0.73
V3	8.92 ± 0.14	9.36 ± 0.03	7.83 ± 0.66	8.51 ± 0.54	7.53 ± 0.26	7.99 ± 0.60	7.65 ± 0.53	7.96 ± 0.60

I+GLS

	Total bacteria		***Bifidobacterium***		***Bacteroides***		***Propionibacterium***	
	SS1	SS2	SS1	SS2	SS1	SS2	SS1	SS2
V1	9.51 ± 0.24	9.67 ± 0.14	8.39 ± 0.54	8.79 ± 0.75	7.92 ± 0.89	7.78 ± 0.86	7.61 ± 1.23	7.88 ± 1.22
V2	9.41 ± 0.13	9.51 ± 0.25	8.28 ± 0.62	8.50 ± 0.65	7.69 ± 0.74	7.84 ± 0.90	7.64 ± 1.08	7.88 ± 0.84
V3	9.00 ± 0.12	9.33 ± 0.29	7.97 ± 0.95	8.36 ± 0.65	7.17 ± 0.55	7.79 ± 0.95	7.26 ± 1.18	7.99 ± 0.80

I+ABX

	Total bacteria		***Bifidobacterium***		***Bacteroides***		***Propionibacterium***	
	SS1	SS2	SS1	SS2	SS1	SS2	SS1	SS2
V1	9.31 ± 0.10	**9.60 ± 0.14***	8.15 ± 0.69	**9.05 ± 0.35***	7.66 ± 0.14	7.76 ± 0.58	8.00 ± 0.57	8.79 ± 0.25
V2	9.31 ± 0.33	9.57 ± 0.27	7.91 ± 1.12	8.96 ± 0.36	7.79 ± 0.97	7.99 ± 1.13	8.07 ± 0.19	8.63 ± 0.45
V3	8.89 ± 0.41	9.23 ± 0.16	7.50 ± 1.13	8.80 ± 0.28	7.38 ± 0.75	7.78 ± 0.75	7.50 ± 0.42	8.59 ± 0.45

There was no significant reduction in bacterial groups, however following treatment with I+RS there was a reduction in the mean cell count for *Bacteroides* ssp., by 0.14 log_{10} cells/mL (P=0.703) and for *Clostridium coccoides* by 0.031 log_{10} cells/mL (P=0.858) in V1. In V2, *Bacteroides* spp*.,* number declined (0.22 log_{10} cells/mL) (P=0.607) and *F. prausnitzii* by 0.28 log_{10} cells/mL (P=0.287). Furthermore, in V3*, C.coccoides* was reduced by 0.53 log_{10} cells/mL (P=0.849) and *Propionibacterium* and *F. prausnitzii by* 0.08 log_{10} cells/mL (P=0.178) and 0.09 log_{10} cells/mL (P=0.207) respectively. During I+ABX fermentation a reduction in *Roseburia* numbers by 0.18 log_{10} cells/mL (P=0.319) occurred and *F. prausnitzii* numbers declined by 0.16 log_{10} cells/mL (P=0.634) in V1. In V2, *Roseburia and F. prausnitzii* were reduced by 0.19 log_{10} cells/mL (P=0.600) and 0.08 log_{10} cells/mL (P=0.821) respectively. During I+GLOS fermentation, there was a small decline in *Bacteroides* spp., and *C. coccoides* numbers in V1: 0.002 (P=0.991) and 0.002 (P=0.458) log_{10} cells/mL respectively.

The potentially pathogenic bacterial group *Desulfovibrio* spp. was reduced in V3 following I+RS treatment by 0.42 log_{10} cells/mL and further to I+ABX fermentation, a reduction of 0.08 log_{10} cells/mL in *Desulfovibrio* spp. numbers was observed in V1 while in V3 *Desulfovibrio* spp. numbers reduced by 0.22 log_{10} cells/mL. Through fermentation of I+GLOS, no reduction *of Desulfovibrio* spp. was observed, in fact in V1, V2 and V3 *Desulfovibrio* spp. numbers increased by 0.45, 0.57 and 0.59 log_{10} cells/mL respectively. *Clostridium histolyticum* declined by 0.44 log_{10} cells/mL during I+ABX fermentation in V3, and this was the only reduction in *C.histolyticum* observed in all three treatments.

There was no significant increase or reduction in *Atopobium* spp*.,* numbers.

SCFA production

SCFAs are expressed in mM ± standard deviations in **Table 3.** V1 simulates the proximal, V2, the transverse and V3 the distal regions of the human colon.

I+RS

	Acetate		Propionate		Butyrate		Total SCFA	
	SS1	SS2	SS1	SS2	SS1	SS2	SS1	SS2
V1	52.21 ± 6.59	74.67 ± 16.21	26.44 ± 1.92	32.13 ± 3.77	18.08 ± 3.62	25.91 ± 2.34	96.75 ± 9.09	132.72 ± 11.10
V2	61.45 ± 7.57	**84.29 ± 5.87***	26.18 ± 3.12	28.38 ± 6.86	21.51 ± 1.24	28.85 ± 5.51	109.14 ± 8.06	141.52 ± 14.94
V3	63.22 ± 14.14	90.21 ± 9.09	26.53 ±2.50	26.34 ± 3.12	23.21 ± 2.30	26.62 ± 6.00	112.96 ± 13.08	143.17 ± 18..01

I+GLS

	Acetate		Propionate		Butyrate		Total SCFA	
	SS1	SS2	SS1	SS2	SS1	SS2	SS1	SS2
V1	61.62 ± 13.51	84.99 ± 12.47	23.81 ± 2.55	28.78 ± 4.16	19.31 ± 7.48	22.50 ± 2.89	104.73 ± 18.99	136.27 ± 8.97
V2	75.28 ± 7.08	**112.22 ± 17.64***	22.58 ± 2.49	26.96 ± 4.54	19.56 ± 2.38	**27.49 4.19***	117.43 ± 6.08	166.66 ± 13.30
V3	76.98 ± 18.77	98.61 ± 26.44	23.10 ± 2.75	26.27 ± 6.06	21.86 ± 3.81	23.90 ± 0.31	121.94 ± 16.53	148.78 ± 29.49

I+ABX

	Acetate		Propionate		Butyrate		Total SCFA	
	SS1	SS2	SS1	SS2	SS1	SS2	SS1	SS2
V1	64.55 ± 1.66	**111.39 ± 16.42***	44.085 ± 7.67	**49.57 ± 6.28***	19.71 ± 6.23	30.28 ± 2.33	128.34 ± 3.28	**191.24 ± 15.93***
V2	75.47 ± 7.46	**121.63 ± 14.61***	25.59 ± 1.49	34.79 ± 3.54	22.90 ± 1.81	33.033 ± 3.81	123.97 ± 6.00	**189.45 ± 15.10***
V3	90.25 ± 11.74	115.28 ± 24.11	30.32 ± 2.51	34.88 ± 4.54	23.10 ± 1.57	28.41 ± 5.79	143.67 ± 10.88	178.58 ± 24.23

Table 3: SCFA production (mM) in V1, V2 and V3 of the gut models before SS1 and after SS2 treatment with I+RS, I+GLOS and I+ABX. Data presented as means of the three gut models ± standard deviations SS1 and SS2 and calculated as mean values over three consecutive days. *P < 0.05 significantly different from SS1.

Total SCFA production increased following fermentation of all 3 treatments, and though there was no two-way interaction, total SCFA production was significantly increased following I+ABX treatment in both V1 (P=0.01) and V2 (P=0.01). An increase of total SCFA was close to significance following I+RS fermentation in V3 (P=0.063).

Acetate production did not increase significantly in V1, however during fermentation of all 3 blends there was a significant elevation in acetate levels: I+RS (P=0.028), I+ABX (P=0.44), I+GLOS (P=0.50).

The only significant increase in propionate production was induced by I+ABX fermentation in V1 (P=0.044) and was also close to significance in V2 (P=0.058). Fermentation of I+GLOS significantly increased butyrate production in V2 (P=0.011) only. Measurement of iso-butyric acid was below the detection limit for calculation. There was also no significant increase in lactic acid production during the fermentations in all three treatments.

Discussion

Blending LC-FOS with ABX elicited significant bifidogenic effects in V1, representing the proximal region of the colon. Significant increases in SCFA production also occurred in V2, as was observed during fermentation of all 3 blends, and though not significant there was an elevation in SCFA production in V3, indicating that a prolonged fermentation had occurred, and that the differences in fermentation characteristics are most likely denoted by their physico-chemical variation, such as chain length, linkages, and ability of bacterial groups to utilise enzymes implicit in metabolism of prebiotics.

An increase in *Bifidobacterium* numbers was desirable as acetate and lactate, produced from their activity is considered beneficial. By reducing pH to create an anti-pathogenic environment, (Clarke *et al*., 2012) and mediating satiety mechanisms (Tolhurst *et al.*, 2012; Canfora *et al.*, 2015; Psichas *et al.*, 2015), acetate and lactate can also take part in cross-feeding with bacterial groups such as *F. prausnitzii* and *Bacteroides* to produce butyrate and propionate. A bifidogenic effect did occur during fermentation of all 3 treatments, most likely through preferential metabolism of inulin (LC-FOS) by bacterial groups such as *Bifidobacterium* spp. (Gibson *et al.*, 1995; Bouhnik *et al.*, 1996; Kolida & Gibson 2007).

However, metabolism of arabinoxylan could account for increased bifidogenic effects reported during I+ABX fermentation bifidobacterial species cannot metabolise the xylan backbone (Rogowski *et al.*, 2015), however some bifidobacterial species such as *Bifidobacterium adolescentis* possess arabinofuranohydrolase-D3 that can cleave arabinosyl side chains (Van den Broek *et al.*, 2005), similarly *Bifidobacterium longum* utilise α-L-arabinofuranosidase to break L-arabinose chains (Margolles *et al.*, 2003). This partial fermentation of arabinoxylan could indicate selective metabolism by bifidobacterial species and therefore be a prebiotic candidate (Vardakou *et al.*, 2008, Van den Abbeele *et al.*, 2013).

Desulfovibrio reduces sulphates to sulphides using electron donors (such as lactate, pyruvate or hydrogen) in the process (Marquet *et al.*, 2009). Although not significant, a reduction of *Desulfovibrio* did occur during I+ABX and I+RS fermentation in V3 and this could be due to bifidogenic effects displacing these bacteria, it may also indicate extended saccharolytic fermentation in this region. The growth in acetate producing *Bifidobacterium* spp., corresponds with significant increases in acetate concentration in V2 during all three treatments, simulating the transverse region of the colon and suggestive of prolonged SCFA output.

Bacteroides comprise ~10%, (and was of interest in this research because of their ability to break down amylose from RS. *Bacteroides thetaiotaomicron* genome was analysed and reported to possess 172 glycosyl hydrolases, and a large number of proteins that associated with polysaccharide metabolism (Xu *et al.*, 2003; Birt *et al.*, 2013) and this ability to degrade a variety of glycosidic bonds, required to metabolise polysaccharides was observed in germ free rats colonised by *Bacteroides thetaiotaomicron* that were fed a high polysaccharide diet (Sonnenburg *et al.*, 2005). Supplementation with high amylose resistant starch 2 (HAMRS-2), was reported to increase butyrate production in the distal region of rats with azoxymethane-induced cancer, when euthanised (Le Leu *et al.*, 2007) and this was most likely due to the complex and organised HAMRS-2 structure exhibiting slow fermentation (Lee *et al.*, 2013; Zhou *et al.*, 2013). However, there was a decline of *Bacteroides* spp*.,* numbers during fermentation in V1 which may be due to increased *Bifidobacterium.* As competitiveness between bacterial groups is relative to specific strains and therefore species interactions and differing responses to prebiotics is a consideration for future research.

Propionibacterium was of interest because of their ability to utilise pentoses from arabinoxylans and colonic lactate to produce propionate through the succinate pathway (Hosseini *et al.*, 2011), favouring decarboxylation of succinate (Delwich, 1958) through transcarboxylase enzymes to produce propionate (Houwen *et al.*, 1991). Propionate is a precursor for *de novo* gluconeogenesis implicated in the attenuation of lipogenesis, through the inhibition of fatty acid synthase expression (Canfora *et al.*, 2015) and is thought to positively impact energy homeostasis. A significant increase in propionate production during I+ABX fermentation in V1 is likely related to the trend for growth in *Propionibacterium* in V1 (P=0.079) during I+ABX treatment.

Increased propionate production was reported in the proximal and distal regions of mice fed wheat arabinoxylan over 28d following euthanasia (Edwards & Eastwood, 1992) and batch culture fermentation of wheat arabinoxylans led to increased propionate production (Karppinen *et al*., 2000), possibly from metabolism of the xylose fraction (Mortensen *et al*., 1988).

SCFAs and propionate, in particular has been implicated in satiety mechanisms through in vitro research (Delmée *et al.*, 2006, Reimer *et al.*, 2012, Tolhurst *et al*., 2012, Chambers *et al*., 2014, Zhou *et al*., 2015), human feeding studies with RS (Willis *et al*., 2009, Bodinham *et al*., 2010, Harrold *et al*., 2014, Mollard *et al*., 2014) and human feeding studies with ABX (Isaksson *et al*., 2011, Hartvigsen *et al*., 2014). This effect could be due to the extended fermentation of these longer, more complex structures effectively increasing the concentration of colonic SCFA and potentially stimulating a greater response of satiety hormones over a sustained period. The overall effect, leading to earlier cessation of an eating episode and reduced food intake to positively impact appetite regulation.

Though all three blends led to bifidogenic effects and a subsequent elevation in SCFA production, it was I+ABX that performed optimally in terms of having the greatest bifidogenic effect, as well as leading increases in *Propionobacterium* leading to a significant rise in SCFAs implicated in appetite regulation: acetate and propionate, and exhibiting sustained fermentation, which was desirable in this research in identifying an optimal blend with the aim of positively impacting appetite regulation.

Conclusion

Blending novel prebiotic fibres results in sustained fermentation, as well as eliciting bifidogenic effects and, in the case of I+ABX an increased growth of *Propionibacterium,* however further testing in human randomised controlled intervention is required to determine if a prebiotic effect occurs.

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Chapter 6:

Investigating the Impact of Chronic Consumption of Inulin Blended with Arabinoxylan on Markers of Appetite Regulation, in Healthy Weight Men.

Abstract

Prebiotics such as inulin can beneficially modify the composition of the microbiota. The gut microbiota is increasingly implicated in the aetiology of the obese phenotype, and saccharolytic fermentation in the gut may influence appetite. By blending arabinoxylan with inulin (I+ABX) it was hoped that consumption would lead to significant bifidogenic effects in the colon and positively regulate satiety in healthy weight men. A double-blind, placebo-controlled crossover study was carried out on 20 healthy weight men who consumed either 9.46g I+ABX or 9.23g flavoured maltodextrin consumed in 2 doses daily for 21 days, followed by 21-day washout, followed by the alternate treatment. Changes to bacterial ecology (FISH-FLOW), SCFA concentration (HPLC), satiety scores (VAS), systemic metabolites (NMR) were assessed and energy intake during an *ad libitum* meal and postprandial appetite were evaluated both before and after treatment with placebo and I+ABX. There was no change in satiety scores following treatment with I+ABX, however there were significant increases in the abundance of bifidobacteria (P=0.017), *Propionibacterium* (P=0.021), as well as elevated acetate production (P=0.009) and reduced food intake (P=0.030). This complimentary blend may be helpful to those in maintaining a healthy weight.

Introduction

Prebiotics modulate gut bacterial composition and include long-chain fructo-oligosaccharide (LC-FOS), and oligofructose (OFS) (Roberfroid, 2007, Gibson *et al*., 2004). Fermentation products of prebiotics include short chain fatty acids (SCFAs) produced by bacterial groups such as *Bifidobacterium.* These play a role in mediating metabolic functions and influence appetite regulation (Psichas *et al*., 2015, Morrison and Preston, 2016, den Besten *et al*., 2013). Acetate and propionate are thought to attach to G-coupled receptors (GPR43) located on entero-endocrine cells that are situated along the length of the colon, and subsequently trigger a release of satiety hormones including glucagon like peptide (GLP-1) and peptide YY (PYY) aiding in reduced food intake.

Inulin type fructans can be hydrolysed from chicory to produce inulin β(2←1) (DP_{av}=12), and further isolated to form OFS (DP_{ave}=4) (Roberfroid, 2007). *Bifidobacterium* preferentially ferment OFS to produce acetate, (Gibson et al., 1995).

Arabinoxylan, a hemi-cellulose comprising a linear xylan backbone with β-(1 → 4) linkages to which α-L-arabinofuranose units are attached as side residues α-(1 → 3) and/or α-(1 → 2) linkages (Izydorczyk and Biliaderis, 1995) are particularly abundant in the cell wall of rye and wheat (REF). Bacterial groups such as *Bifidobacterium* can utilise Arabinofuranosidases to cleave L-arabinofuranosyl residues on the arabinoxylans (Rivière *et al*., 2014). Metabolism of L-arabinofuranosyl residues by strains of *Bifidobacterium* mainly occurs in the proximal region of the colon, potentially leaving the resultant xylan backbone to be metabolised by other bacterial groups with different enzymatic glycosidases, such as *Bacteroidetes* further along the colon (Dodd *et al*., 2011). Increased metabolic activity of *Propionibacterium* can increase the concentration of metabolic end products such as propionate implicated in appetite regulation (Van den Abbeele *et al*., 2013).

It is proposed that a blending prebiotic inulin and arabinoxylan (I+ABX), delivered in a single product will have an enhanced prebiotic effect, feeding a more diverse array of colonic saccharolytic bacteria. It is thought this may attenuate the rapid fermentation rate of the LC-FOS (inulin) and thereby lead to a slow steady release of acetate and propionate in the gut. A 3-stage continuous colonic model with separate but connected vessels simulating the anatomically distinct regions of the colon was utilised to investigate the fermentation

characteristics of 3 novel blends. I+ABX produced the greatest concentration of acetate and propionate in the vessels simulating the transverse and distal region, indicating an elongation of fermentation compared with inulin alone. Following *in vitro* assessment of the fermentation characteristics of this prebiotic blend (Chapter 5), it is suggested that the enhanced production of propionate and acetate in the colon may influence appetite regulation in man, and therefore the impact of chronic consumption of I+ABX on markers of appetite regulation are tested here in a population of healthy weight men. A cross over study design was employed, with three-week intervention periods to assess the effect of a chronic intake of I+ABX on appetite, with use a visual analogue scale (VAS) to measure self-reported satiety in participants before and after consumption of I+ABX and an *ad libitum* lunch to measure changes in energy intake. Changes in bacterial ecology were assessed via 16sRNA FISH-FLOW, and fermentation metabolites by NMR.

Methods

Study Design

A randomised, double blind, crossover acute within chronic appetite study was conducted to investigate the satiety inducing properties of a blended prebiotic fibre inulin + arabinoxylan (I+ABX). This study was approved by the Reading University Ethics Committee (UREC 16/23) and registered with clinicaltrials.gov.uk (Identifier: NCT02846454). There were two 21-day treatment periods and a 21-day washout in between (**Figure 1**). Participants were screened in a short visit to the Hugh Sinclair Unit of Human Nutrition (HSUHN), followed by a familiarisation visit and 4 subsequent study visits at the beginning and end of each treatment period.

Study Days

Participants would arrive at 8am and remain in the unit until 1.40pm on day 0, day 21, day 42 and day 63 of the intervention. The day prior to study visits the participants were asked to keep a 24h record of food and activity. A standardised dinner meal of 400g macaroni cheese **(Table 1)** was provided to be eaten before 9pm, after which time participants were asked to fast for 10 hours prior to the study day. Participants were also asked to refrain from drinking alcohol and taking part in strenuous exercise, as this could affect appetite. Conditions were

controlled during the study day, with meals eaten in a cordoned off area, to minimise external stimuli and distractions. Participants were provided with a breakfast at 8.45am. The standardised breakfast meal comprised of toasted white bread, jam and butter and was the equivalent to 10% SACN daily recommendation energy intake for males (SACN, 2011). Lunch was provided *ad libitum* at 1pm and consisted of cheese and tomato pizza **(Table 1)**. Weighed plates of pizza were bought out every 5 minutes, and previous plates removed rather than providing the food as a buffet style, which has been shown to impact the quantity of food consumed (Wansink *et al*., 2015). Water consumption was standardised on the visit on day 1 and the same volume given to volunteers each subsequent visit days.

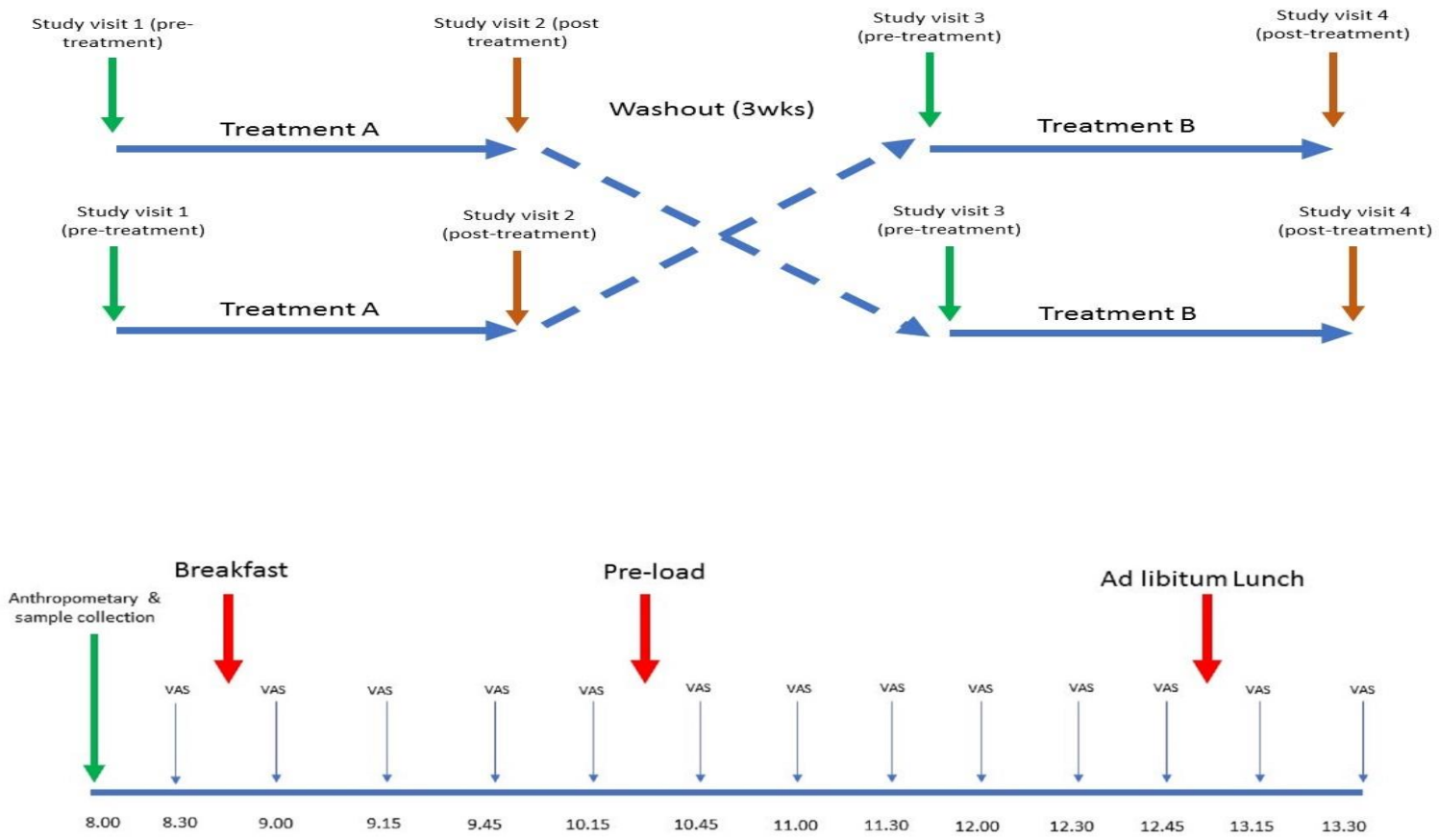

Figure 1: Time line showing the duration of the study (top) and time line of the study day (bottom): indicating the times that visual analogue scale (VAS) questionnaires for satiety scores were completed, also shows when anthropometrics, sample collection and meal times occurred.

Dietary Intervention

Participants were asked to consume either product 1, or product 2 at 10.30am and 3pm (± 30 minutes) daily throughout the 21-day period, and during the study day. The powdered drink was added to 150mL water and mixed in a shaker. Drinks were matched for energy, taste and viscosity and contained either 9.46g of I+ABX or 9.23g flavoured maltodextrin **(Table 1)**.

Table 1: Nutritional composition of study meals and products consumed during the study period.

Meals	Product	Carbohydrate	Fibre	Protein	Fat	Weight (g)	Energy (kcal)
Breakfast	Hovis Best of Both bread	30.4	3	7.6	1.8	76 (2x38)	172
	Hartley's strawberry Jam	12.1	0	0.04	0	20	48.8
	Flora original margarine	0	0	0	3.8	10	35
Lunch	Sainsbury's Cheese and tomato pizza	29.5	2.6	15	10.5	100	278
Standardised dinner prior to study day	Sainsbury's Macaroni cheese	16.9	1.9	7.6	7.4	400	168
Placebo - maltodextrin	Maltodextrin	19.21	0.23	5.97	0	9.23	34
I+ABX	I+ABX	0.41	6.83	1.33	0	9.46	20.7

Participants

Out of the 153 people that responded, 40 people were screened and 33 were eligible to take part. A total of 20 healthy weight males completed the study, which was advertised on social media, free-ads, and poster boards in and around University of Reading campus.

Participants attend a short screening visit, in which full details of the study was disclosed to the potential participant and informed consent was obtained. Following this, anthropometric measurements were taken including: height measured by wall mounted stadometer (m), body composition (Tanita 'BC 418ma' Analyser, Tanita Inc, USA), and blood pressure (OmoronM2). A blood sample was then taken by venepuncture. Participants also completed a three-factor eating questionnaire (TFEQ), which helps to establish if cognitive restraint is part of the habitual eating habit (Löffler *et al*., 2015), as well as the Pittsburgh sleep quality questionnaire (Beaudreau *et al*., 2013), which gives information about sleeping habits. Following the screening visit 2 x 24hr dietary recalls were conducted on 2 non-consecutive days in 1 wk, and this information was used for dietary analysis, to give an indication of fibre intake.

Eligibility was assessed from the results of the screening questionnaires, blood sample and dietary analysis, and this was based on the following inclusion criteria: aged 19–55y, normal weight (BMI: 18.5-24.9 kg/m^2) resting blood pressure (<160/90 mmHg), fasted blood haemoglobin (>125g/l males), gamma GT (<80 IU/l), cholesterol (<6.5 mmol/l), triglycerides (<1.5 mmol/l) and glucose (<5.5 mmol/l), no recent blood donation, sleep duration < 5 hours, cognitive restraint in eating habits (<13 TEFQ), no recent surgery or current disease or taking medication, no history of drug or alcohol abuse, non-vegetarian or those regularly consuming >25 g/day fibre (AOAC definition), no anti-biotic use in the past 6 months or dietary supplementation with prebiotic/probiotic enriched products for at least 3 weeks prior to commencing the study.

Anthropometrics

On each study visit, Anthropometric measurements were taken, using the same equipment as screening and included BMI, hip-waist circumference, body fat percentage. Additionally, blood pressure was taken 3 times and the average recorded. A recent food intake and physical

activity questionnaire was completed by all fasted subjects on each visit. The details of participant baseline anthropometric data are reported in **Table 2.**

Table 2: Participant demographics were collected at the screening process to assess eligibility for the study. Specific markers were measured to ensure they were within a range associated with healthy bodily function, through use of exclusion criteria.

	Participants (n=20)	
	Mean ± SD	**Range**
Age (y)	35.05 ± 9.33	22-55
Height (cm)	1.76 ± 0.08	163-188
Weight (kg)	72.1 ± 9.48	59.8-92.7
BMI (kg/m2)	23.24 ± 2.27	19.9-26
Body fat (%)	15.84 ± 4.66	7-22.3
Haemoglobin	144.56 ± 35.46	136-154
Gamma GT - liver enzyme (IU/L)	22.68 ± 11.06	12.4-45
Cholesterol (mmol/L)	4.8 ± 1.94	3.12-6.3
Triglycerides (mmol/L)	0.93 ± 0.47	0.40-1.47
Fasted blood glucose (mmol/L)	4.96 ± 1.77	2.75-5.46
Systolic mean blood pressure (mm/Hg)	125.79 ± 11.64	105-148
Dystolic mean blood pressure (mm/Hg)	72.42 ± 8.58	59-92
Factor 1 (cognitive restraint of eating)	5.61 ± 3.13	0-11
Factor 2 (disinhibition)	4.88 ± 2.87	0-11
Factor 3 (hunger)	2.88 ± 3.21	0-10
Exercise/wk (h)	2.00 ± 0.45	0-2
Sleep (h/night)	7.2 ± 1.77	6.00-8.00
Energy intake (Kcals/d)	2496.24 ± 595.78	1497-3500
Protein (g/day)	113.94 ± 38.48	68.5-214.77
Fibre (AOAC) g/day	18.75 ± 5.71	8.13-25.9

VAS analysis

An unstructured 100mm line visual analogue scale (VAS) was used to measure satiety and hunger on a computer monitor using Compusense-at-hand software (version 8.8), developed in Guelph, Ontario, Canada by Compusense Inc. This software randomised and collated information about satiety scores. A total of 13 VAS questionnaires were completed by each participant during each study visit every half hour, except after meals when the questionnaire would be given every 15 minutes, totalling 13 questionnaires per visit. The computer monitors were in a cordoned off part of the HUSSN and participants used the same computer each visit to carry out these questionnaires.

Bacterial enumeration with FISH-FLOW

Enumeration of faecal bacterial groups was carried out by fluorescent *in-situ* hybridisation (FISH) and flow cytometry (FISH-FLOW). Participants bought the samples in on the morning of the study day, collected in a pot, and kept in an anaerobic container with a gas generating kit (AnaeroGen™; Oxoid), whereby the atmospheric O_2 is absorbed with the simultaneous generation of CO_2 so that within 30 minutes O_2 levels reach approximately 1% and CO_2 between 9% and 13%. for no more than 1.5hrs after being voided. A small volume (10g) was used to make a 10% w/w faecal homogenate in 1 x PBS (0.1 M; pH 7.4), this was homogenised in a stomacher (Steward 400) for 2 min at 240 paddle beats per minute.

A 375µl volume was taken from the 1:10 dilution, this was centrifuged for 5mins at 13000 x g for 5min, the supernatant then removed and pellet re-suspended with 1125µL of 4% v/v paraformaldehyde. Following incubation at 4˚C for 4 hours, samples were centrifuged at 13000 x g for 5 min and washed twice in 1ml filtered PBS. Washed cells were then re-suspended in 150 µl filtered PBS and 150µl ethanol 99% and stored at -20˚C as described by Grimaldi *et al,* 2016.

For permeabilisation, 75µL of the fixed sample was mixed with 500µl of cold PBS at 4°C and centrifuged at 1136 × *g* for 3 min, the supernatant removed, and pellet resuspended with 100 µL of filtered TE-FISH (Tris/HCl 1M pH 8, EDTA 0.5M pH 8, distilled H_2O) filtered with 0.22µm filter (Millipore, Bedford, MA). This was followed by hybridisation steps in which pellets were

resuspended in 150µL of hybridisation buffer (Chambers *et al*., 2014) (5M NaCl, 1M Tris/HCl pH8, 30% formamide, ddH_2O, 10% SDS), then vortexed and centrifuged at 1300 × g for 3 min). Pellets were then resuspended in 1 mL of HB and 50µL aliquoted into Eppendorf tubes, for individual bacterial groups to be enumerated using specific probes as detailed in **Table 3** (Wallner *et al*., 1993, Daims *et al*., 1999, Langendijk *et al*., 1995, Harmsen *et al*., 2002, Manz *et al*., 1996, Franks *et al.*, 1998, Walker *et al*., 2005, Devereux *et al*., 1992 , Hold *et al*., 2003). For the control, no probes were added to the 50µl sample. NON EUB338 probe was used to control for non-specific binding to EUB338. Simultaneously EUB338 I, II & III linked at their 5' end either to Alexa488 and Alexa647 was used to target total bacterial species, as EUB338 I alone is insufficient for the detection of all bacterial groups and also using EUB I, II, III together allows for more accurate quantification (Daims *et al.*, 1999). In each eppendorf, 4 µL of Eub338 I-II-III linked to Alexa488 was added for total bacteria and 4µL of the specific probe linked with Alexa647 at the 5' end, then incubated in a heating block at 35°C for a minimum of 12 hours to enable hybridisation.

Table 3. Oligonucleotide probes used during FISH for enumeration of bacterial groups.

Probe Name	Target species	Sequence 5' to 3'	Reference
Non Eub	Non bacteria	ACTCCTACGGGAGGCAGC	Wallner *et al.,* 1993
Eub338‡	Most bacteria	GCTGCCTCCCGTAGGAGT	Daims *et al*., 1995
Eub338II‡	Most bacteria	GCAGCCACCCGTAGGTGT	Daims *et al*., 1995
Eub338III‡	Most bacteria	GCTGCCACCCGTAGGTGT	Daims *et al*., 1995
Bif164	*Bifidobacterium* spp.	CATCCGGCATTACCACCC	Langendijk *et al*., 1995
Lab158	*Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis;* all *Enterococcus, Vagococcus, Melisococcus, Catellicoccus, Tetragenococcus, Pediococcus* and *Paralactobacillus* spp.	GGTATTAGCAYCTGTTTCCA	Harmsen *et al*., 2002
Bac303	Most *Bacteroidaceae* and *Prevotellacea*	CCAATGTGGGGGACCTT	Manz *et al.,* 1996

Erec482	Most *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb)	GCTTCTTAGTCARGTACCG	Franks *et al*., 1998
Rrec584	*Roseburia - Eubacterium rectale*	TCAGACTTGCCGYACCGC	Walker *et al.*, 2005
Ato291	*Atopobium, Colinsella, Olsenella* and *Eggerthella* spp.; *Cryptobacterium curtum; Mycoplasma equigenitalium* and *Mycoplasma elephantis*	GGTCGGTCTCTCAACCC	Harmsen *et al*., 2002
Prop853	*Propionibacterium* (Clostridial cluster IX)	ATTGCGTTAACTCCGGCAC	Walker *et al.*, 2005
Fprau655	*Faecalibacterium prausnitzii*	CGCCTACCTCTGCACTAC	Devereux *et al.*, 1992
DSV687	Most *Desulfovibrionales* and *Desulfuromonales*	TACGGATTTCACTCCT	Hold *et al*. 2003
Chis150	*Clostridium histolyticum* (*Clostridium* cluster I and II)	TTATGCGGTATTAATCTYCCTTT	Franks *et al*., 1998

Following hybridisation, samples were centrifuged for 3min at 13000 x g and supernatant removed. The negative control no probe was re-suspended with cold PBS (4˚C) and analysed using the flow using the BD Accuri™ C6 flow cytometer according to the manufacturer's instructions to determine background noise and subtract this from the analysis. Samples containing probes were then re-suspended with the same volume as negative control. Specific and total bacterial groups were then enumerated taking into account a dilution factor (DF) which was calculated from different volumes used during preparation of the samples and events/µl determined from NON EUB338 and EUB I-II-III probes that were analysed by flow cytometry.

Dietary analysis

Weighed food diaries were completed in the 24h period prior to each study visit, which were then collected at the beginning of each study visit (n=4). Dietplan 6 was used for analysis to compare the intake of individual macronutrients (g/24h) and energy intake (Kcal/24h) both before and after treatment with placebo and I+ABX.

SCFA analysis

The concentration of total and individual SCFAs was measured using HPLC (Agilent 1260 Infinity) as previously described by Grimaldi *et al*., 2016 From the faecal homogenate, 320µl of the supernatant was filtered using 0.22µm filter (Millipore, Bedford) and mixed with 120µl internal standard (I/S) which was 2-ethylbutiric acid (10mM) giving a ratio of 1:4, and this was then added to HPLC vials. By adding the same amount of I/S to each sample and standard and using the ratio of the area of the peak analyte and I/S, the peaks can be corrected, and quantitative analysis is improved instead of keeping track of absolute peak area which may have volumetric losses in preparation and injection. A 20µl volume was then injected into the HPLC system with a run time of 45 minutes to allow all peaks required to be measured. Agilent (Chemstation) software was then used to integrate the peaks, using calibration curves of individual SCFAS: lactic, acetic, propionic and butyric acid of increasing concentration (12.5, 25, 50, 75, 100mM).

NMR

Urine collection and storing

Urine samples were collected from each fasted participant on the morning of each of the four study visits, on days: 0, 21, 42, 63. Urine samples were kept cold in transit to the unit with freezer blocks and cool bag. Once the sample was received, approximately 45mL was transferred into a 50mL falcon tube where it was spun for 12000 g at 4˚C for 10 mins and subsequently aliquoted into a labelled 1.5mL eppendorf and stored at -80˚C pending NMR analysis.

Nuclear Magnetic Resonance Spectroscopic Analysis

Based on the hypothesis that variation in metabolites will occur between treatment and control, an untargeted metabolic phenotyping approach was chosen to detect as many metabolites as possible (as opposed to detecting a defined set as is the case with targeted metabolic phenotyping approaches). The spectral data acquired following NMR analysis enables quantification of metabolite concentration as well as information on chemical structure to assist with assignment of metabolites. Based on the chemical composition of the molecule, a characteristic pattern of peaks is generated, where peak intensity is reflective of concentration and the number and multiplicity of peaks (splitting pattern) enables identification. In this study, 2D NMR experiments were conducted as well as 1D, allowing for overlapping peaks to be viewed, that may otherwise be hidden in the 1D axis (Alonso *et al*., 2015).

Store urine sample were defrosted in a fridge 4˚C for 30 minutes. Once thawed, 400 µl was taken from each sample and added to 200 µl phosphate buffer and transferred to a 5mm NMR tube. Phosphate buffer (1Ltr): comprising D_2O (1Ltr), 28g of 2M disodium hydrogen phosphate (Na_2HPO_4), 4.8g of 0.04M sodium dihydrogen phosphate (NaH_2PO_4), 172mgTrimethylsilylpropanoic acid (TSP) ($C_6H_{14}O_2Si$) and 195mg sodium azide (NaN_3) was prepared. The pH was adjusted to 7.4 with 5M HCl or NaOH. Buffer was added to the urinary samples to maintain a narrow range, since variations could cause peak shifts in the NMR spectra. Heavy water (D_2O) was used in the phosphate buffer to serve as a lock signal for the spectrometer and thereby reducing interference. Also added to the buffer solution was TSP

which produces a singlet chemical shift at 0ppm (for calibration of acquired spectra) as well as sodium azide as a preservative.

Quality controls (QC) were included in the experimental run to assess for potential instrument or technical variation and to provide confidence in the reproducibility of the acquired data. A composite QC sample was created by taking a 20ul volume from each participant sample and mixed together in a vial. This QC sample was run every 10 participant samples. The samples were randomised to address order biases, and 60 samples were run at a time (15min per sample) using an automated sample injector.

The NMR analysis was carried out as previously described by Dona *et al.,* (Dona *et al*., 2014). The 1D NMR experiment used to acquire data included a pulse sequence with pre-saturation of the water peak. Urine samples inherently contain a large volume of water, and it is therefore necessary to suppress this peak to focus on other peaks of biological interest, therefore a probabilistic quotient normalization approach was employed, based on calculating the most probable dilution factor by looking at the distribution of quotients of the amplitudes of a test spectrum by those of a reference spectrum. This method has been demonstrated to be a robust approach to correct for differential dilution in complex biological mixtures commonly measured in NMR-based metabolomics studies (Dieterle *et al*., 2005).

Data processing

To begin with, the NMR spectra were processed using Bruker Topspin software (version 3.5, Bruker, Karlsruhe, Germany) where spectra were phased, baseline corrected and referenced to the TSP singlet peak at 0ppm. To prepare for multivariate statistical analysis, data were imported into the Matlab environment where they were digitised into 32000 spectral descriptors (variables). Data were aligned, normalised, using the probabilistic quotient normalisation approach (Dieterle *et al*., 2006) and regions corresponding to the water signal (4.5-6.0ppm) and other regions not containing biological information (such as the TSP peak) were removed.

Data analysis

Since NMR spectroscopic data can include peaks corresponding to one metabolite as well as multiple peaks corresponding to the same metabolite, it is most appropriate to use a multivariate statistical analysis approach to model the spectroscopic data, which enables observation of patterns based on the entire metabolite profile. Digitised spectral data was imported into the SIMCA-P software programme (version 14.1, Umetrics AB, Umea, Sweden) to conduct the multivariate analysis. Initially, unsupervised principal component analysis (PCA) was carried out to gauge an initial inspection of trends in the samples and define any outliers to be excluded from the analysis (based on those outside of the ellipse in the PCA scores scatter plot, which represents a 95% confidence level). A supervised model was then used to explore trends further, using orthogonal projections to latent structures discriminant analysis (OPLS-DA). OPLS-DA models were evaluated using R^2, an estimate of goodness of fit of the model for the data, and Q^2 as an estimate of the goodness of prediction. Models were constructed to identify trends before and after treatment, and with placebo and I+ABX treatment. Discriminatory metabolites correlating with these classes were interpreted using the "loadings line plot" feature in the SIMCA-P software programme. This enabled visualisation of regions of the spectra that correlated with each class in a format like that of the original NMR spectra, to ease in identification of chemical shift and multiplicity. Furthermore, this plot enabled identification of covariance (direction) of specific metabolites as well as correlation (importance of a metabolite in discriminating one group from the other). Chemical shifts and multiplicity of potentially discriminatory metabolites were confirmed by referring to the original NMR data, and then verified by referencing against chemical standard spectra in in-house databases or the Human Metabolome Database (HMDB) (Wishart *et al*., 2007). Any peaks that could not be assigned using these approaches are labelled "unassigned".

The PCA score plots of NMR data offer a profile of each participant and respective visit (2 timepoints). The plots are classed by either placebo or I+ABX. One outlier was identified as lying quite far away from the elipse, and so this was removed from further analysis.

Statistical Analysis

A linear mixed model with repeated measures for crossover designed studies was used to compare differences in appetite, energy intake, changes to bacterial ecology and SCFA measurements. Fixed factors in the model were visit, treatment, timepoint, and visit* treatment*timepoint. Random factors were age and BMI. Area under the curve (AUC) values were calculated for appetite data using the trapezoid method and a two-way ANOVA repeated measures. Specifically, segmental appetite AUC values were calculated for key time periods: breakfast to morning preload, 45min post preload, 75 min after morning preload following by pre-lunch period >30 min and post-lunch period 30min. These time segments were chosen to explore the gastric, pre-absorptive and post absorptive effects of I+ABX. Data were analysed using SPSS version 21.0 for Windows (SPSS Inc., USA). A P value of < 0.05 was considered statistically significant. Paired t-tests were used to analyse pre-treatment values.

Results

Fasting measures

There were no significant differences in fasting satiety or hunger scores measured using VAS either prior to treatment (P=0.731) or post-treatment (P=0.174) **(Figure 2).**

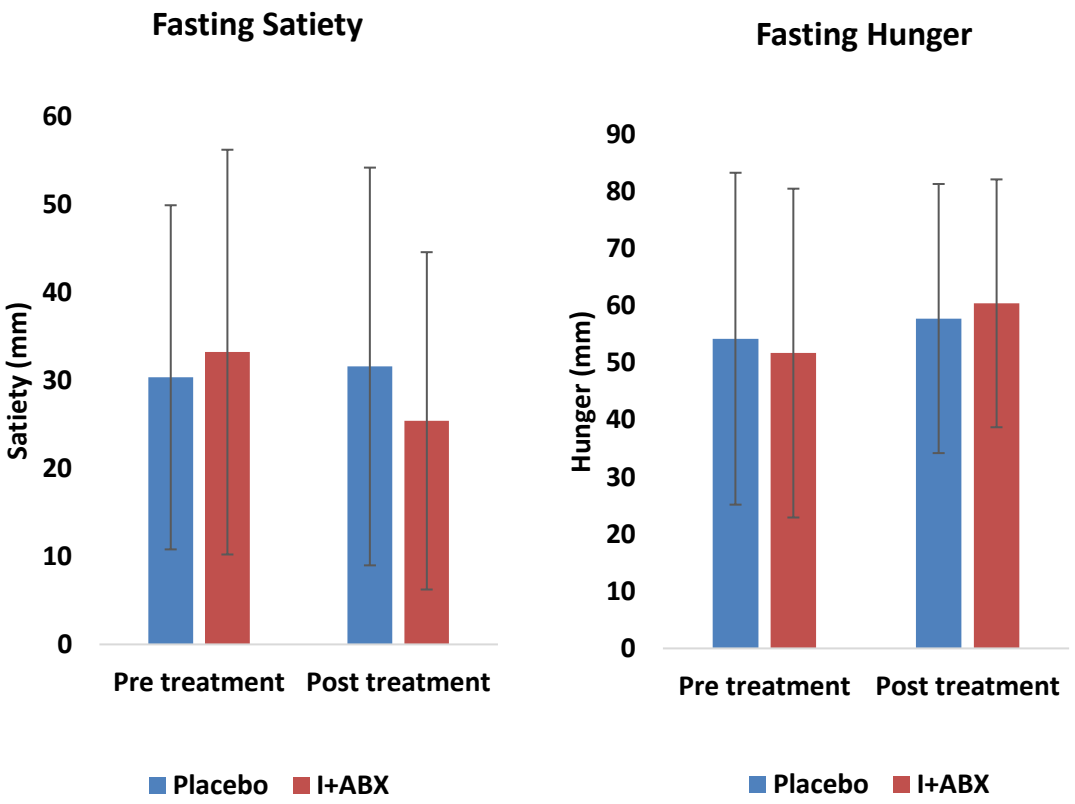

Figure 2: Fasting hunger and satiety ratings Before and after treatment with placebo and I+ABX. Data presented as estimated marginal means ± SD and using 2-way ANOVA repeated measured, no statistical difference was observed between treatments. Statistical significance set at $P<0.05$.

Post Breakfast Satiety

AUC values were measured for the 75min period following consumption of the standardised breakfast. There were no significant differences between satiety scores pre-treatment with placebo or I+ABX (P=0.930), nor was there an effect of time on AUC satiety between placebo mean I+ABX (P=0.936), treatment (P=0.139) or time*treatment (P=0.557). AUC hunger was also measured, however there was no difference in hunger scores pre-treatment with placebo or I+ABX (P=0.809), nor was there an effect of time (P=0.917), treatment (P=0.257) or time*treatment (P=0.219) **(Figure 3).**

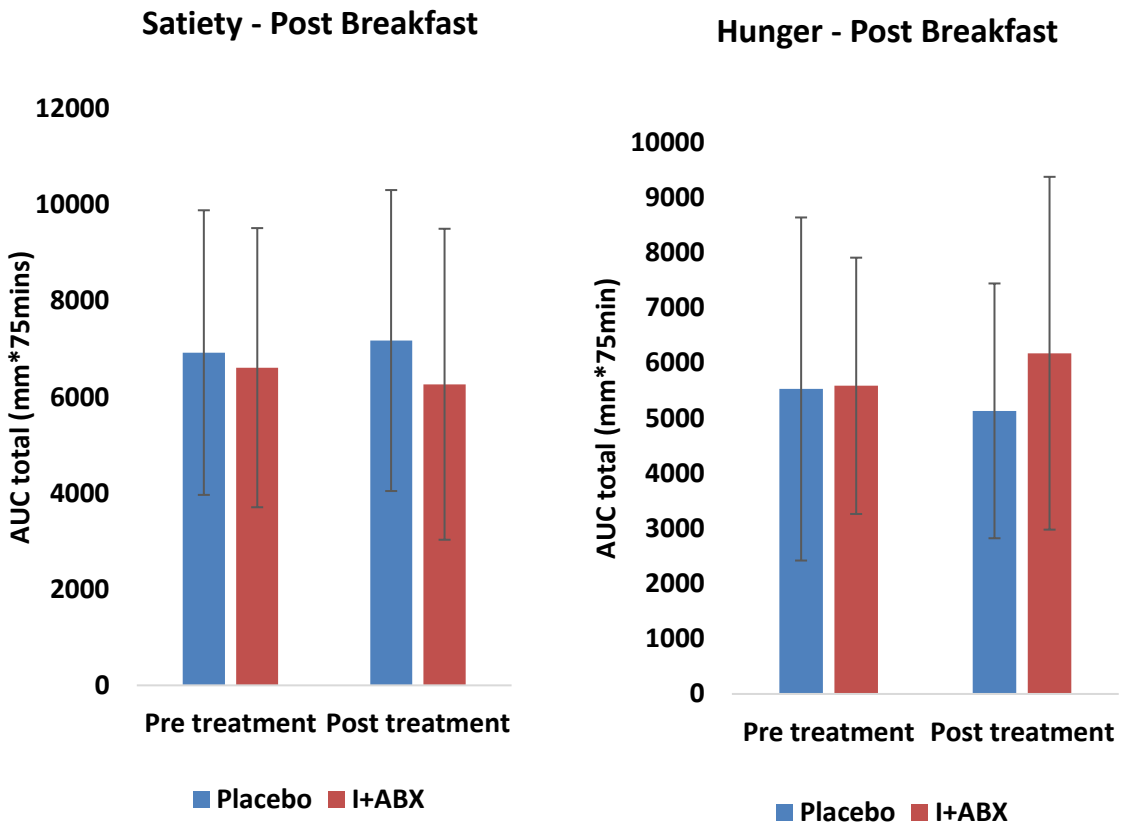

Figure 3: Mean AUC satiety and hunger scores 75mins post breakfast period, measured both before and after treatment with placebo or I+ABX. Data presented as estimated marginal means ± SD and using two-way ANOVA repeated measured, there was no statistical difference between treatments set at $P<0.05$.

Preload effects

AUC values satiety and hunger evaluated 115mins following consumption of the preload (placebo or I+ABX). This timeframe was considered sufficient to determine any pre-absorptive effects such as gastric distension (Benelam, 2009). No different in AUC satiety between treatments at day 0, (P=0.176) and there was no significant effect of time (P=0.745) treatment (P=0.358) or time*treatment (P=0.275) between treatments. There was also no significant difference in AUC hunger scores prior to treatment with placebo or I+ABX (P=0.216) and no significant effect of time (P=0.762), treatment (P=0.182) or time*treatment (P=0.917) **(Figure 4).**

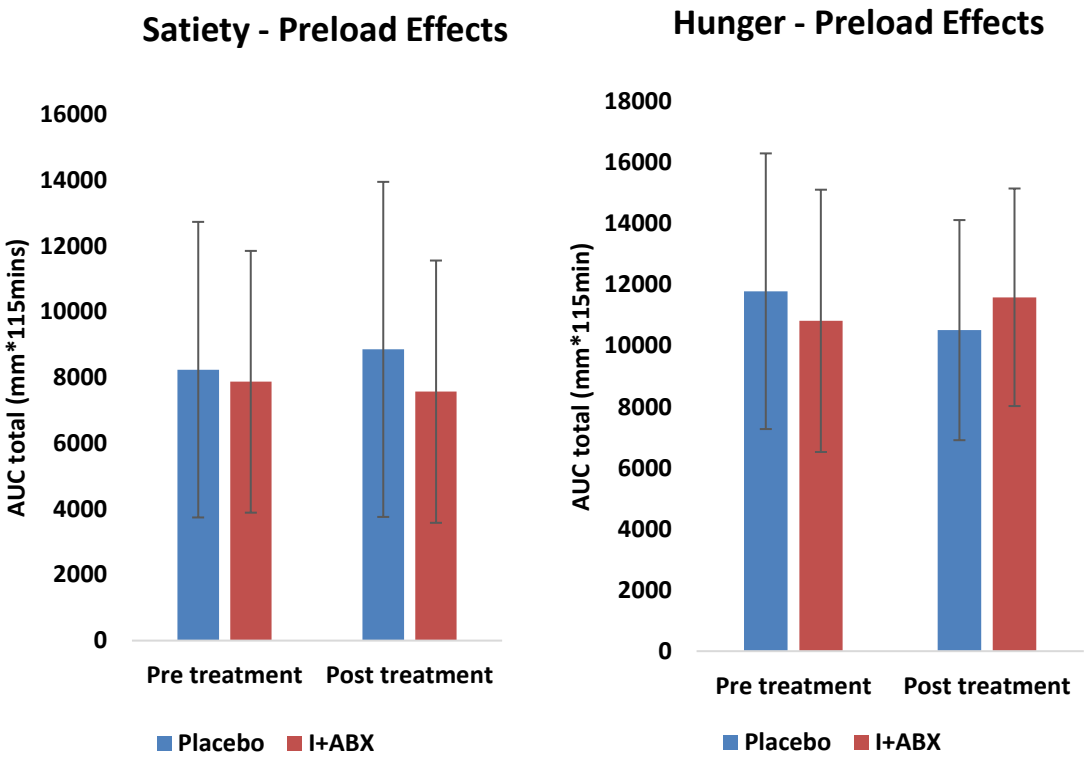

Figure 4: Mean AUC satiety and hunger scores measured 115mins post preload consumption, both before and after treatment with placebo or I+ABX. Data presented as estimated marginal means ± SD and using two-way ANOVA repeated measured, there was no statistical difference between treatments set at $P<0.05$.

Pre-lunch effects

AUC satiety and hunger during the 30min period prior to lunch was evaluated. No different in mean AUC satiety scores prior to treatment with placebo or I+ABX (P=0.693) and there was no significant effect of time (P=0.512) treatment (P=0.808) or time*treatment (P=0.765) between treatments. There was also no significant difference in AUC hunger scores prior to treatment (P=0.653) and no significant effect of time (P=0.819), treatment (P=0.676) or time*treatment (P=0.380) **(Figure 5)**.

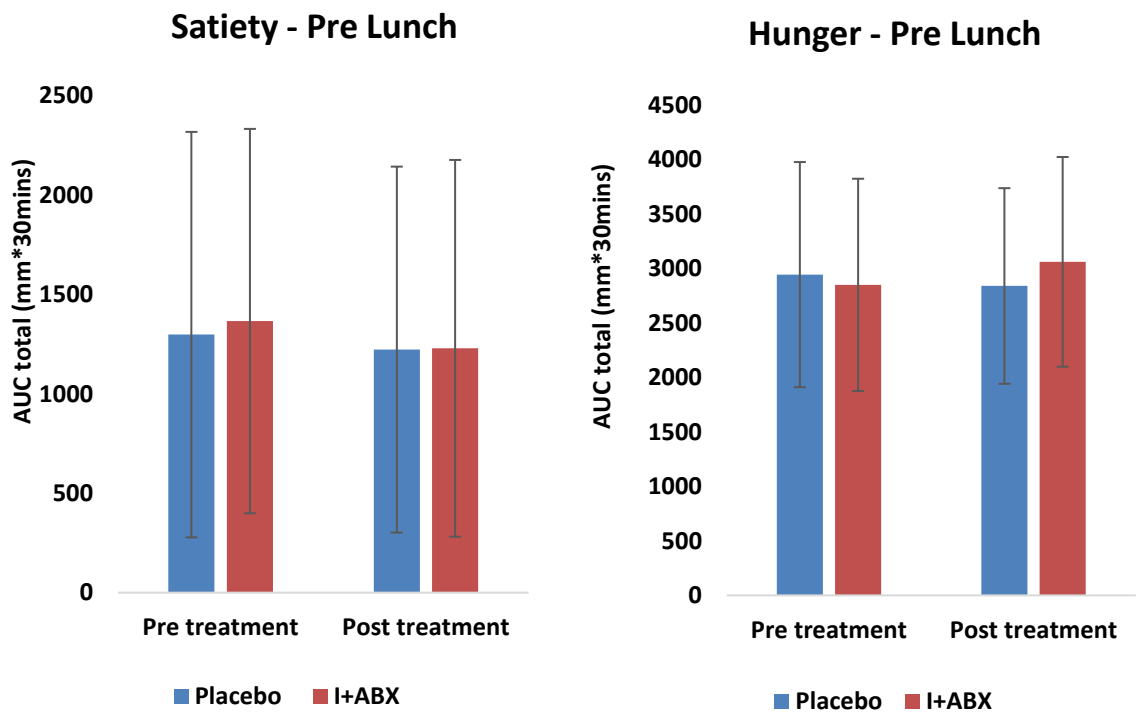

Figure 5: Mean AUC satiety and hunger scores measured during the 30mins prior to consumption of the *ad libitum* lunch meal, both before and after treatment with placebo or I+ABX. Data presented as estimated marginal means ± SD and using two-way ANOVA repeated measured, there was no statistical difference between treatments set at $P<0.05$.

Post Lunch Satiety

AUC satiety and hunger were evaluated in the 30min period post *ad libitum* lunch meal. There was no significant difference in AUC satiety scores between prior to both treatments (P=0.693), but there was an effect of time (P=0.006) treatment (P<0.00) and time*treatment (P<0.00). There was a mean difference of AUC_{total} 696.16 between placebo and I+ABX, with satiety scores being reduced significantly post lunch post-placebo treatment.

There was no significant difference in AUC hunger scores prior to either placebo or I+ABX (P=0.127) and no significant effect of time (P=0.235), treatment (P=0.152) or time*treatment (P=0.782) **(Figure 6)**.

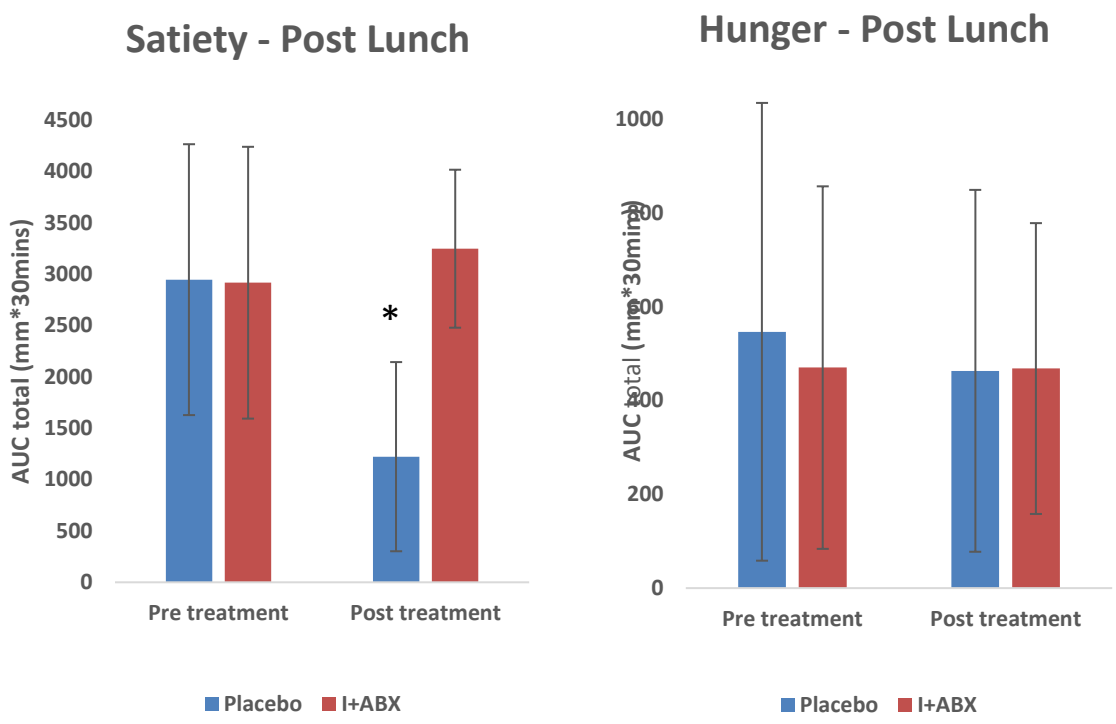

Figure 6: Mean AUC satiety and hunger scores measured during the 30mins following consumption of the *ad libitum* lunch meal, both before and after treatment with placebo or I+ABX. Data presented as estimated marginal means ± SD and using two-way ANOVA repeated measured, there was no statistical difference between treatments set at $P<0.05$.

Ad libitum food intake

Prior to treatment with placebo or I+ABX there was no difference in energy intake (EI), however there was a significant difference in time*treatment post treatment ($P<0.05$). EI increased by 23.91 Kcal ($P=0.204$) following treatment with placebo and decreased by 38.39Kcals ($P=0.030$) following treatment with I+ABX **(Figure 7).**

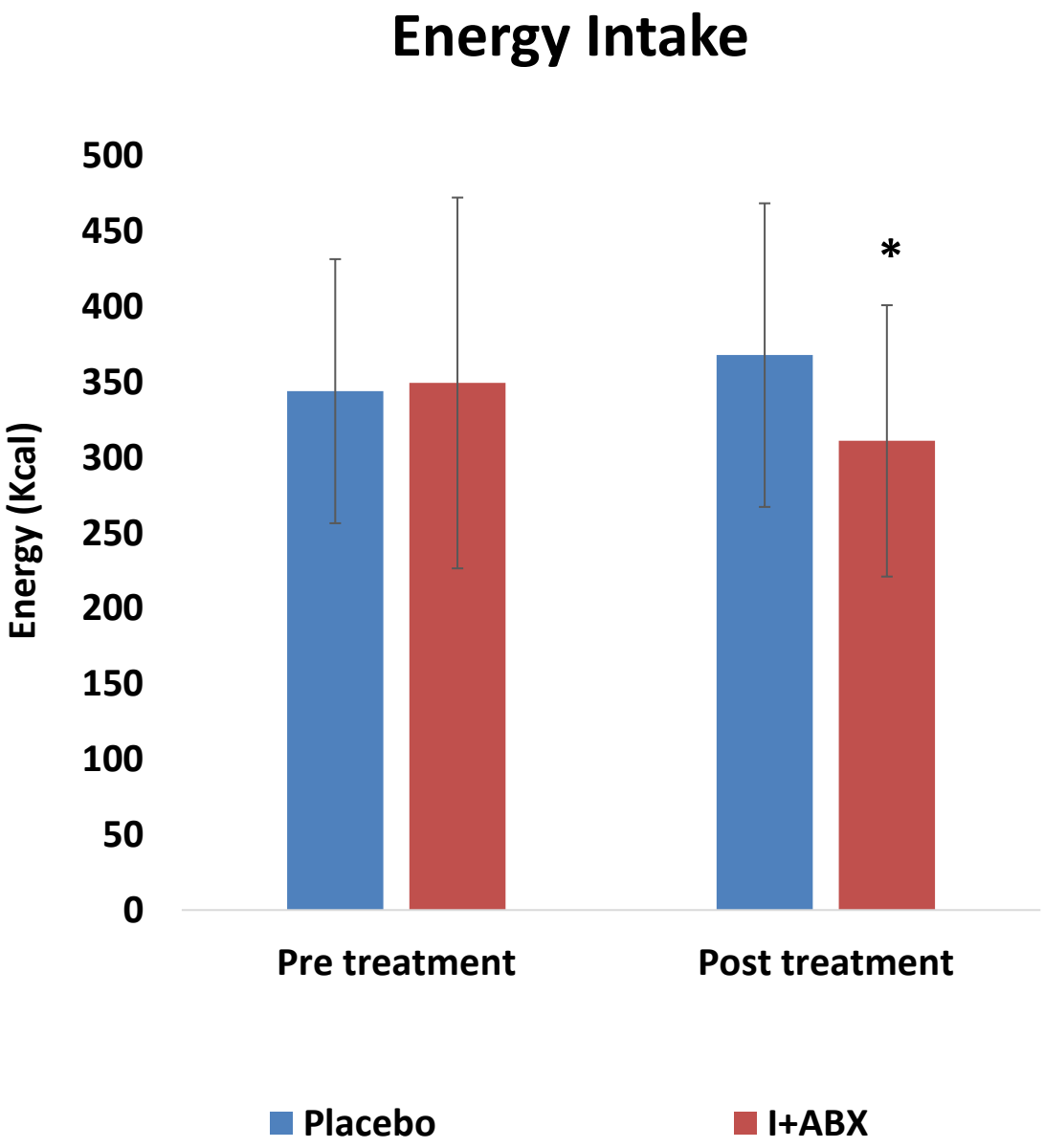

Figure 7: Energy intake during *ad libitum* lunch period. Significant differences between treatments (P<0.05) denoted by an asterisk (*).

Dietary analysis

There was a mean difference of 52Kcal energy consumption in the 24h prior to the pre-treatment visits that was close to being statistically different (P=0.62), however, there was a 418Kcal mean difference in energy intake in the 24h prior to post-treatment visit (P=0.04). During this 24h period, there was a 318Kcal increase in energy consumption following placebo supplementation, which was significantly greater than the 14Kcal increase in energy consumption that occurred following I+ABX supplementation **(Table 4)**.

There was no significant difference in consumption of fat (P=0.146), protein (P=0.21) or carbohydrate (P=0.35) in the 24h prior to the pre-treatment visits.

In the 24h prior to the post-treatment visits however, there was a difference in consumption of protein (P=0.077), fat (P=0.056) and carbohydrate (P=0.71) that was close to significance, and which relates to increased consumption of individual nutrients rather than a reduction, which was more desirable.

There was however, a small reduction in the consumption of individual macronutrients following I+ABX treatment, with a mean 3.44g reduction in carbohydrate consumption over 24h period, compared with pre-treatment over 24h.

Fat and protein consumption increased following treatment with both placebo and I+ABX, however this increase was greater following placebo treatment than with I+ABX.

Table 4. Weighed food diaries collected at the beginning of each study visit (n=4) from volunteers were analysed using Dietplan 6 to compare the intake of individual macronutrients (g/24h) and energy intake (Kcal/24h) both before and after treatment with placebo and I+ABX. One-way ANOVA and paired t-tests were used to determine statistical differences, with significance set at $P<0.05$ and denoted by an asterisk (*).

	PRE-I+ABX	**POST I+ABX**	**PRE-PLACEBO**	**POST-PLACEBO**
PROTEIN (g/24h)	95.41 ± 32.96	97.08 ± 40.19	123.91 ± 81.35	137.83 ± 60.45
FAT (g/24hr)	100.66 ± 25.77	103.75 ± 24.9	130.75 ± 76.65	152.5 ± 67.98
CARBOHYDRATE (g/24h)	229 ± 50.14	225.56 ± 72.40	273.41 ± 108.76	285.41 ± 108.03
ENERGY (Kcal/24h)	2510 ± 228.2	2524 ± 205.04	2562 ± 289.52	**2942 ± 254.21***

Faecal bacteria enumerated (FISH-FLOW)

There was no significant difference in bifidobacterial numbers prior to treatment with placebo or I+ABX (P=0.627) with a 0.05 log_{10} cells difference, however there was a significant mean increase of 0.15log_{10}cells/dry weight bifidobacteria (P=0.017) following chronic consumption of I+ABX.

Propionibacterium growth was not statistically different prior to treatment with either placebo or I+ABX with a 0.015 log_{10} cells/dry weight difference, however there was a significant difference at the end of treatment with an increase of 0.16 log_{10} cells/dry weight (P=0.021) following I+ABX consumption **(Table 5).**

Table 5: Results from faecal analysis using FISH-FLOW. Significant difference as estimated marginal mean between treatments ± SD. Significant set at (P<0.05) and marked with an asterisk (*).

	Placebo		**I+ABX**	
	Day 0	**Day 21**	**Day 0**	**Day 21**
Total bacteria (EUB I II III)	9.85±0.62	9.8±0.6	9.96±0.44	10.06±0.45
***Bifidobacterium* genus (Bif164)**	8.05±0.46	7.88±0.61	8.11±0.65	8.26±0.47
***Lactobacillus* genus (Lab158)**	7.44±1.81	7.15±1.8	7.74±0.45	7.78±0.54
***Propionobacterium* (Prop853)**	8.08±2	7.98±1.95	8.5±0.55	8.66±0.49
Bacteroidetes	7.57±1.87	7.38±1.86	8.01±0.54	7.88±0.56
***Faecalibacterium Prausnitzii* (Fprau655)**	8.12±1.99	8.17±0.96	8.56±0.46	8.68±0.46
***Eubacterium rectale* (Rrec584)**	7.95±1.99	7.89±1.94	8.45±0.49	8.45±0.6
***Clostridium cluster XIVa* (Erec482)**	8.29±2.02	8.3±2	8.84±0.48	8.91±0.53
***Desulfobrio* (DSV)**	7.28±1.81	6.93±1.77	7.6±0.53	7.52±0.62
***Clostridium histolyticum* (Chis150)**	7.4±1.81	7.21±1.75	7.76±0.49	7.86±0.47

There was a ~2% reduction of *Faecalibacterium prausnitzii* following consumption of both placebo and I+ABX. Potentially pathogenic bacterial group *Desulfibrio* (DSV) was reduced by ~1% following placebo, however, there was no reduction in DSV following I+ABX intervention **(Figure 8)**. I+ABX may have attenuated the growth of proteolytic bacterial group *Bacteroides* with a ~2% reduction in abundance during consumption of I+ABX, *Atopobium* was also reduced by ~1%, so the abundance may have shifted to a 1% and 2% increase in saccharolytic *Lactobacillus* and *Propionibacterium* respectively **(Figure 9)**.

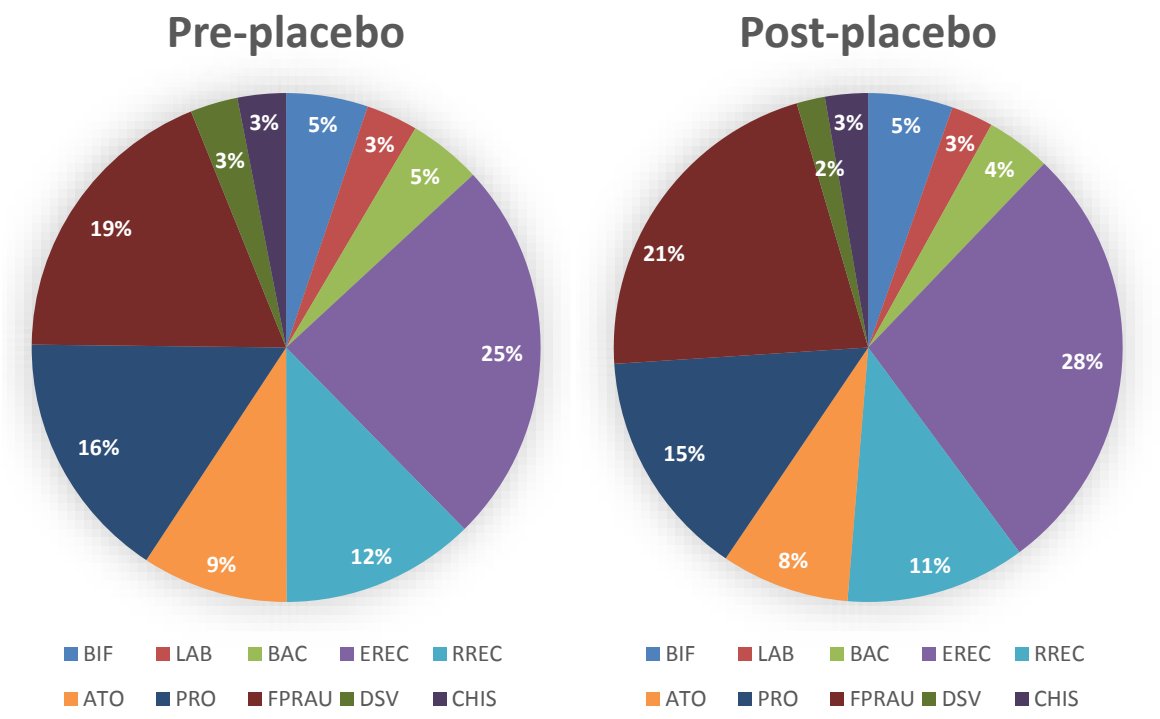

Fig 8. Comparison of the mean relative abundance of bacterial groups before and after treatments with placebo

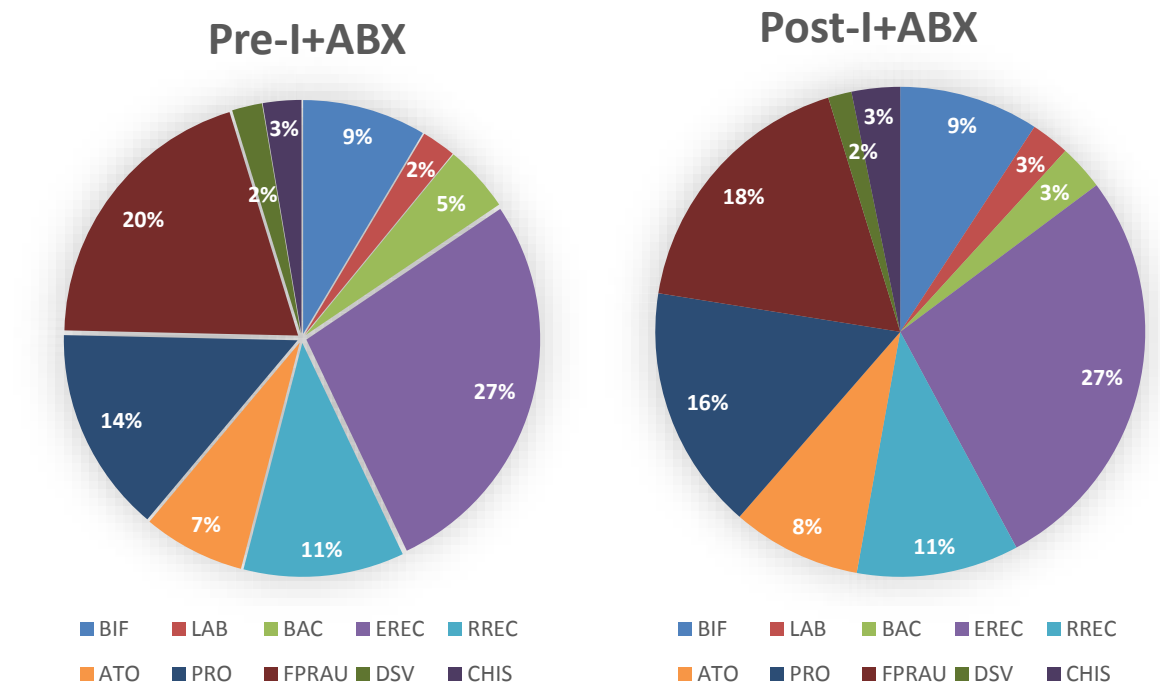

Figure 9. Comparison of the mean relative abundance of bacterial groups before and after treatment with I+ABX.

Faecal SCFAs measured using HPLC

There was no significant difference in total faecal SCFA concentration occurred between treatments at baseline (P=0.879), however there was a significant difference post-treatment (P=0.016) with an increase of 52.04mM/Kg following I+ABX intervention **(Table 6)**.

Prior to treatment there was no significant difference in acetate concentration between placebo and I+ABX (P=0.772), however there was a significant difference between treatments post-treatment (P=0.009) with a 33.81mM/Kg increase in acetate production following I+ABX consumption.

There was no significant difference in propionate production pre-treatment with placebo and I+ABX (P=0.909). Following consumption of I+ABX there was a mean increase in 4.558mM/Kg compared with placebo, however this was not significant (P=0.582).

There was also no significant difference in butyrate production between placebo and I+ABX pre-treatment (P=0.909). Following consumption of I+ABX there was a mean increase in 10.594mM/Kg compared with placebo, however this was not significant (P=0.141).

There was no difference in isobutyrate production between treatments or time points.

Table 6: Results from HPLC analysis of SCFA concentration (mM/Kg dry weight faeces) before and after treatment. Significant differences as estimated marginal mean between treatments ± SD and set at (P<0.05) and marked with an asterisk (*).

	Placebo		**I+ABX**	
	Pre	**Post**	**Pre**	**Post**
Total SCFA	218 ± 80.36	213 ± 79.8	227 ± 91.25	273 ± 81.66
acetate	115 ± 43.58	106 ± 35.67	116 ± 46.9	**146 ± 49.51***
propionate	55 ± 25.15	58 ± 32.91	56 ± 27.38	64 ± 20.67
buyrate	41 ± 21.39	40 ± 26.49	47 ± 25.41	54 ± 23.86
isobutyrate	6 ± 4.2	7 ± 7.3	7 ± 4.43	8 ± 6.61

NMR results

There was no obvious trend identified from inspecting the unsupervised principal component analysis (PCA) plots following treatment with either placebo **(Figure 10)** or I+ABX **(Figure 13)** with no specific group in either quadrant observed. Therefore, the supervised model was produced by firstly carrying out orthogonal projections to latent structures discriminant analysis and using the S-line correlation determined from this to identify trends in this experiment and then comparing metabolic profile with treatments, placebo and I+ABX: baseline vs post treatment. Baseline samples were represented as negative on the S-line, whereas post treatment by the positive values. The height of the peaks denotes the concentration of the metabolite and the intensity of colour represents the correlation with the group (red is highly correlated near to 1, green is closer 0 representing no correlation). These models have good Q^2 predictability, and comparable R^2 goodness of fit.

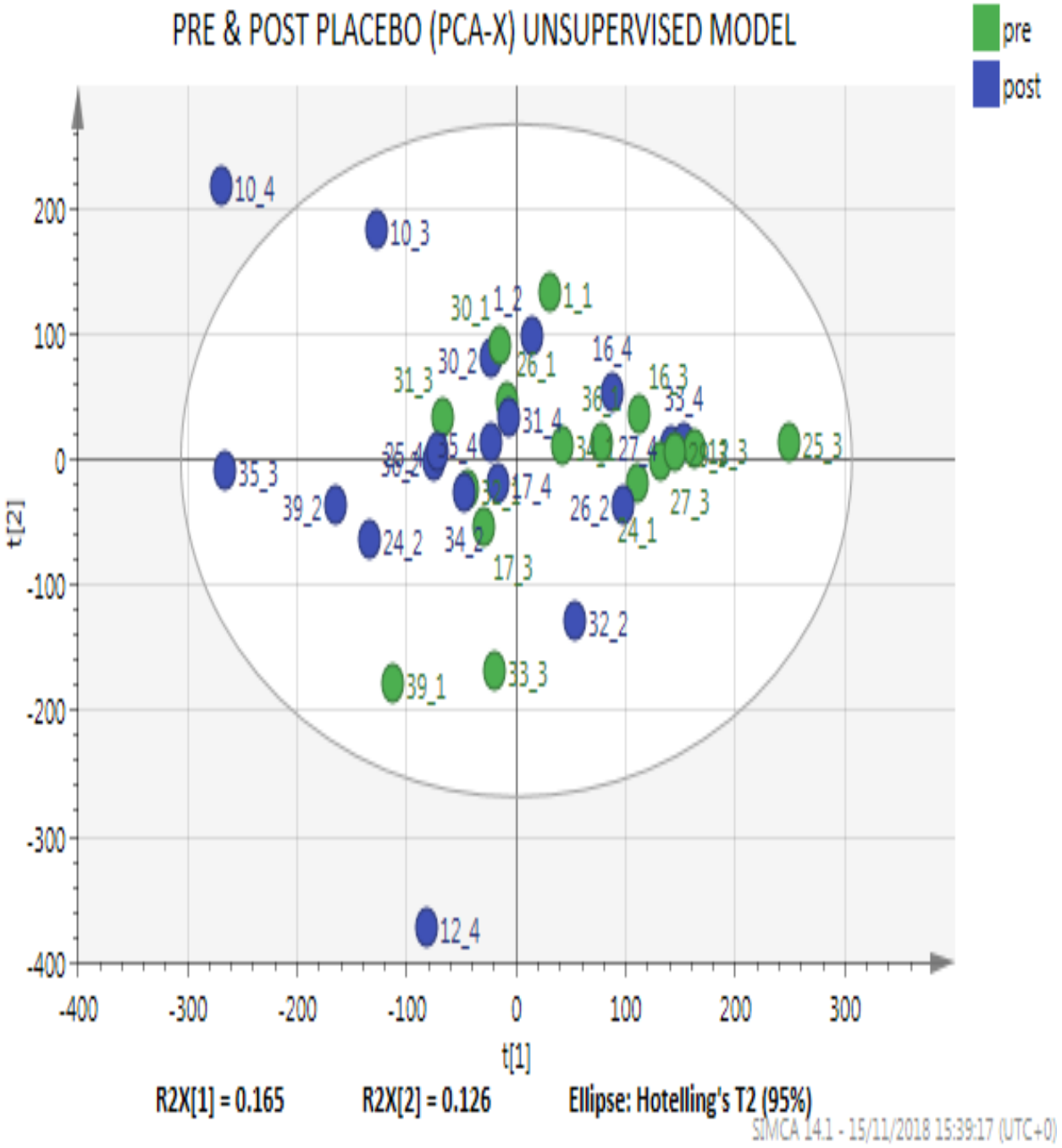

Figure 10: Principal component analysis (PCA) scores scatter plot of the first two principal components to visualise any inherent trends or patterns in the data. Baseline (pre-treatment) is compared with placebo post treatment.

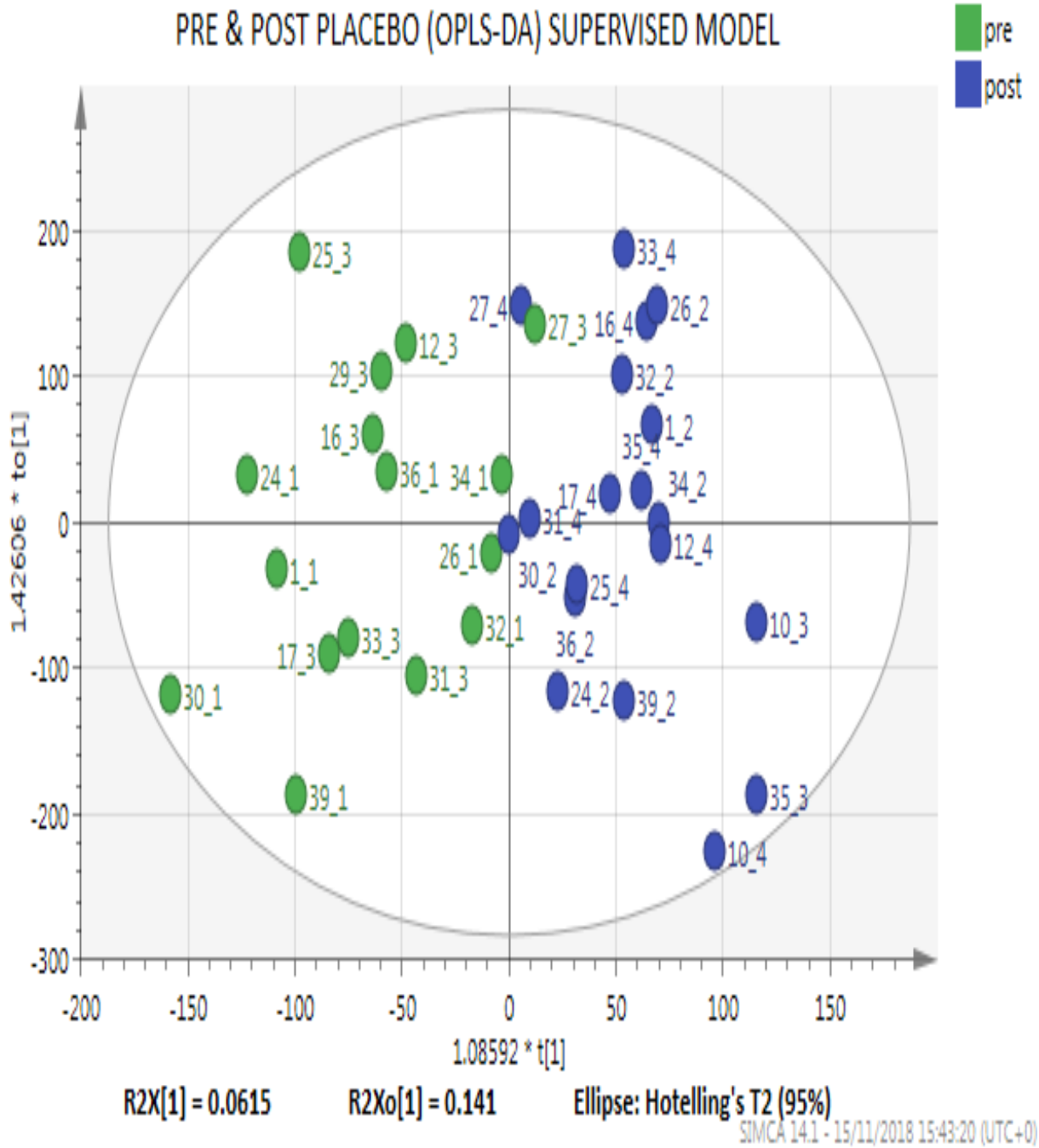

Figure 11: OPLS-DA scores scatter plot where the predictive component is the x axis, and orthogonal component is the y axis. This supervised model comparing before (green) and after (blue) treatment with placebo aimed to maximise separation between classes to view discriminatory metabolites.

There appears to be no signicant effect of treatment with placebo on systemic metabolic profile **(Figure 10)**. However, levels of creatinine were similar before and after treatment with placebo, denoted by the large peak, which indicates a high concentration, nevertheless, as it is green in colour this denoted that there is little or no correlation between creatinine production both pre or post placebo treatment, therefore the placebo appears to have had no or little effect on creatinine levels **(Figure 12)**.

Trimethylamine-N-oxide (TMAO) produced from metabolism of meat, fish and dairy products, was weakly correlated with the post placebo group (0.24) as was 3-methyl-histidine (0.26) **(Table 7)**.

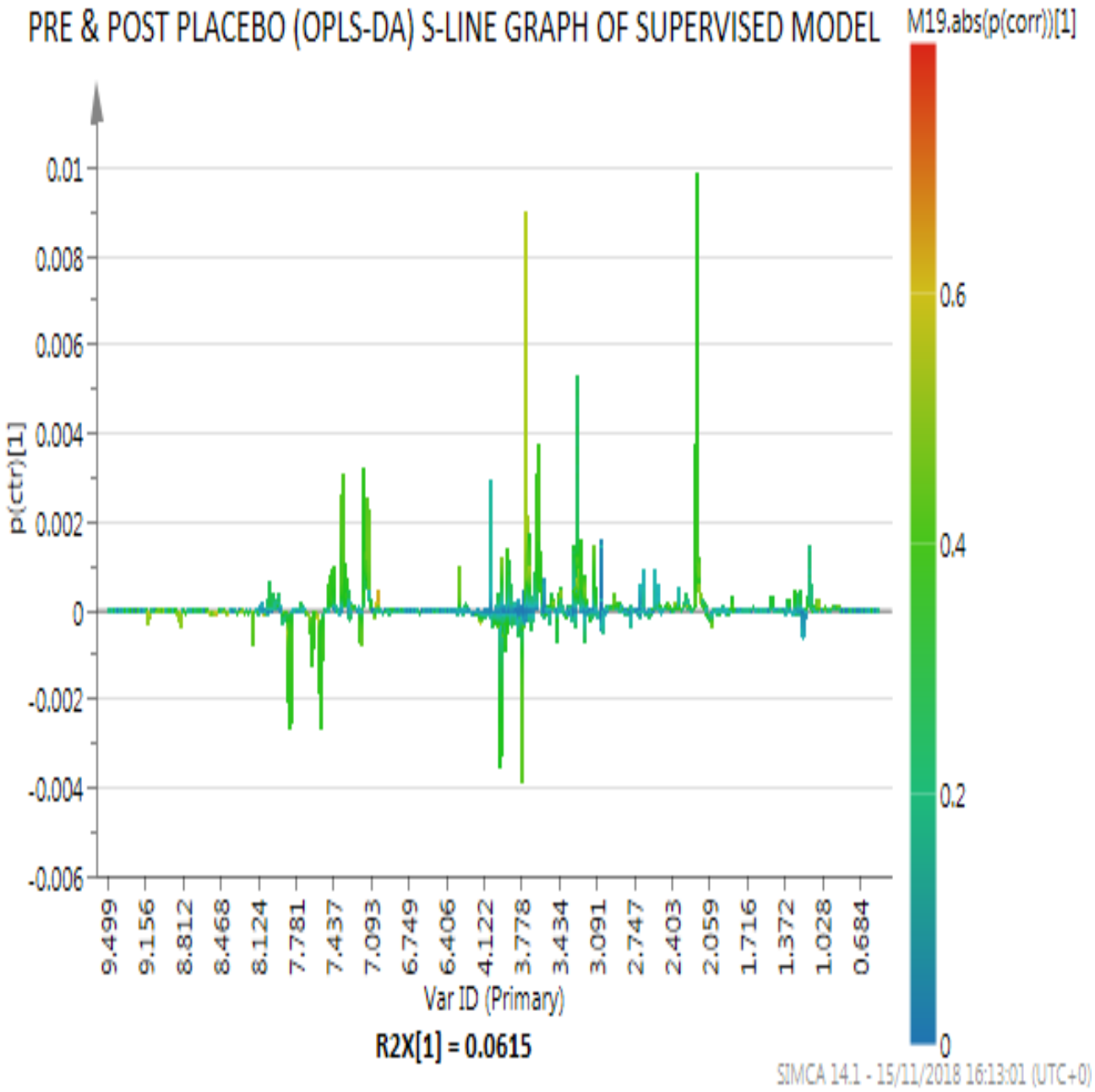

Figure 12: OPLS-DA analysis loadings line plot of NMR spectra obtained before treatment (pointing down) and post treatment with placebo (pointing up). The colour indicates the strength of the correlation with class.

Table 7: Discriminatory metabolites identified following OPLS-DA. Chemical shift position and multiplicity allows identification of metabolites, and correlation value determines if the metabolite is more correlated with either pre or post treatment with placebo.

Correlation	Chemical shift (ppm)	Multiplicity	Metabolite	Function
0.24	3.27	singlet	Trimethylamine-N-oxide (TMAO)	Produced by bacteria from breakdown fish, meat, dairy and eggs. High levels have been associated with heart disease, diabetes and bowel cancer
0.544	3.735	singlet	Unassigned	
0.42	3.97	singlet	Unassigned	
0.26	7.03	singlet	3-Methyl-L-histidine	Biomarker for meat consumption - chicken, biomarker of myofibrillar proteolysis, which may contribute to skeletal muscle loss
0.434	7.13	singlet	Unassigned	
0.401	7.36	doublet	Unassigned	
0.361	7.55	triplet	Hippurate	Carboxylic acid usually those that consume phenolic compounds
0.363	7.83	doublet	Hippurate	Carboxylic acid usually those that consume phenolic compounds

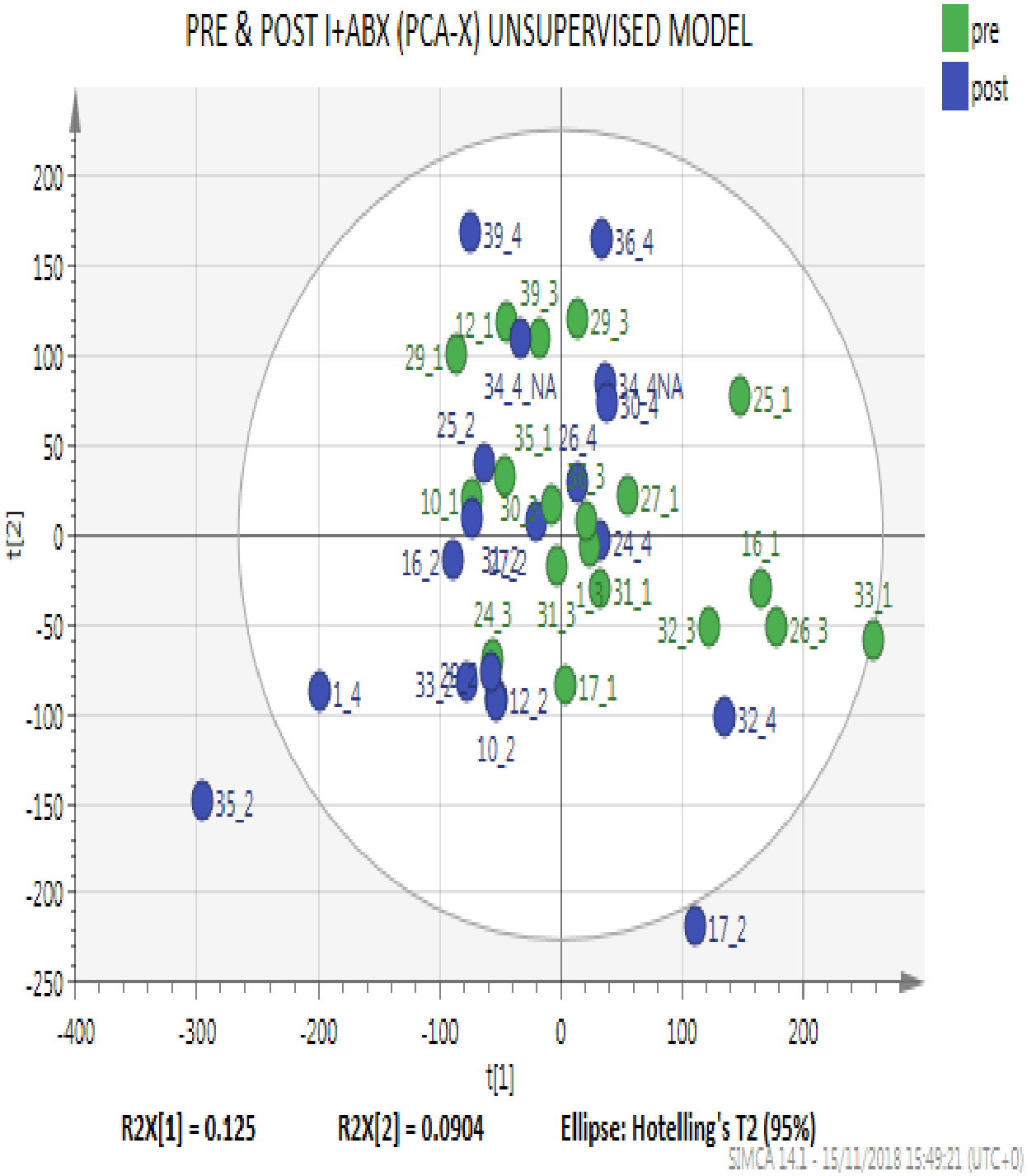

Figure 13: Principal component analysis scores scatter plot with predictive component in the x axis, orthogonal component in the y axis comparing before and after treatment with I+ABX.

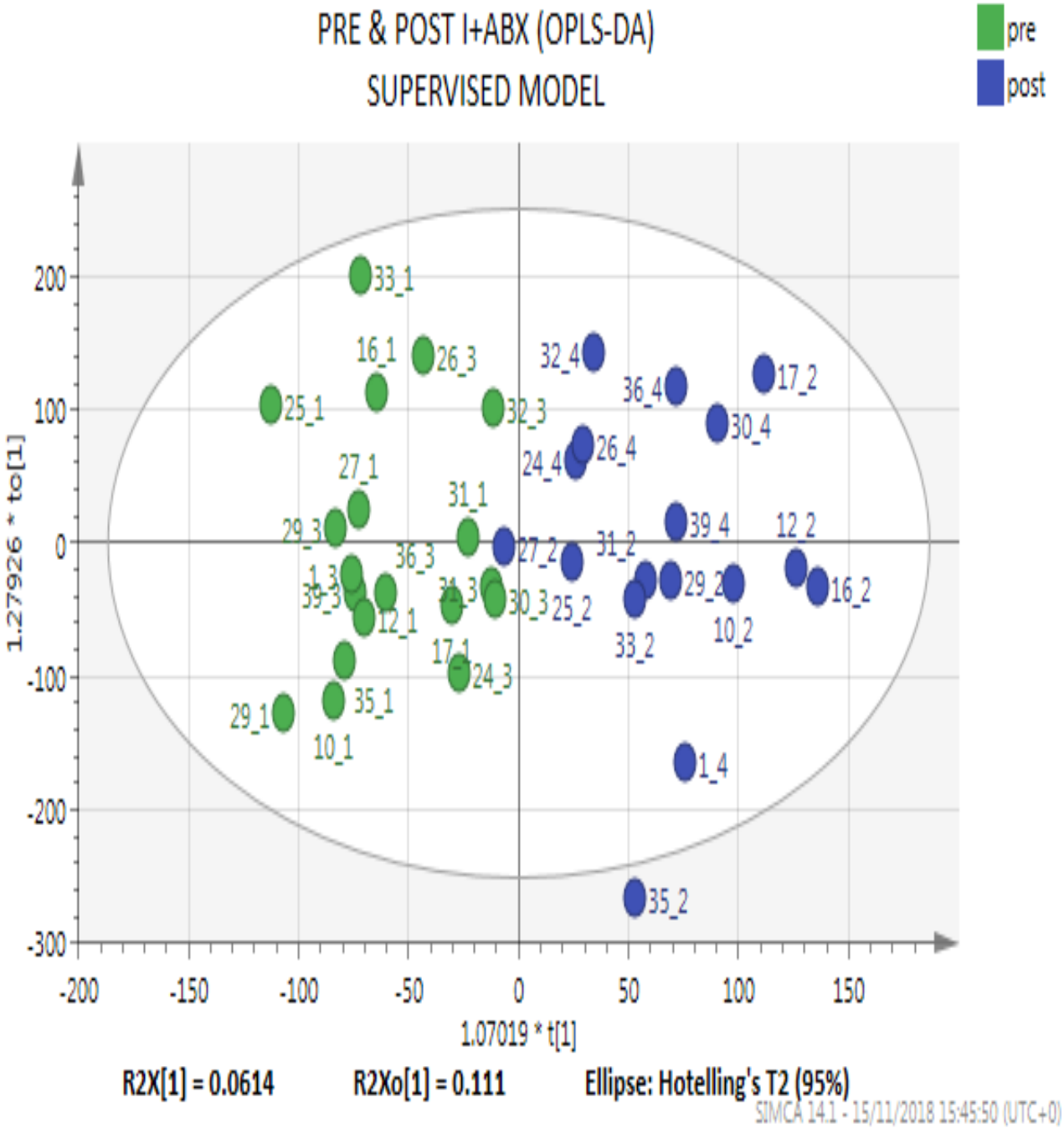

Figure 14: Orthogonal partial least squares discriminant analysis (OPLS-DA) scores with predictive component in the x axis, orthogonal component in the y axis comparing before (green) and after (blue) treatment with I+ABX.

Levels of dimethylamine, though small, were more strongly associated with pre-treatment of I+ABX, based on the correlation (0.54) **(Figure 15)**, than post I+ABX treatment **(Table 8).**

With reference to **Table 8**, creatinine production appears to be associated more with the pre-treatment of I+ABX, based on the weak correlation (0.34) than post I+ABX treatment.

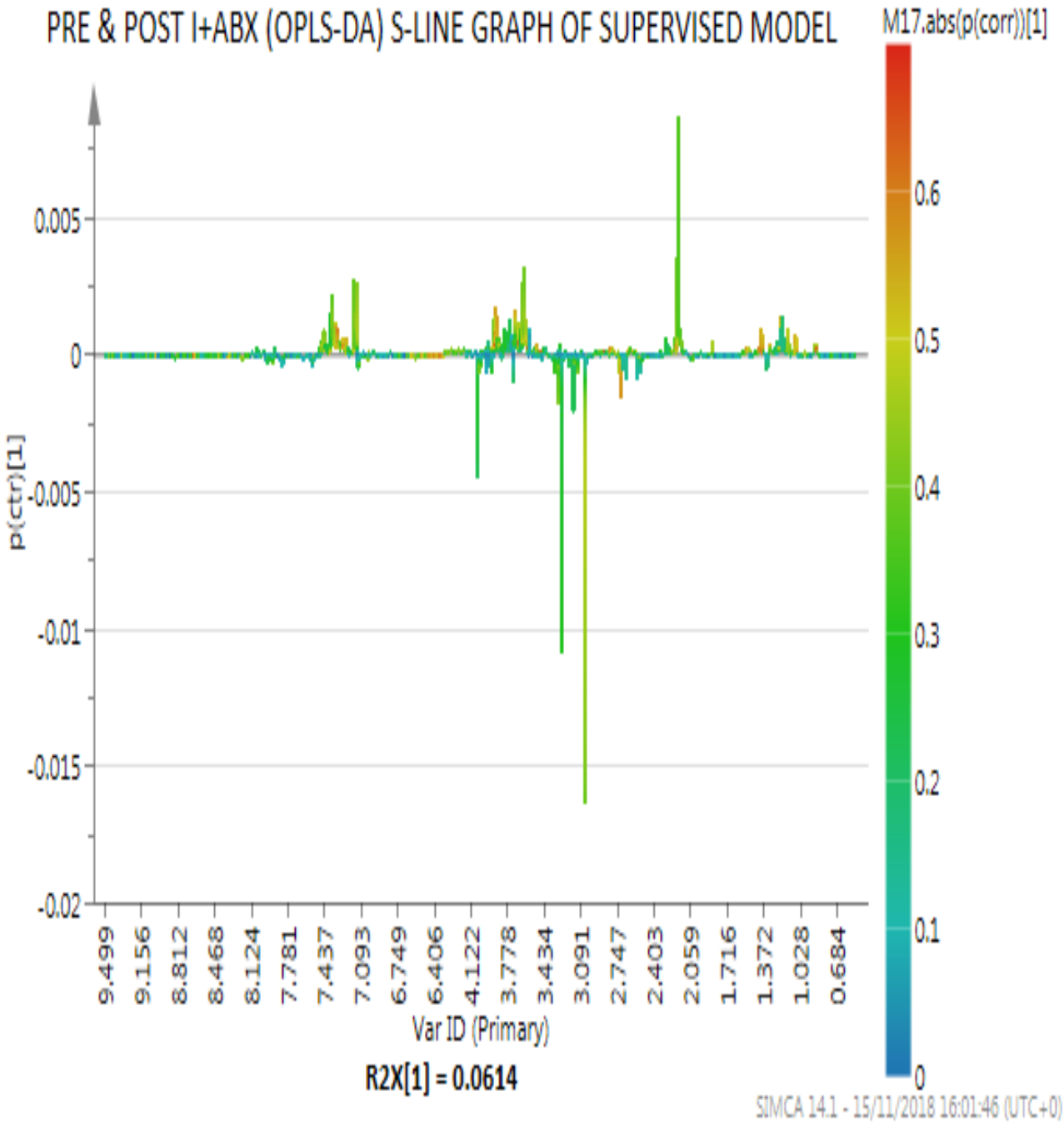

Figure 15: Orthogonal partial least squares discriminant analysis (OPLS-DA) loadings line plot of NMR spectra obtained before treatment with placebo (pointing down) and post treatment with I+ABX (pointing up). The colour indicates the strength of the correlation with class.

Table 8: Orthogonal partial least squares regression discriminant analysis (OPLS-DA) data. Information about the chemical shift allows identification of metabolites and determine if the metabolites is more correlated with either pre or post treatment with I+ABX.

Correlation	Chemical shift (ppm)	Multiplicity	Metabolite	Function
0.489	2.72	singlet	Dimethylamine	Ammonia type gas, possibly toxic effects in larger concentrations
0.344	3.05	singlet	creatinine	Waste product of protein metabolism or muscle breakdown and creatine to creatinine can be toxic.
0.41	3.88	singlet	Unassigned	
0.25	3.273	singlet	Trimethylamine-N-oxide (TMAO)	Produced by bacteria from breakdown fish, meat, dairy and eggs. High levels have been associated with heart disease, diabetes and bowel cancer
0.368	7.14	doublet	Unassigned	

Discussion

Investigating the impact of blended I+ABX on markers of appetite regulation is novel, as much of the research that explores the satiety inducing effects of prebiotics have involved individual fibres. Inulin (LC-FOS) is a well-documented prebiotic (Gibson *et al*., 1995, Kolida & Gibson 2007, Rycroft *et al*., 2001) and there is some evidence that prebiotic supplementation can induce increased satiety and reduce energy intake, thought to be mainly through stimulation of SCFAs implicated in appetite regulation (Kaji *et al*., 2011, Morrison & Preston, 2016). Most satiety studies with prebiotics are acute in design, and prebiotic induced compositional changes to the microbiota are transient and poorly characterised. Here, the impact of chronic consumption of I+ABX on markers of satiety in healthy weight men is investigated. It was anticipated that blending prebiotics might induce significant prebiotic effects.

The results from the satiety scores obtained throughout the study visits suggest that I+ABX did not have a significant impact on subjective satiety, or hunger. This may be due to inter-individual variation or participant bias. The use of VAS as a measurement of subjective satiety was previously validated by Flint *et al*., as results are reproducible, although best utilised in a crossover design. In Flint *et al*., study, a difference of 10 mm on fasting and 5 mm on mean satiety ratings detected with 18 participants. A crossover design was used in this study, and it was hoped therefore that this type of variation would be minimised, however Flint *et al*., also suggests large variation can be reported, as it is subjective feelings that are being measured, which are prone to variation (Flint *et al*., 2000). Furthermore, the daily placebo dose (9.23g) contained 5.97g pf protein, whereas the daily dose of I+ABX (9.46g) contained only 1.33g protein. Increased protein content of the placebo treatment could have led to higher satiation during this treatment as protein is known to be the most satiating of the macronutrients (Astrup, 2005), and this could have obscured increases in satiety scores induced by I+ABX induced satiety, including those related to subjective satiety.

There was a significant decrease in food intake of 38.39 Kcal (P=0.030) recorded during the *ad libitum* lunch meal following chronic consumption of I+ABX, compared with placebo that is not explained by the self-reported feelings of hunger and fullness. This conflict has previously been reported in previous human studies investigating the satiating effects of RS2, Wheat bran, B-GLU and PDX (Bodinham *et al.*, 2010, Freeland *et al*., 2009, Keogh *et al*., 2003

Olli *et al.,* 2015, Ranawana *et al.,* 2013*)*. The subjective satiety scores may not corroborate the significant energy reduction reported here, however other objective measurements such as significantly greater acetate production, increased propionate production and growth of SCFA producing *Bifidobacterium* and *Propionibacterium* strengthens this result. There is growing evidence that SCFAs are implicated in appetite regulation by acting as signalling molecules to stimulate satiety hormonal response, that subsequently trigger anorexigenic pathways, resulting in reduce food intake (Lin *et al*., 2012, Chambers *et al*., 2014, Cani *et al*., 2006, Greenway *et al*., 2007, Da Souza *et el*., 2014). Although it may be considered a limitation of the study that satiety hormones were not measured, the significant elevation in acetate concentration by 33.811mM/Kg dry weight faeces ($P<0.00$), produced, from stimulation in growth in bifidobacterial numbers, indicates that the mechanism by which food intake has been significantly reduced is through prebiotic induced changes to composition of the microbiota and subsequent elevation in SCFA production.

The analysis from the weighed food diaries, completed in the 24h prior to each study visit both before and after treatment, show that energy consumption (Kcal) increased in the 24h period post-treatment visit, however this increase was significantly greater following placebo supplementation than with I+ABX, according to self-reported data. Additionally, fat and protein consumption increased following both treatments, again this increase was more associated with the placebo treatment. Weighed food diaries can be accurate and provide detailed information if carried out correctly, however they can be time consuming and require compliance for effective reporting (Laville *et al*., 2017). In research, it has been reported that participants that are required to record their dietary habits are prone to modifying their diet, as they may be more conscious of what they consume, leading to under/over-reporting of foods as a result (Ortega *et al*., 2015). In this study however, under reporting does not seem to be an issue, but there was an increase in consumption. It may be that as volunteers were fasted for a minimum of 10 hours prior to the study visit, and because food intake was so tightly controlled during the study day, the volunteers' response may have been to overcompensate as a result and consume more either consciously or otherwise.

Consuming a greater amount of energy or macronutrients such as protein or fibre has been reported to be more satiating than carbohydrate or fat consumption, and an effect that may persist, potentially masking satiety induced by treatment (Chambers *et al*., 2015). There was

a 13.47g increase in protein consumption recorded in the 24h prior to the post-placebo visit, compared with 24h prior to the pre-placebo visit. However, this appears to have had little effect on food intake during the *ad libitum* meal the following day during the post-placebo visit as there was a 23.91 Kcal (P=0.204) increase in food intake. Conversely, protein consumption only increased by 1.67g during the 24h prior to the post-I+ABX visit, yet food intake was significantly reduced by 38.39Kcals (P=0.030) the following day, during the *ad libitum* meal post-I+ABX. Therefore, in this study the I+ABX treatment appears to have had a greater effect on food reduction than increased energy/protein consumption has. This may be further evidence that supplementation with I+ABX has led to prebiotic induced changes to microbiota composition leading to greater satiation.

Though consumption of most individual macronutrients increased during 24h period prior to post-treatment visits, carbohydrate intake reduced by 3.44g during the 24h period prior to post-I+ABX visit, though the result was not significant. However, the increase in fibre consumption from the I+ABX supplementation, might explain this small reduction as previous studies have reported sustained satiety following fibre supplementation (Bodinham *et al*., 2010, Greenway *et al*., 2007), and again this could be due to prebiotic induced changes to microbiota composition, leading to elevated SCFA concentration, thereby inducing satiety mechanisms. This indicates that consumption of I+ABX might be useful tool in those on a lower carbohydrate diet, or those following a lower glycaemic diet.

A limitation of this dietary analysis is that not all food diaries were completed, some were missing, leaving an uneven number of set and therefore care been taken when presenting these findings. A total of 13 volunteers completed a whole set for the placebo arm, and 15 from the prebiotic arm. Also, as diet was only considered during the 24h period prior to visit, was only recorded for 24h, it may not have been long enough to ascertain any habitual changes to diet.

Previous studies have reported some increases in SCFA production, but results are mixed regarding the reporting of significant increases to SCFA production following chronic consumption of individual prebiotics. This is most likely because SCFAs are quickly utilised by colonocytes as energy or transferred via the hepatic portal vein to the liver (Cummings and Overduin, 2007) leaving only around 5% of SCFAs measured in faecal samples (Cummings and

Macfarlane, 1991). However, in this study there was a significant increase in acetate levels following I+ABX treatment, which is reflective of the significant increases observed through *in vitro* fermentation studies carried out previously. Similar research by Koecher *et al*., observed LC-FOS blended with gum acacia and pea fibre, delayed SCFA production in 24h batch fermentation compared with LC-FOS alone, and in humans there was a 5mmol/L increase in total SCFA production (Koecher *et al*., 2013).

Treatment with I+ABX in this study led to a significant Increase of 0.15 log_{10}cells/dry weight faeces bifidobacteria cells (P=0.017), as well as a 0.16 log_{10} cells/dry weight increase in the numbers of *Propionibacterium* (P=0.021). This was surprising as the power calculation suggested that 30+ participants were required to observe a 0.22 log_{10} cell difference in bifidobacteria following chronic consumption of prebiotic fibre such as inulin (Bouhnik *et al*., 2004, Tuohy *et al*., 2001), however only 20 participants completed the study in total. In my previous exploration of 3 novel prebiotic blends, using a three-stage *in vitro* continuous culture model system, fermentation of I+ABX induced a significant increase in bifidobacterial growth compared with other two blends: I+RS, and I+GLOS. As there were significant bifidogenic effects following consumption of I+ABX reported in this study, it may be that combining the two distinct but complimentary substrates has led to an additive prebiotic effect, despite the power calculation not being fulfilled, it would suggest that combining LC-FOS with ABX has enhanced or added to the bifidogenic capabilities of LC-FOS.

Van den Abeele *et al*., 2013 reported that inulin and arabinoxylan, exhibit two distinct pathways of fermentation, with LC-FOS stimulating *Bifidobacterium adoloscentis* growth and ABX stimulating *Bifidobacterium longum* growth (Van den Abeele *et al*., 2013). Differences in their physico-chemical structure have most likely led to this distinction in fermentation profile with LC-FOS being shorter, less complex and lower molecular weight than arabinoxylan. Genomic studies of bifidobacterial strains reported that the *Bifidobacterium longum* genome encodes for a greater number of carbohydrate utilising enzymes, such as glycosyl hydrolases, and sugar ABC transporters compared with *Bifidobacterium animalis* (Pokusaeva *et al*., 2011) hence their ability to degrade more complex substrates, such as ABX. Unfortunately, within phyla characterisation was not possible, due to conservation of the 16rRNA sequence; however, it is worth considering these analyses in future work.

Urinary samples are subject to variation, with some being more dilute than other, and this can result in large variation between samples, that can impact or skew analysis. Therefore, probabilistic quotient normalisation of samples was carried out during analysis (Dieterle *et al*., 2006), to account for this type of variation. It is reported to be a robust method of dealing with variation in results based on the concentration of samples. Creatinine is a metabolite produced from protein metabolism, and though creatinine levels not be reported as toxic in healthy individuals, those consuming an excess of the recommended 0.8g/kg daily intake, may be at risk from kidney damage (Poortmans and Francaux, 2000). Creatinine was more associated with placebo treatment than with I+ABX treatment. Similarly, trimethylamine-N-oxide (TMAO) which is a metabolite produced by colonic bacteria, via metabolism of fish, meat and dairy products, was also more associated with the placebo group as well as with participants on pre-treatment with I+ABX. TMAO is associated with increased risk of developing heart disease (Heianza *et al*., 2017) and in a genomic systems analysis, TMAO was genetically associated with colorectal cancer (Xu, Wang and Li, 2015) Dimethylamine, a potentially toxic metabolite found in seafood, was quite strongly associated with the placebo compared with I+ABX. 3-methyl-histidine, a biomarker of muscle breakdown (Sheffield-Moore *et al.,* 2014) was also more associated with placebo than I+ABX. The results of metabolites in this model, but only in this model, it is an indication however, that I+ABX has persisted towards the distal region as this is where the metabolites of protein digestion are produced.

Conclusion

Chronic supplementation with inulin blended with arabinoxylan may be useful as a weight management tool, and there may be other health benefits elucidated with further research testing the impact of consumption in other study populations such as those at risk from metabolic syndrome and diabetes.

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Chapter 7 –

General Discussion

Introduction

As the rate of obesity has increased, along with the downward trend for consumption of fruit and veg, particularly in lower socio-economic populations (Booth *et al.*, 2017) and is a major public health concern. As there is some evidence of the efficacy of prebiotic supplementation in maintaining a healthy weight, through modification of gut microbiota, leading to increased SCFA production, which is implicated in appetite regulation (Kaji *et al.*, 2011, Morrison & Preston, 2016) developing foods or ingredients that may help those at risk, by maintain a healthy weight is important.

Experimental findings

A 48h batch culture was used to screen 11 individual prebiotics/candidate prebiotics to explore the impact of *in vitro* fermentation on bifidobacterial growth, and other commensal bacterial groups, and to identify substrates that elicited the greatest increases in acetate and propionate production, particularly in the later stages of the culture (24-48h). This would suggest a longer fermentation, which was desirable, as the aim was to develop a blend that was hoped would extend carbohydrate metabolism towards the distal region of the colon in humans, leading to sustained SCFA production that might therefore positively impact on appetite regulation. The substrates performed optimally in batch culture were blended with LC-FOS and tested further in a realistic 3-stage colonic model. During colonic fermentation, it was anticipated that growth in *Bifidodobacterium, Propionibacterium*, and *Bacteroides* would occur and subsequently elevate acetate and propionate levels, principally in the vessels simulating conditions in the transverse region (V2), and the distal region (V3), as it was predicted and desirable that blending would induce a more sustained fermentation towards V3.

As well-researched prebiotics (Gibson *et al*., 1995, Kolida, Meyer & Gibson, 2007). LC-FOS was expected to elicit bifidogenic activity during batch culture fermentation, and the results reflect this. LC-FOS was the only substrate to exert sustained bifidogenic effects during 0-24h (P=0.20) and 24-48h (P=0.01), corroborated by the significant acetate production (P=0.026), therefore LC-FOS was chosen to be blended with other substrates.

The fermentation of GLOS, through 48h batch culture led to bifidogenic effects (P=0.02) and increased numbers of *Roseburia* (P=0.03), which was reflected in the significant acetate production (P=0.01) and propionate (P=0.01). Previous research into the fermentation profile of GLOS have reported bifidogenic effects (Wichienchot *et al*., 2006), however data was limited, particularly in humans. Colonic model fermentation increased bifidobacterial numbers, however this was not significant, however there was elevated butyrate and acetate levels that were significant, in V2, therefore I+GLOS did perform somewhat as expected, exhibiting some sustained SCFA production towards V3.

RS2 was chosen to blend with LC-FOS, due to effects observed in batch culture fermentation, whereby *Propionibacterium* growth between was close to significance 24-48h (P=0.06) and was the only substrates to do so. This growth reflected in the elevated propionate levels (P=0.039). *Bacteroides* numbers did not significantly increase as observed in previous research, (Upadhyaya *et al.*, 2016, Lyte *et al.*, 2016).However, there was growth of butyrate producing bacteria *Roseburia* between 0-24h and 24-48h, though not significantly, potentially due to variation in starting inoculum. Gut model fermentation of RS blended with LC-FOS (I+RS) led to significant increased bifidogenic effects in V1, as well as an increase in acetate in V2 (P=0.028), which is reflective of the increased bifidobacterial growth, so there is evidence of sustained fermentation in V3, simulating the distal region of the colon.

MV-ABX fermented to produce significant bifidogenic effects during the earlier stages of the culture (0-24h) (P<0.00), and *Bacteroides* growth was more prevalent later in the culture (24-48h) (P=0.03), and this reflected in the significant rise in acetate (P<0.00) and propionate (P=0.039). When blended with LC-FOS, gut model fermentation elicited a significant increase of *Bifidobacterium* (P=0.02) in V1 and *Propionibacterium* was close to significance (P=0.084), and this activity is reflected in the elevated and significant increases in acetate in V1 (P=0.04), and propionate in V1 (P=0.044), close to significance in V3 (P=0.059). Bifidogenic effects did occur in V2 and V3 with increases of 1.00Log_{10}cells and 1.3log_{10}cells respectively, however variation most likely meant it was not a significant growth.

I+RS and I+ABX outperformed I+GLOS exhibiting a sustained fermentation towards V3, however I+ABX was the frontrunner, due to the levels of propionate produced, including those in V3, which was desirable. The significant bifidogenic effects and increased

Propionibacterium reported *in vitro*, were also observed in the human appetite study, as were significant acetate production. Although to a lesser extent, however, which to be expected as *in vitro* conditions were highly controlled compared to human studies. It was anticipated that consumption of I+ABX would result in a significant increase in satiety scores, particularly as food intake was reduced significantly following chronic consumption of I+ABX, however the results do not corroborate the objective measurements in this study. There was variation, observed by the large error bars in the results, unfortunately VAS, as a subjective measurement is prone to error and disparity has been reported in appetite studies between objective measurement of energy intake and subjective satiety scoring (Bodinham *et al.*, 2010, Hess *et al.*, 2011, Ranawana *et al.*, 2013).

The evidence from both *in vitro* and human study indicate that blending LC-FOS with ABX is complimentary and may induce additive prebiotic ability over LC-FOS alone. When blended together, they elicited bifidogenic effects, though their mechanism of action is different, whereby LC-FOS has been shown to stimulate *Bifidobacterium longum* growth, ABX stimulates *Bifidobacterium adoloscentis,* this indicates a different method of fermentation between the two so rather than competing against each other, there is a synergy between the two, that appears to be complimentary. Using statistical power calculation, it has been observed that at a significance level of 5% (one sided) a log change of 0.22 can be detected at a power of 90% with 30 volunteers (Costable *et al*, 2010). However, it is interesting to note that in the human study, though the power calculation of 33+ participants was not satisfied as only 20 participants completed, there was still significant bifidogenic effects and *Propionibacterium* growth, which could be an illustration of the benefits of blending complimentary fibres with prebiotic oligosaccharides. Reduced food intake was significant in the human study, which may be due to the increased acetate levels, which is thought to be beneficial metabolic marker of appetite regulation (Morrison & Preston, 2016). This indicates that I+ABX might be a useful if developed as a tool for weight maintenance in those at risk from becoming obese.

Limitations

Though it was anticipated that increasing bifidobacterial numbers would lead to increased SCFAs, implicated in appetite regulation through stimulation of satiety hormone such as GLP-

1 and PYY these hormones were not measured. I am therefore unable to determine if the increased acetate production that I observed in the human study, had any benefit or correlation with production of satiety hormones. Though the reduced food intake does help to corroborate increases satiety, as the result from VAS did not support the reduced food intake, it would have been beneficial to have another objective measurement such as satiety hormones to strengthen the evidence that was significant in the human study.

LC-FOS and ABX have been shown to have distinct fermentation profiles (Van den Abbeele *et al.*, 2013). However, in this study I was unable to observe this, because the method (FISH-FLOW) used to enumerate bacterial groups is unable to identify bacterial groups at species level. Though FISH-FLOW enables quantification of bacterial groups, which was beneficial, I was unable to identify which strains of bacteria were the functional groups.

There was no increase in satiety scores in the human study, which was initially surprising. The ability of I+ABX ability to induce satiety is therefore inconclusive, and while this is a can occur with appetite studies (Bodinham *et al.*, 2010, Hess *et al.*, 2011, Ranawana *et al.*, 2013), it was hoped that the bifidogenic effects and increased acetate observed would significantly increase satiety and reduce hunger. As Flint et al conceded however, though VAS is a reproducible method of measuring satiety, it is prone to error, as is the case when measuring subjective feelings (Flint et al., 2000)

I was also surprised to observe that both *Bifidobacterium* and *Propionibacterium* increased significantly following chronic consumption of I+ABX in healthy weight men given the power calculation was not satisfied, as usually 30+ subjects are required to observe a $0.22\log_{10}$ cells difference in bifidobacterial numbers (Costable et al.,2010). I think, however the synergistic partnership of LC-FOS and ABX, with their complimentary but distinct fermentation profiles (Marzorati et al., 2016) has elicited an additive prebiotic effect.

Chronic studies testing the satiety inducing effects of prebiotics have shown increased satiety with this method (Anderson *et al.*, 2002, Cani *et al*., 2009, Hull, 2012). In particular, when using a crossover design, as I did, so each participant is their own control, thought to reduce variability (Flint *et al.*, 2000), however, this was not the case in this research. The use of VAS as a method of measurement of subjective satiety may be a weakness as the results conflict

with the objective measurements; however, there is not much of an alternative to this method at present. Although 18 single gender subjects have been reported to be sufficient to observe satiety increases (Flint *et al*., 2000), as only 20 participants completed, it may be that there was an insufficient participant numbers to observe an increase, and so this is also a limitation of the study. Extending the time frame to include more people in the study may have had benefit in satiety results. The protein content per 9.23g daily dose was 5.94g, compared with 1.33g protein content of I+ABX in the 9.46g daily dose. Protein is one of the most satiating of the macronutrients (Astrup, 2005) and may have obscured some of the satiety inducing effects of I+ABX, including subjective satiety measured by VAS. Reducing the protein content of the placebo to ensure this increase in protein does not lead obscuring the satiety inducing ability of I+ABX.

In the batch culture fermentation, a growth of bacterial groups, such as *Bifidobacterium* was observed, however some of these were not statistically significant, due to large SE observed across the batches, thought to be due to inter-individual differences in starting composition in faecal inoculate. All three donors were from different parts of Europe, which may have caused different responses to the same prebiotic. Geographical/racial variation in the composition of gut microbiota is mainly attributed to varying environmental exposure such as diet (Gupta *et al.*, 2017). Differences have been reported in microbial composition between lower and higher BMI (Andoh *et al.*, 2016) and the BMI of the three donors ranged from normal to overweight (19.5-28kg/m^2) which may have contributed to this SE.

It may be that choosing donors with a very similar phonotype (a healthy BMI, and potentially low fibre consumers) and then screening potential donors to determine the initial composition of the microbiota, and choosing those with similar composition, might help with reducing variability.

Future work

The reduced energy intake of 34.28 kcal in the human arm of this research indicates a potential for weight management, however if consumed for a longer period, say over 12 weeks, there might be additional weight loss effect. However, given that appetite and markers of appetite regulation are very difficult to assess, compounded by neurological impact – it is important to have studies that recognise this neurological impact of satiety in conjunction with prebiotic efficacy and potentially work in collaboration with these disciplines to more fully understand the multi-mechanistic and complex physiological and neurological processes involved in appetite regulation and satiety mechanisms.

The aim of this research focused on the satiety inducing ability of blending I+ABX, through increased saccharolytic metabolism. However, there is scope for investigation of LC-FOS with other dietary fibres or substrates, to manipulate the ratio of SCFAs produced by bacterial groups, depending on the SCFA of interest. In the 3-stage colonic fermentation experiments I carried out*, in vitro* fermentation of I+GLOS induced a significant increase in butyrate, in the vessel simulating the transverse region of the colon, most likely through the stimulation of butyrate producing bacteria such as *Roseburia*. SCFA and in particular butyrate is thought to have an anti-carcinogenic effect, through inhibition of histone deacetylases (HDACs) and regulation of cell growth (Brouns *et al*., 2002) and as colon cancer, is the second biggest cancer killer in the UK (Marshall, Webb & Hall, 2016, Goncalves *et al*., 2013), developing a blend that might increase butyrate rate levels in the distal region of the colon might significantly increase butyrate production, could be useful tool in anti-carcinogenic strategies.

In this research, consumption of I+ABX may have led to an attenuation of proteolytic metabolites such as 3-methylhistidine, high levels of which have been associated with kidney damage (Elia *et al*., 1981). This attenuation is most likely through an increase saccharolytic metabolism occurring towards a region more associated with proteolytic breakdown and therefore reducing the effects of metabolites associated with proteolytic metabolism, such as amines, SO_4, further metabolomic research could highlight systemic metabolic effects of blending, to understand more fully if increasing sacchorlytic metabolism towards the distal region has an effect on metabolites associated with kidney damage.

IBS is a chronic condition that affects 2/10 people in the UK, mainly women (Gray, 2018). Though there has been some success in alleviating symptoms of IBS including: bloating, abdominal pain by supplementing the diet with LC-FOS as part of the FODMAP diet (Rao *et al.*, 2015), consuming LC-FOS at higher doses >5g/d can result in increased bloating (Bonnema *et al*.,, 2010, Hernot *et al*.,2009). It might be that those suffering with IBS could benefit from blending inulin with more slowly fermenting fibres, as a more sustained and less rapid rate might be achieved, which could have the effect of reducing some of the symptoms of IBS and reduce bloating in general. During *in vitro* fermentation LC-FOS blended with acacia gum, fermentation was slower than LC-FOS alone (Marzorati *et al.,* 2015). Furthermore, Goetze *et al* reported reduced incidence of belching in men and women following consumption of 10g OFS blended with acacia gum, compared with OFS alone (Goetze *et al.*, 2008).

Those with metabolic syndrome or at risk from metabolic syndrome might benefit from consumption of I+ABX. Metabolic syndrome is defined as a cluster of conditions that includes high blood pressure, central adiposity, increase cholesterol levels as well as high blood sugar (Alberti *et al.*, 2005). Recently two sensory receptors: Olfactory receptor 78 (Olfr78) and G protein couple receptor 41 (Gpr41) were identified as receptors for SCFAs and may help modulate blood pressure (Pluznick et al., 2014) As there was a significant increase in acetate observed in the human study, this could contribute a positive effect to blood pressure. Furthermore, in a mouse study, SCFAs were implicated in reducing blood pressure by activating G-couple receptor 41, expressed in blood vessels (Pluznick *et al*., 2014).

Furthermore, the reduced energy intake of 34.28Kcal that occurred during the human arm of this research indicates a potential for I+ABX to be used in weight management, and if consumed at a higher dose, for over 12 weeks, there might be additional benefit of weight loss effect in those that are overweight or obese. Prebiotic fibres can slow the transit time of food, reducing insulin spikes that cause disruption to glucose homeostasis, as well as improving lipogenesis and cholesterol synthesis (Morrison & Preston, 2016), which are metabolic processes that are disrupted through the development of metabolic syndrome, therefore further investigation as to any additional benefit to this population is worth consideration.

Conclusion

Blending LC-FOS with ABX may provide some additional prebiotic activity, evident from the increased SCFA production and elongation of carbohydrate metabolism suggested by the attenuation of potentially toxic metabolites of protein. I+ABX may be a useful tool in weigh management but may also have other metabolic benefits to help that further research might elucidate.

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