University of Reading

Effect of inulin type fructans on protein fermentation by gut bacteria: *in vitro* and *in vivo* studies

Xuedan Wang

Supervisors: Prof Glenn Gibson

Prof Bob Rastall

Date: 26th January 2018

Declaration

I declare that this thesis is my own work and the contribution of others has been properly and fully acknowledged.

Xuedan Wang, 2018

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Acknowledgement

I would like to thank my supervisors Professor Glenn Gibson and Professor Bob Rastall for their support and guidance during my PhD. I would also like to thank Dr. Adele Costabile for her help and patience during my first year of study with endless support in the lab, and Dr. Anisha Wijeyesekera for her help on NMR data analysis.

Thank you to all the technical support team in the Food Science department and CAF lab in the Chemistry department.

I thank to all my friends who were with me for this tough period of my life.

A special thank goes to my parents: without their love, I would not be able to go this far.

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General abstract

In Europe and Northern America, protein intake is high, whilst fibre intake is relatively low. With large amounts of protein entering the colon, bacterial proteolysis may have some negative effects through potentially toxic end-products. Prebiotics could have the potential to reverse negative consequences of gut bacterial protein fermentation.

Single stage, pH controlled, anaerobic, stirred batch culture systems simulating the distal colon were applied first with faecal inoculum from both omnivore and vegetarian volunteers. Fermentation of different protein sources with and without supplementation of inulin type fructans (ITF) were tested. A significant increase of bifidobacteria was observed with the addition of the ITF together with lower concentrations of protein fermentation metabolites (BCFA and ammonia).

Three-stage continuous colonic model systems simulating the whole colon were then studied with both omnivore and vegetarian volunteers. Casein, with and without two different doses of ITF were assessed. A significantly higher number of bifidobacteria and reduction of bacteroides and *Desulfovibrio* spp. were found with ITF addition. Furthermore, production of metabolites from protein fermentation (BCFA and ammonia) was significantly lowered with ITF.

To confirm the health benefit of ITF on high protein population *in vivo*, 43 volunteers were recruited to complete a randomised, double blind, cross over trial. A significant increase in bifidobacteria and a decrease in *Desulfovibrio* spp. was confirmed with the addition of prebiotic treatment. Stool frequency was significantly higher with ITF as compared to the placebo group, with a trend towards softness as based on the Bristol scale. Total bacteria and bifidobacteria changes during interventions were significantly correlated with stool frequency.

In conclusion, all three phases of the project found favourable bacterial and metabolic changes with ITF supplementation. ITF had inhibitory effects on colonic microbial

proteolysis, and could exert health benefit for high protein consumers, especially those who also consume low fibre diet.

Abbreviations

°C	Degree Celsius
1D	One Dimensional
^{1}H-NMR	proton nuclear magnetic resonance
AOAC	American Official Association of Chemists
BCFA	branched chain fatty acids
CCK	Cholecystokinin
CD	Crohn's disease
CFU	colony forming unit
CGC	capillary gas chromatography
D_2O	deuterium oxide
DNA	Deoxyribonucleic acid
DP	degree of polymerisation
DRV	dietary reference value
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations
FFA	free fatty acid receptor
FISH	florescence *in situ* hybridisation
FOS	fructo-oligosaccharides
xg	relative centrifugal force
GABA	gamma aminobutyric acid
GC	gas chromatography
GCMS	Gas Chromatography and Mass spectrometry
GI	gastrointestinal
GLP-1	Glucagon-like peptide
GOS	galacto-oligosaccharides
GPCRs	G protein-coupled receptors
GPR	G protein receptor
h	hours
H_2O	water

HCl hydrochloric acid

HPAEC-PAD high performance anion exchange chromatography with pulsed amperometric detection

HPLC high pressure liquid chromatography

IBD Inflammatory Bowel Disease

IBS Irritable Bowel Syndrome

IL interlukine

ITF inulin type fructans

l litre

LAB lactic acid producing bacteria

LPS lipopolysaccharides

M molar

mg milli grams

MHz mega hertz

Min minutes

mL milli litre

mm milli metre

mM milli molar

ms milli second

Na^+ sodium

NaCl sodium chloride

NaOH sodium hydroxide

OPLSA-DA Orthogonal projection to latent structure discriminant analysis

PFA paraformaldehyde

PBS phosphate buffer solution

pH potential of hydrogen

ppm parts per million

PRI Population Reference Intake

PYY peptide YY

RDA recommended dietary allowance

RNA ribonucleic acid

rRNA ribosomal ribonucleic acid

RS resistant starch

s second

SACN Scientific Advisory Committee on Nutrition

SCFA short chain fatty acid

SD standard deviation

SRB sulphate-reducing bacteria

SS steady state

TMA trimethylamine

TMAO trimethylamine-N-oxide

Tris/HCl Tris hydrochloride

TSIM trimethylsilylimidazole

TSP 3-(trimethylsilyl)-[2,2,3,3,-2H4]-propionic acid

U unit

UC ulcerative colitis

UREC University of Reading Research Ethics Committee

V vessels

VOC volatile organic compounds

WHO World Health Organization

XOS xylooligosaccharides

µg micro second

µL micro litre

µm micro metre

Chapter 1

1.1 Prebiotic Ingredients in Probiotic Dairy Products

X. Wang and R.A. Rastall

1.1.1 Introduction

The human body functions as a complex ecosystem with more micro-organisms being present than human cells. The gastrointestinal tract is the biggest and most important habitat for micro-organisms due to the abundance of nutrients in the form of digesta that flows through the lumen without being absorbed by the small intestine. According to several human intestinal metagenomic studies, the most widely represented phyla of micro-organisms in the human gut are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Verrucomicrobia, Fusobacteria, and Euryarchaeota methanogens (Arumugam *et al.*, 2011, Eckburg *et al.*, 2005). The gastrointestinal tract's physiochemical status affects the microbiota to a large extent. Due to the low pH of the stomach, only a few microorganisms can survive there. Although pH fluctuates in the small intestine as a result of the secretion of alkaline pancreatic juices and acid bile, numbers of bacteria increase gradually and reach high number in large intestine. On the other hand, the microbiota composition and metabolism affect host health in various ways, for instance by influencing immunity, mineral absorption, energy intake regulation and lipid metabolism. A good symbiosis between the human body and its microbiota is essential for human health, whereas dysbiosis, which can be caused by antibiotic therapy, drugs, diseases, injury, surgery, stress, or aging, is problematic. Diet

plays an important role on the gut microbiota, therefore food ingredients and supplements that can promote beneficial bacteria can confer health benefits.

Prebiotics are defined as "selectively fermented ingredients that result in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit upon host health" (Gibson *et al.* (2010). Prebiotics not only boost the growth of beneficial bacteria in the gastrointestinal (GI) tract, but also have the potential to inhibit pathogens, improve mineral absorption, increase satiety and improve wellbeing.

1.1.2 Criteria for an ingredient to be classified as a prebiotic

Prebiotics were originally defined by Gibson and Roberfroid (1995) as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health". Since then the definition has been revisited several times and criteria have been proposed to qualify a compound as prebiotic. There are three criteria as proposed by Gibson *et al.* (2004):

- Non-digestibility: resistant to gastric acid, hydrolysate enzymes, and gastrointestinal absorption.
- Fermentable by gut microbiota.
- Selectivity: selectively boost the growth or/and the activity of beneficial colonic bacteria.

Current recognised prebiotics are all carbohydrates: non-digestible oligosaccharides that contain different oligomers of saccharides with different degree of polymerisation.

Inulin-type fructans are oligo- or poly-saccharides composed principally of fructose. D-fructose molecules are linked by β (2→1) linkages and the chain is terminated by a D-glucose molecule bonded with fructose by an α(1↔2) linkage; inulin often refers to molecules with more than 10 degrees of polymerisation (DP) whereas fructooligosaccharide (FOS) or oligofructose (OF) often refers to molecules with 2~10 DP. Inulin is naturally present in many plants such as chicory, artichoke, leek, banana, asparagus, and onion. Fructooligosaccharides also occur in natural food as well as being produced from sucrose or inulin. These molecules can stay intact until reaching the colon, where they principally simulate the growth of bifidobacteria (Kelly, 2008).

Galactooligosaccharides (GOS) are oligosaccharides with DP less than 10 that consist of one or more galactose residues and a terminal glucose, or they are disaccharides of galactose (Gosalbes *et al.*). Galactooligosaccharides can be produced from lactose and in the GI tract can enhance the growth of bifidobacteria while supressing the growth of clostridia; this has been shown both *in vitro* and *in vivo* (Rycroft *et al.*, 2001, Vulevic *et al.*, 2013, Giovannini *et al.*, 2014).

Other carbohydrates that have been investigated as candidate prebiotics include human milk oligosaccharides, resistant starch (RS), isomaltooligosaccharides, lactosucrose, xylooligosaccharides (XOS), lactulose, soya/soybean oligosaccharides, glucooligosaccharides, arabinoxylan, arabinogalactans, pectin/pectic oligosaccharides, seaweeds/microalgae and β-glucans.

1.1.3 Health benefits of prebiotics and their mechanisms of action

Commensal bacteria in the human gut can utilise undigested carbohydrates or proteins as energy sources. Microbial breakdown of carbohydrates produces short chain fatty

acids (SCFA) (e.g. acetate, propionate, and butyrate), ethanol, formate, lactate, succinate, carbon dioxide, methane and hydrogen through various pathways. Protein fermentation also generates SCFA, ethanol, gases and other organic acids together with branched chain fatty acids (BCFA), such as iso-butyrate and iso-valerate, ammonia (NH_3), and amines. Fermentation of aromatic amino acids leads to the production of phenolic and indolic compounds, whereas fermentation of sulphur containing amino acids often generates hydrogen sulphide (H_2S). In general, SCFA are believed to be involved in various health benefits (Russell *et al.*, 2013); however NH_3, phenolic and indolic compounds, and H_2S are believed to have a negative impact on human cells, as indicated by various *in vitro* cell studies (Windey *et al.*, 2012). Commensal bacteria in the human gut can be saccharolytic, proteolytic or both. Consumption of prebiotics usually promotes increased growth of beneficial saccharolytic bacteria and an increase in saccharolytic activity of the gut microbiota, resulting in increased SCFA production

1.1.3.1 *Short chain fatty acids (SCFA) and human metabolism*

Bacteria are present throughout the gastrointestinal tract; however they are more abundant in the large intestine than the small intestine. This coincides with the SCFA concentration within the human body: SCFA are at low concentrations in the terminal ileum and high in the colon, which confirms the role of colonic bacteria in SCFA production (Cummings *et al.*, 1987). Within the colon, SCFA are produced primarily in the ascending colon where substrates are abundant and to a lesser extent in the descending colon where most substrates have been utilised by the bacteria in the early part of the colon and are scarce. Although the concentration of SCFA in the human gut varies between individuals, the ratio of acetate, propionate, butyrate and BCFA is generally around 50:20:20:10 (Cummings *et al.*, 1987, Macfarlane and Macfarlane,

2003). Whether considering the amount of substrates reaching the lower gut and then being fermented, or the requirement to sustain the survival of colonic bacteria, the amount of SCFA produced in the human colon is considerable, however, human faecal output contains only low amounts of SCFA (Cummings, 1981). This is due to SCFA absorption in the colon, which not only happens in humans but also in other mammalian species such as rat, horse, and pig. McNeil *et al.* (1978) studied 46 human subjects' rectal SCFA absorption by connecting dialysis bags filled with different testing solutions to the volunteers' rectum. The fluid in the dialysis bags was measured before and one hour after connecting: this showed that absorption of SCFA occurs together with the excretion of bicarbonate and that absorption of SCFA is not related to the pH level in the lumen.

After absorption by the colon, butyrate is present in low concentrations in portal blood due to utilisation of butyrate as an energy source by colonic epithelial cells. The remaining SCFA are transported to the liver (Cummings *et al.*, 1987). Colonocytes oxidise acetate, propionate, butyrate, glucose and glutamine to generate energy; however butyrate is the preferred substrate for colonocytes. The presence of butyrate inhibits the oxidation of other energy sources with the preference order being: butyrate>acetate>propionate>glucose>glutamine (Clausen and Mortensen, 1995). After being absorbed by the colon, SCFA are transported to the liver via portal blood. Most propionate and butyrate is metabolised by the liver, however, acetate is further transported by the venous blood to peripheral tissues (Bloemen *et al.*, 2009). In venous blood, more than 90% of SCFA is acetate, which can join the tricarboxylic acid cycle in peripheral tissues by forming acetyl-CoA and then providing energy (Cummings *et al.*, 1987). Around one third of the acetate absorbed from the colon will be taken by the cells in peripheral tissues providing energy (Cummings *et al.*, 1987).

1.1.3.2 *Mineral absorption*

Prebiotic consumption can directly lead to an increase in mineral absorption. Feeding rats with FOS can improve the absorption by the gut of multiple minerals including calcium, magnesium, and iron (Ohta *et al.*, 1995, Delzenne *et al.*, 1995). Supplementation with prebiotics, such as GOS, lactulose and other resistant carbohydrates, to rats also revealed similar findings: calcium absorption by the gut was enhanced (Brommage *et al.*, 1993, Chonan *et al.*, 1995) The GOS study also measured bone ash and found that rats fed with GOS had higher levels, implying that prebiotics can improve bone health (Chonan *et al.*, 1995).

Most studies on prebiotics and mineral absorption have focussed on calcium as it is crucial for bone health, especially in children and women. Most calcium in the human body is distributed in bones and the adult calcium levels are maintained by a dynamic equilibrium of calcium deposition and resorption. Bone calcium in elderly people decreases, particularly in postmenopausal women. Calcium deficiency in children causes rickets, while low bone mass in the elderly causes osteoporosis and elevated risk of fracture (Greer *et al.*, 2006, Jackson *et al.*, 2006). Calcium is absorbed by both active absorption, which is vitamin D dependent, and in the small intestine by passive absorption.

Many human trials have been carried out and these have confirmed that prebiotics stimulate mineral absorption. Such studies have investigated FOS, inulin, GOS and lactulose or have targeted specific groups (male and female adolescents, and postmenopausal women), (van den Heuvel *et al.*, 2009, van den Heuvel *et al.*, 1999b, van den Heuvel *et al.*, 2000, Whisner *et al.*, 2013, van den Heuvel *et al.*, 1999a).

The mechanism underlying prebiotic stimulation of mineral absorption is not fully known; however, it may involve protonation of the minerals which increases passive absorption across cell membranes. A feeding study involving 10 ileostomy subjects fed with FOS, inulin and sucrose did not show changes in mineral absorption indicating that the effect of prebiotics occurs in the large intestine (Ellegard *et al.*, 1997).

1.1.3.3 *Energy intake and appetite regulation*

There is increasing interest in the potential role of the microbiome in human energy metabolism. In an acute study that used inulin as a fat replacer in a sausage patty, a significant decrease of 1521kJ energy intake was seen (P=0.039) compared to the full-fat patty; this was similar to results obtained in a study using inulin in yoghurt (Archer *et al.*, 2004, Perrigue *et al.*, 2009). Twenty one volunteers had two consecutive days of FOS and β-glucan supplement and did not show any difference in satiety compared with the control intervention (Peters *et al.*, 2009). However, five volunteers who were on a supplementation of 16 g of FOS for 2 weeks showed an enhancement of satiation compared with another 5 volunteers who had placebo (Cani *et al.*, 2006).

It is hard to quantify satiety and satiation; however, gut hormones that regulate energy intake can be quantified. There are a number of hormones that are secreted by the gastrointestinal tract that can affect energy intake and satiety. They are produced by different cells and have various functions on satiety regulation. Table 1.1 shows a list of gut hormones and how they affect satiety.

Table 1.1 Gastrointestinal hormones

Name	Secretion site	Produced by	Hormone signals targeting organ	Effect on food intake

Ghrelin	Stomach	Gastric oxyntic cells	Vagus and hypothalamus	Increase
Cholecystokinin (CCK)	Small intestine	I-cells	Vagus and brainstem	Decrease
Glucagon-Like Peptide-1 (GLP-1)	Ileum and colon	L-cells	Vagus and brainstem	Decrease
Polypeptide YY (PYY)	Ileum and colon	L-cells	Hypothalamus	Decrease
Oxyntomodulin	Ileum and colon	L-cells	Hypothalamus	Decrease
Table compiled from Cummings & Overduin (2007).				

Bioactive glucagon-like peptide-1 (GLP-1) has two forms: GLP-1(7-37) and GLP-1(7-36), which can both be obtained from the biologically inactive 37 amino acid peptide GLP-1(1-37) by cleavage of the peptide chain. Both forms of bioactive GLP-1 have alanine at position 2, so they can be inactivated by dipeptidyl peptidase-4 which breaks down alanine containing peptides. The molecules can then be cleared through the kidneys. Glucagon-like peptide-1 (GLP-1) is produced in L-cells in the gut, but the receptor for GLP-1 is present in pancreatic islets mainly β cells, kidney, lung, heart and nervous system. In addition, GLP-1 can increase gastric emptying time by communicating with the nervous system. Glucagon-like peptide-1 not only increases insulin secretion, but also enhances the storage of insulin and stimulates insulin gene expression; it can also mediate glucose levels by glucagon secretion inhibition and activation of glucose disposal sensors (Drucker, 2006). Seven days of FOS supplementation significantly increased gastric transit time and higher plasma GLP-1 levels in nine gastroesophageal reflux disease patients (Piche *et al.*, 2003). A placebo controlled study with 48 volunteers on a 12 weeks' intervention confirmed FOS can regulate energy intake, decrease ghrelin and increase plasma peptide YY (PYY) (Parnell and Reimer, 2009).

Gut hormones involved in energy homeostasis can be regulated by prebiotics via metabolites, such as SCFA. Infusion of SCFA directly into rat colon increased PYY, and in pigs both PYY and GLP-1 reached higher concentration after SCFA infusion (Cherbut *et al.*, 1998, Cuche *et al.*, 2000). Short chain fatty acids also have their own receptors, which can affect energy uptake and storage. G protein receptor 40 (GPR40), GPR41, GPR43 are also known as free fatty acid receptor 1 (FFA1), FFA3, FFA2. Free fatty acid 1 is activated by long chain fatty acids; however, FFA2 and FFA3 can be activated by SCFA. Both of these SCFA receptors are expressed in a variety of human tissues including the colon (Tazoe *et al.*, 2009, Karaki *et al.*, 2008). Free fatty acid 2 is more highly expressed in immune cells, and FFA3 has the highest expression in adipose tissues (Brown *et al.*, 2003). For FFA2, the agonist strength is propionate = butyrate = acetate > valerate, while for FFA3, propionate = valerate = butyrate > acetate (Brown *et al.*, 2003). Free fatty acid 2 is expressed in colon L cells and can activate PYY secretion, while FFA3 stimulates the secretion of leptin in adipose tissues (Karaki *et al.*, 2008, Xiong *et al.*, 2004).

Propionate and its potential for energy regulation were observed in a human study: inulin-propionate ester (10 g d^{-1}) was fed to 60 overweight adults recruited on a randomised, double-blind, placebo-controlled, parallel study. Acute measurements revealed that inulin-propionate ester significantly increased plasma concentration of PYY and GLP-1 after 6 hours compared to inulin; after 24 weeks supplementation, the inulin-propionate ester group gained less weight and had less low density lipoprotein (LDL) -cholesterol and total cholesterol compared to the inulin group (Chambers *et al.*, 2015).

1.1.3.4 *Lipid metabolism*

High concentrations of LDL-cholesterol and triacylglycerol in the blood are risk factors for cardiovascular disease. Triacylglycerol levels can be reduced by prebiotics partly by their regulation of gut hormones and fat intake. Short chain fatty acid receptor FFA2 is involved in mediating plasma fatty acid level by inhibiting lipolysis and simulating adipogenesis (Stoddart *et al.*, 2008). Prebiotics can modulate microbiota composition with a bifidogenic effect, and anaerobic growth of lactobacilli and bifidobacteria can assimilate cholesterol with bile salts (Pereira and Gibson, 2002b). In rats prebiotic supplementation can inhibit hepatic lipogenesis by downregulating lipogenesis enzymes (Kok *et al.*, 1996). Intervention with FOS resulted in reduction of blood glucose and LDL-cholesterol in 18 diabetic subjects compared to 10 diabetic subjects who received sucrose (Yamashita *et al.*, 1984). Other studies, mainly on inulin type fructans, have produced inconsistent results where reduction of LDL-cholesterol or triacylglycerol was not always observed. These studies, however, targeted different groups and used different doses of prebiotics (Canzi *et al.*, 1996, Davidson *et al.*, 1998, Pedersen *et al.*, 1997)

1.1.3.5 *Immune function modulation of prebiotics*

An immune system that functions properly is able to protect the human body from invasion of pathogens and other antigens; however, immunity disorders can lead to problems, such as allergy, and inflammatory disease. Prebiotics can modulate immune function from two perspectives: they enhance the defence against pathogenic infections and reduce unwanted inflammatory events. Short chain fatty acids receptors, which are discussed in Section 1.1.3.4, may help to modulate immunity in the human body: FFA2 expression is highest in immune cells which indicates SCFA may be involved in host defences (Le Poul *et al.*, 2003).

Prebiotic defence against pathogens can prevent acute gastroenteritis or shortens its duration. Two research groups have studied two different prebiotics for their effect on travellers' diarrhoea: GOS had significant improvement on both the occurrence ($P<0.05$) and the length of travellers' diarrhoea ($P<0.05$); however, a study on FOS improved the general wellbeing of volunteers but did not show improvement on diarrhoea (Cummings *et al.*, 2001, Drakoularakou *et al.*, 2010). Another study used a synbiotic containing FOS and two strains of probiotics also failed to find any impact on traveller's diarrhoea (Virk *et al.*, 2013). The rationale behind any effect is that prebiotics often shorten gastrointestinal transit time resulting in excretion of pathogens before they have had time to grow. Furthermore, supplementation with prebiotics boosts the growth of beneficial bacteria that compete with the pathogens.

Inflammatory bowel disease (IBD) describes two gastrointestinal disorders ulcerative colitis (UC) and Crohn's disease (CD), which present as abnormal inflammation. These disorders are believed to be related to gut microbiota dysbiosis, therefore by modulating gut bacteria composition, prebiotics have the potential to improve wellbeing or even ease the symptoms of IBD. Fructooligosaccharides (15 g daily) reduced CD activity in 10 patients was observed by Lindsay *et al.* (2006); however, there was no significant finding with another study feeding 103 patients 15 g FOS daily (Benjamin *et al.*, 2011). Fourteen UC patients and seventeen CD patients received 10 g lactulose every day for 4 months, and although no disease parameter improved, quality of life scores increased with lactulose compared to before the intervention (Hafer *et al.*, 2007). A synbiotic with FOS, inulin and '*Bifidobacterium longum*/Synergy 1' (presumed to be *Bifidobacterium longum* subsp. *longum*/Synergy 1) resulted in a significant improvement in disease parameters in a randomised placebo controlled trial with 18 UC patients (Furrie *et al.*, 2005). There are not many human

trials into the effects of prebiotics on IBD, and those trials that had positive results did not have high number of patients, more research needs to be done.

Atopic dermatitis is an allergic skin disease that mostly happens in early infancy and childhood. There is evidence that feeding infants with mixed prebiotics can reduce incidence of atopic dermatitis; however, the severity of ongoing atopic dermatitis was not improved by GOS as indicated by the results of a randomised control trial with 107 infants (Bozensky *et al.*, 2015, Moro *et al.*, 2006, Gruber *et al.*, 2010)

1.1.3.6 *Colorectal cancer risk and prebiotics*

Since prebiotics can benefit human gut health in many aspects, their effects on colorectal cancer have also been investigated. Feeding colon cancer patients with prebiotic for 12 weeks did not result in any significant difference in cancer related biomarkers compared to control group (Rafter *et al.*, 2007). However, an observational study with a large population and a 9 year follow up revealed that dietary fibre consumption and colorectal cancer risk were negatively correlated (Bingham *et al.*, 2003). Direct studies of the mechanism by which prebiotics may lower human colorectal cancer risk are few in number, but many *in vitro* or animal studies have been done. Three groups of mice had six weeks supplementation of inulin, FOS or cellulose were then challenged with carcinogen, and abnormal crypt foci were significantly lower in the prebiotic group compared to the control group (Buddington *et al.*, 2002). Prebiotics may not be able to cure colon cancer, but they show potential for reducing the risk of colorectal cancer incidence. Two mouse studies found FOS, long chain inulin, and a mixture of both could affect the onset of colon cancer by inducing apoptosis (Hughes and Rowland, 2001, Femia *et al.*, 2002). Burns and Rowland (2004)

found that fermentation by probiotics and faecal genotoxic water with FOS or inulin helped human cells resist genotoxicity.

Prebiotics may reduce the genotoxicity of faecal water by modulating the metabolism of the gut microbiota. Proteolysis by gut bacteria can generate carcinogens or co-carcinogens. Fermentation of aromatic amino acids, for instance, generates indolic or phenolic compounds, which are generally considered as carcinogens. Supplementation of prebiotics can shift the microbiota to a more saccharolytic one by increasing the number of bifidobacteria and lactobacilli, thereby reducing production of such carcinogenic compounds. The primary bile acids are synthesised in the liver from cholesterol, and some of them can escape intestinal absorption to be metabolised by colonic bacteria. Secondary bile acids are produced by bacterial groups, such as bacteroides, clostridia, lactobacilli, bifidobacteria, and *Eubacterium*, by deconjugation and dihydroxylation (Ridlon *et al.*, 2006). Secondary bile acids can lead to a loss of mucosal cells in the colon due to disruption of the cell membrane and, furthermore, induce hyper-proliferation of mucosal cells that helps the development of colon cancer (Nagengast *et al.*, 1995). Prebiotics can possibly reduce secondary bile acids production by changing microbial metabolism and reducing colonic transit time. Glucuronidation is one of the most important and widely present detoxification pathways in human body. β-glucuronidase activity of some bacteria, which can deconjugate toxins, leads to a longer transit time of toxins; this leads to an increase of cytotoxicity and genotoxicity.

Prebiotics possibly exert apoptosis induction by SCFA production. In healthy subjects, colonic epithelial cells, which are derived from stem cells, start from the bottom of crypts. They move to the proliferation region and then move upwards until undergoing programmed cell death at the top of crypts; epithelial cells become increasingly more differentiated during this process towards apoptosis. Short chain fatty

acids are believed to enhance cell differentiation and cell apoptosis, with butyrate being the most effective (Hague *et al.*, 1995). Butyrate can act as a histone deacetylase inhibitor, which can selectively modulate gene expressions involved with the cell cycle (Boffa *et al.*, 1978, Sambucetti *et al.*, 1999).

1.1.3.7 *Gut permeability*

The gut forms a semipermeable barrier preventing the translocation of antigens, proinflammatory compounds and toxins from the digestive lumen to the sterile organs and tissues; reduced gut barrier function is related to infection, carcinogenesis and other chronic diseases. Supplementation with FOS for 2 weeks did not result in improvement in gut barrier function among 34 healthy volunteers (Ten Bruggencate *et al.*, 2006). However, a human study feeding volunteers with inulin enriched pasta for 5 weeks revealed decreased gut permeability (Russo *et al.*, 2012). An *in vitro* study indicated that a prebiotic along with a probiotic exerted better improvement of barrier function compared to the prebiotic alone (Commane *et al.*, 2005).

The mechanism behind this could be SCFA modulation of cell signalling pathways. The colonic mucosa has a layer of cells joined by tight junctions composed of specific proteins, for instance, claudins and occludin, which forms a physical barrier. Direct application of mixed SCFA to rat intestinal wall resulted in reduced permeability with higher concentrations of acetate exerting better effects (Suzuki *et al.*, 2008). Butyrate is believed to be an epithelial cell differentiation inducer and differentiated mature epithelial cells have enhanced expression of tight junction proteins. Bordin *et al.* (2004) found that butyrate treatment of several cell lines resulted in an increase in tight junction protein expression.

1.1.3.8 *Colon motility and faecal bulking with application to constipation*

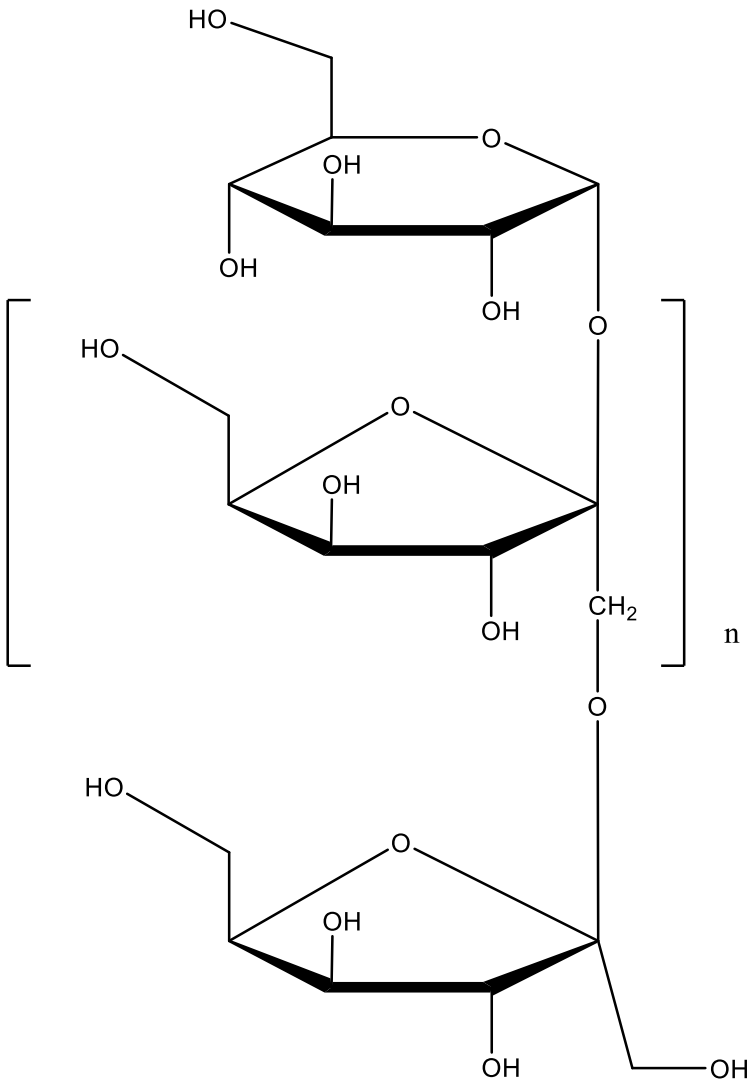
Generally, prebiotics increase faecal weight, colon motility and decrease colonic transit time which can exert benefits, such as soothing constipation. Short chain fatty acids produced by fermentation may also play crucial roles in colon motility: propionate increased muscle contraction frequency in rats possibly linked to the SCFA receptors FFA2 and FFA3 (Tazoe *et al.*, 2008), and reported studies on colon motility in relation to prebiotic food supplementation are shown in Table 1.2.

Table 1.2 Human studies of colon motility and faecal bulking with prebiotic supplementation				
Prebiotics and control or placebo	Duration of the treatment	Targeted group and subjects number	Results	References
Low RS (5 g) and high RS (39 g)	3 weeks - a crossover study	11 healthy human subjects	Increased faecal weight	Phillips *et al.* (1995)
GOS (9 g d^{-1})	2 weeks - a crossover study	14 elderly females suffering constipation	Increased defecation frequency	Teuri and Korpel (1998)
GOS (15 g d^{-1})	2 weeks - before and after treatment	12 healthy human subjects	Increased defecation frequency	Teuri *et al.* (1998)
Control diet, wheat bran diet and wheat bran + RS diet	3 weeks - a crossover study	12 healthy human subjects with family history of colorectal cancer	Increased faecal weight and defecation frequency	Muir *et al.* (2004)
Inulin (13 g d^{-1})	3 weeks - a crossover study	15 wheelchair bound adults	Increased faecal weight and no difference in defecation frequency	Dahl *et al.* (2005)
Inulin (20 g) and resistant maltodextrin	20 d - a placebo controlled parallel study	32 constipation sufferers	Increased defecation frequency and improvement of constipation	Roman *et al.* (2008)
Polydextrose (8 g d^{-1})	3 weeks - a placebo controlled parallel study	45 healthy human subjects	Increased defecation frequency and no difference in faecal weight	Hengst *et al.* (2009)
Inulin (20 g d^{-1})	3 weeks - a crossover study	12 healthy male subjects	No difference in both faecal weight and defecation frequency	Slavin and Feirtag (2011)
Inulin and guar gum mixture (15 g)	3 weeks - a placebo controlled parallel study	60 female constipation sufferers	Increased defecation frequency but no difference between control and prebiotic groups	Linetzky Waitzberg *et al.* (2012)
Abbreviations:- GOS = galactooligosaccharides; RS = resistant starch.				

1.1.4 Inulin-type fructans as prebiotics

Inulin type fructans, which include FOS with DP 2-10 and inulin with DP>10, are comprised of a number of fructose residues sometimes with a glucose at the terminal: D-fructose molecules are linked by β (2→1) linkages and when there is a glucose, the chain is terminated by a D-glucose molecule bonded with fructose by an α (1↔2) linkage (see Fig. 1.1). Fructan is a generic term used for all molecules that contain one or more fructosyl-fructose links such as inulin and laevan, which mainly has β (2→6) linkages. In this section, inulin type fructans are discussed.

Figure 1.1 The chemical structure of FOS (n=2-9) and inulin (n>9)



Both inulin and FOS are widely present in nature and have been in the human diet for a very long time. Due to variations in dietary habits, people consuming Western diets obtain 1-10 g of inulin type fructans, while people consuming an American diet obtain 5.1 g on average with 2.6 g of inulin and 2.5 g of FOS (van Loo *et al.*, 1995, Moshfegh *et al.*, 1999). The occurrence of these fructans in the human diet is mainly in plant-based foods, especially onion, Jerusalem artichoke, chicory and asparagus. Table 1.3 shows a list of plant foods that are rich in inulin in the Western diet. In addition, people from India, Japan, and other parts of the world also consume dahlia tuber and thistles roots, which also contain inulin type fructans (van Loo *et al.*, 1995).

Table 1.3 Inulin rich plant foods in the Western diet and their inulin content			
Name	Inulin content (100 $g^{-1)}$	Degrees of polymerisation of its inulin	Characteristics
Onion	1.1-7.5 g	2-12	Depolymerise during storage
Asparagus	2-3 g	---	---
Jerusalem artichoke	16-20 g	2-50	---
Leek	3 g	12	---
Rye flour	0.5-1 g	---	Resistant to baking
Garlic	9.8-16 g	2-50	---
Dandelion	12-15 g	---	---
Artichokes globe	2.5-9.5 g	≥ 19	High DP range
Barley	0.5-1 g	---	---
Banana	0.3-0.7 g	---	---
Wheat	1-4 g	50% of its inulin ≤5	Resistant to baking
Chicory root	15-20 g	2-65	Resistant to roasting: > 70% of its inulin is still present after roasting
Footnote: --- = data was not reported. Data adapted from van Loo *et al.* (1995).			

The degree of polymerisation of these plant fructans is relatively low with a maximum DP<200 and they also tend to be less branched, whereas fructans from bacterial origin can be both highly branched and highly polymerised with a maximum DP up to 100 000 (Roberfroid, 2005b). The food industry uses chicory root to produce inulin and its derivatives because the dry weight of chicory root contains > 70% of inulin (van Loo *et al.*, 1995).

1.1.4.1 *Determination of inulin type fructans*

Both capillary gas chromatography (CGC) and high pressure liquid chromatography (HPLC) can be used for the determination of short chain fructans, such as FOS. Sample preparation for HPLC is quick and straightforward as diluted samples can be injected

into the HPLC. van Loo *et al.* (1995) used two packed Aminex HPX87K columns with water (H_2O) (pH 9.5 adjusted by potassium hydroxide - KOH) as eluent and the separation was performed at 85°C. Sample preparation for CGC included adding internal standard, dilution with water, drying, formation of oxime by hydroxylamine-HCl, derivatisation with trimethylsilylimidazole (TSIM), extraction of silylated fructans by addition of isooctane, and centrifugation; separation was performed by a capillary column together with a flame-ionisation detector with an helium gas flow of 9 mL min^{-1} (van Loo *et al.*, 1995). The American Official Association of Chemists (AOAC) validated two methods to quantify fructans: AOAC method 997.08 and AOAC method 999.03, analysis by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and spectrophotometry, respectively. Both depend on enzymatic treatment to hydrolyse the polysaccharide and release sugars for analysis. Method 997.08 of the AOAC needs three chromatography readings from one sample to quantify fructans: the quantity after direct hot water extraction, quantity after amyloglucosidase treatment of the first extraction and quantity after fructozyme treatment of the second extraction (Hoebregs, 1997). Method 999.03 of the AOAC contains two enzymatic treatments: hydrolysis of starch and sucrose by a mixture of enzymes followed by reduction with borohydride, then hydrolysis of fructans by a fructanase mixture followed by spectrophotometric determination of the reducing sugars with *para*-hydroxybenzoic acid hydrazide (McCleary *et al.*, 2000).

1.1.4.2 *Production of inulin type fructans*

As discussed above, chicory roots are the main raw material for inulin production on an industrial scale. The extraction of inulin from chicory roots is carried out by diffusion in hot water, purification and then spray drying, in a process similar to extraction of

sucrose from sugar beet. The inulin produced by simple extraction is native inulin or standard inulin which has DP from 2 to 65 and average DP 12, such as Orafti® ST from Beneo in Belgium. Native inulin can be refined by physical separation to obtain high polymerised inulin (inulin HP) with DP 10-60 and average DP 25, such as Orafti® HP from Beneo in Belgium.

The partial enzymatic hydrolysis of inulin can produce FOS with DP 2-7 and an average DP of 4, by means of an endoinulinase (EC 3.2.1.7) or an exo-inulinase (EC 3.2.1.80) together with the endoinulinase (Roberfroid, 2007, Roberfroid, 2005a). Fructooligosaccharides can also be synthesised from sucrose by fructosyltranferases (EC 3.2.1.99 and EC 3.2.1.100), which catalyse the transfer of fructofuranosyl residues from sucrose (Gibson *et al.*, 2006). The transfer of fructofuranosyl residue from sucrose to sucrose, the growing fructan chain, or water, can produce GF2 (where G represents glucose, F represents fructose, and 2 represents the number of fructose moieties), extend the fructan chain, or breakdown sucrose respectively. The yield of FOS from synthesis is around 55%-60% and separation is needed to obtain purified FOS (Gibson *et al.*, 2006).

Long chain inulin and short chain FOS may exert different health benefits, therefore, Beneo provide a product known as Synergy 1®, which is a mixture of short chain FOS and long chain inulin HP.

1.1.4.3 *Physical and chemical characteristic of inulin type fructans and application in the food industry*

Chicory root inulins are white odourless powders, while FOS can be a powder obtained by spray drying or a viscous syrup with 75 g 100 g^{-1} dry matter obtained by evaporation (Franck, 2002). Inulin type fructans do not possess any off flavour or aftertastes; FOS

and native inulin have a slightly sweet taste due to the small amount of glucose, fructose and sucrose present. Water solubility negatively correlates with inulin chain length: at 25°C, more than 75% of FOS can dissolve in water, whereas only 2.5% of inulin HP can dissolve in water. Although inulin and FOS can resist general food processing, such as heat, the linkage between fructoses can breakdown at very low pH. Dissolving inulin in water or any other liquid results in a gel with a creamy structure that can be used as a fat replacer (Franck, 2002). Inulin can support most gelling agents, stabilise foam and emulsions and improve the taste and texture of bakery and cereal products (Franck, 2002). It has been used in many foods, such as baked goods, baby food, beverages, breakfast cereals, candy, dairy products, frozen desserts, soups and sauces and table spreads.

1.1.4.4 *Prebiotic effects of inulin type fructans*

One of the main characteristics of prebiotics is their resistance to digestion in the GI tract. Humans and other mammals lack the enzyme to breakdown the β (2→1) linkages within inulin and FOS. Human studies have confirmed that most inulin and FOS are not absorbed by the small intestine: 88% of FOS and 89% of inulin were recovered in the ileostomy effluent of 10 ileostomy patients (Ellegard *et al.*, 1997). However, gut bacteria do possess enzymes that hydrolyse β (2→1) linkages. and McKellar and Modler (1989) found that three strains of bifidobacteria have cell associated β-fructosidases that hydrolyse inulin type fructans, although different strains may have different preference for chain length. Enrichment culture of different species of bifidobacteria also revealed their growth by metabolisation of FOS (Gibson and Wang, 1994). Twenty-eight strains of lactobacilli, bifidobacteria, and *Streptococcus thermophilus* were examined in MRS agar together with FOS: 12 out of 16 strains of

lactobacilli, 6 out of 7 strains of bifidobacteria, and none of the *Streptococcus thermophilus* strains were capable of utilising FOS (Kaplan and Hutkins, 2000). Bifidogenic effects of inulin and FOS were observed by Wang and Gibson (1993) in an *in vitro* single vessel fermentation simulating the human colon, which was achieved by inoculation with human faecal slurries. A number of human studies have been carried out with different dosages, treatment durations, numbers of volunteers and different target groups to investigate the microbiota composition changes associated with inulin or FOS supplementation. All of the studies found there was an elevated bifidobacterial count, which in some cases was significant (Hidaka, 1991, Williams *et al.*, 1994, Gibson *et al.*, 1995, Buddington *et al.*, 1996, Kleessen *et al.*, 1997, Bouhnik *et al.*, 1999, Kruse *et al.*, 1999). Gibson *et al.* (1995) put eight volunteers on a controlled diet for 45 d, feeding them with 15 g sucrose for the first 15 d, then 15 d with 15 g FOS. Four volunteers continued to complete another intervention for 15 d with 15 g inulin. The authors did not observe any change in total faecal bacteria; however, both FOS and inulin boosted growth of bifidobacteria significantly. Furthermore, decreased level of bacteroides, clostridia, and fusobacteria were seen in this diet-controlled study.

1.1.4.5 *Health benefits of inulin type fructans*

Inulin type fructans are the best studied prebiotics and many human studies have been carried out to investigate various health benefits, such as increased mineral absorption, effect on energy regulation, effect on lipid metabolism, improvement of immunity, potential to prevent colorectal cancer and gut function improvement.

Most of the mineral absorption studies have focused on calcium intake with an emphasis on postmenopausal women and girls. Supplementation of inulin or FOS in postmenopausal women resulted in increased in absorption and bone health (based on

bone turnover markers and other biomarkers) in different studies using various methods, dosage of inulin or FOS and products (Tahiri *et al.*, 2003, Holloway *et al.*, 2007, Adolphi *et al.*, 2009, Slevin *et al.*, 2014, Kruger *et al.*, 2015). Inulin type fructans have also been shown to increase calcium absorption in adolescents, both male and female (van den Heuvel *et al.*, 1999a, Griffin *et al.*, 2002, Griffin *et al.*, 2003, Abrams *et al.*, 2005, Abrams *et al.*, 2007a). However, one study showed contradictory results: 10 g d^{-1} of FOS for 36 d did not reveal any improvement in calcium absorption, but subjects in this study were girls low calcium intake (van den Heuvel *et al.*, 2009). A further two studies have been carried out in healthy adults and these resulted in improvement of calcium absorption (Coudray *et al.*, 1997, Abrams *et al.*, 2007b). Enhancement of magnesium absorption by inulin type fructans was observed in both postmenopausal women and young girls (Tahiri *et al.*, 2001, van den Heuvel *et al.*, 2009).

As discussed in Section 1.1.3.4, satiety and energy intake regulation is another possible health benefit from prebiotics. Inulin type fructans showed improvement of satiation in some human intervention studies and some studies have measured hormones, which are related to energy regulation and revealed enhancement of satiation (Cani *et al.*, 2006, Antal *et al.*, 2008, Parnell and Reimer, 2009, Tarini and Wolever, 2010, Russo *et al.*, 2011a). Although inulin and FOS showed potential to regulate energy intake, 8 g d^{-1} of FOS for 12 weeks failed to reduce weight in overweight and obese children (Liber and Szajewska, 2014), therefore, the long term effects on weight management needs further investigation.

Lipid lowering effects of inulin type fructans were studied in 18 subjects with diabetes: reductions of blood glucose, total cholesterol, and LDL-cholesterol were seen in those who received 8 g d^{-1} of FOS for 14 d (Yamashita *et al.*, 1984). Two studies

with 4 week interventions of FOS or inulin did not find any difference in the lipid profiles of healthy subjects (Luo *et al.*, 1996, Pedersen *et al.*, 1997). Seventeen healthy volunteers had either placebo or a mixture of inulin and FOS for 6 months and this resulted in only a trend for total cholesterol and LDL-cholesterol reduction in the inulin study group (Forcheron and Beylot, 2007). Many other studies in different target groups, such as healthy subjects, hypercholesterolemia and individuals with type 2 diabetes, found that inulin significantly improved lipid profile, at different doses, study durations and monitored parameters (Davidson *et al.*, 1998, Brighenti *et al.*, 1999, Jackson *et al.*, 1999, Causey *et al.*, 2000, Letexier *et al.*, 2003, Russo *et al.*, 2008, Dehghan *et al.*, 2013).

Elderly people tend to have weaker immune function, hence there is an interest in improving their health by prebiotic supplementation. Nineteen elderly individuals were recruited in a study supplementing 8 g d^{-1} of FOS for 3 weeks and their immunity was compared before and after the intervention: there was a reduction of phagocytic activity after the treatment, however, a decrease of IL-6 was observed with FOS supplementation (Guigoz *et al.*, 2002). Another cross-over study had 43 elderly subjects being given a synbiotic comprising the probiotic '*Bif. longum*' (presumed to be *Bif. longum* subsp. *longum*; Mattarelli *et al.*, 2008) and FOS enriched inulin. Pro-inflammatory cytokines were significantly lower in the synbiotic treatment group (Macfarlane *et al.*, 2013). This inhibition of pro-inflammatory cytokines was also seen in healthy adult volunteers aged 18-24 supplemented given a combination of xylose oligosaccharides (XOS) and inulin (Lecerf *et al.*, 2012a). FOS enriched inulin improved seasonal influenza vaccination in middle aged-humans, as seen by increased antibody titres after vaccination (Lomax *et al.*, 2015). However, supplementation of FOS in

infant cereal did not show any change in subjects' immune function (Duggan *et al.*, 2003).

A synbiotic featuring '*Bif. longum*' (presumed to be *Bif. longum* subsp. *longum*) and FOS enriched inulin not only reduced UC patients' inflammatory cytokines, but also improved epithelial tissue regeneration (Furrie *et al.*, 2005). Studies on Crohn's disease have not shown any change (Lindsay *et al.*, 2006, Benjamin *et al.*, 2011). Apart from these studies on inflammatory bowel diseases, one study showed that intervention with 24 g d^{-1} of inulin for 3 weeks decreased inflammation in patients who had ileal pouch-anal anastomosis (Welters *et al.*, 2002).

Some animal models have suggested that inulin can reduce cancer risk, and Pool-Zobel (2005) reviewed data from animal models that investigated the effects of inulin type fructans on colorectal cancer risk. Inulin type fructans can reduce faecal water genotoxicity and reduce secondary bile acid production in humans (Klinder *et al.*, 2004, Boutron-Ruault *et al.*, 2005). However, experimental results with human colorectal cancer patients are controversial: different studies have monitored genotoxicity, proliferation, immune parameters or aberrant crypt foci. Only a limited number of studies with inulin type fructans have shown significant changes in colorectal cancer patients (Rafter *et al.*, 2007, Roller *et al.*, 2007, Limburg *et al.*, 2011).

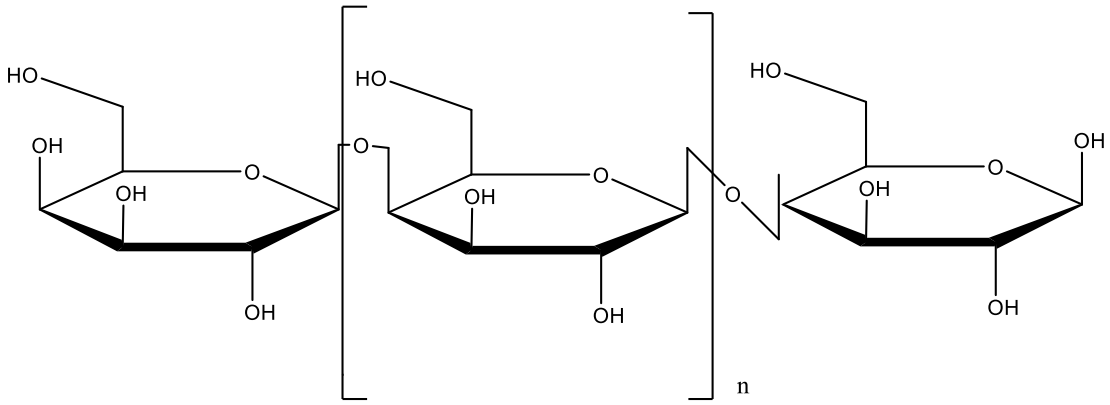
1.1.5 Galactooligosaccharides (GOS) as prebiotics

Studies of infant faecal microbiota composition found different patterns depending on feeding regime: (a) bifidobacteria tend to be higher in breastfed infants compared to formula-fed or formula-breast mixed fed infants, (b) clostridia and some facultative microbes are lower in breastfed infants, and (c) proteolytic metabolism is less active in

breastfed infants (Stark and Lee, 1982, Benno *et al.*, 1984, Mevissenverhage *et al.*, 1987, Harmsen *et al.*, 2000b, Heavey *et al.*, 2003). This led to the identification and isolation of bifidogenic compounds in human milk. Human milk contains more oligosaccharides compared to cow's milk, and some of them have a galactose-glucose structure (Kobata and Ginsburg, 1969, Kobata and Ginsburg, 1972, Yamashit.K and Kobata, 1974, Yamashita *et al.*, 1976b, Yamashita *et al.*, 1976a, Yamashita *et al.*, 1977b, Yamashita *et al.*, 1977a). The ability of GOS to act as a substitute for these human oligosaccharides has attracted interest in further researching into their prebiotic and health beneficial effects.

Galactooligosaccharides or *trans*-GOS are oligosaccharides comprised of a number of galactose monomers with a glucose molecule at the terminus with DP 3-10 and disaccharides comprising of two galactose monomers. Galactoses are often linked by β (1→4) and β (1→6) linkage, while β (1→2) and β (1→3) linkages occur less frequently in GOS (Fig. 1.2).

Figure 1.2 Chemical structure of GOS (n=1-8)



1.1.5.1 *Production and determination of galactooligosaccharides*

The GOS are synthesised by transgalactosylation in an enzymatic catalysis from lactose by β-galactosidase (EC 3.2.1.23). β-Galactosidase catalyses the transfer of a galactosyl

residue from lactose to a sugar chain or water, and can either extend the sugar chain and produce GOS or break down lactose, respectively. During enzymatic transgalactosylation, the GOS production rate is influenced by the enzyme source, substrate concentration and reaction conditions (temperature and pH). Generally, GOS production is favoured by high substrate concentration although different enzyme sources have different reaction condition preferences and can form different glycoside linkages.

Various organisms can produce β-galactosidase, including: *Aspergillus oryzae*, *Kluyveromyces marxianus* subsp. *lactis*, and *Cryptococcus laurentii* in eukaryotic organisms; *Sulfolobus solfataricus* and *Pyrococcus furiosus* in Archaea; *Bifidobacterium* spp., *Lactobacillus* spp., *Bacillus* spp., *Str. thermophilus*, *Escherichia coli* and *Enterobacter cloacae* in bacteria. Table 1.4 shows the main characteristics of some GOS products in the market.

Table 1.4 Commercially available galactooligosaccharides (GOS) products

Name	Format	GOS content (g 100 g^{-1} - dry weight)	Enzyme source	Glycoside bond	Reference
Cup Oligo H-70	Syrup	≥70	*Cryptococcus laurentii*	β (1→4)	(Ohtsuka *et al.*, 1990)
Cup Oligo P	Powder	≥70	*Cry. laurentii*	β (1→4)	(Ohtsuka *et al.*, 1990)
OLIGOMATE® 55N	Syrup	≥55	*Sporobolomyces singularis* and *Kluyveromyces marxianus* subsp. *lactis*	β (1→4) and β(1→6)	(Asp *et al.*, 1980, Gorin *et al.*, 1964)
OLIGOMATE® 55NP	Powder	≥55	*Spo. singularis* and *K. marxianus* subsp. *lactis*	β (1→4) and β(1→6)	(Asp *et al.*, 1980, Gorin *et al.*, 1964)
Vivinal® GOS	Syrup	≥59	*Bacillus circulans*	β (1→4)	(Yanahira *et al.*, 1995)
	Syrup (easy drying syrup)	≥72			
	Powder	≥69			
Bimuno	Syrup	≥57	*Bifidobacterium bifidum*	β (1→3)	(Rabiu *et al.*, 2001)
	Powder	≥80			
Purimune™ BIOLIGO GL 5700 IMF GOS	Syrup	65	*B. circulans*	β (1→4)	(Yanahira *et al.*, 1995)
Floraid® GOS Syrup	Syrup	39	*Aspergillus oryzae*	β (1→6)	(Toba *et al.*, 1985)

Floraid® GOS	Powder	39	*A. oryzae*	β (1→6)	Culhane & Tanugraha (2013)

The AOAC published a validated method (method 2001.02) to determine GOS by HPAEC-PAD. This method requires enzymatic treatment with β-galactosidase to hydrolyse the oligosaccharides and release sugars for analysis. Before the enzymatic reaction, an initial solution is taken and heated to 100°C for 10 min to deactivate the enzyme. This is then analysed by HPAEC-PAD to determine the concentration of lactose and monosaccharides. The remaining solution with active β-galactosidase is incubated at 60°C for 30 min, and then sugar analysis is performed by HPAEC-PAD (Slegte, 2002). Galactooligosaccharide content can be calculated by subtracting the initial galactose and lactose concentrations from the final galactose concentration. This method needs efficient deactivation of β-galactosidase in the initial solution to avoid underestimating GOS concentration.

1.1.5.2 *Application of galactooligosaccharide in the food industry*

Galactooligosaccharide is categorised as Generally Regarded As Safe (Boudry *et al.*) in the United States of America (USA) and regarded as a Food for Specific Health Use (FOSHU) in Japan. It has been used in infant formula to enhance bifidobacteria growth in an attempt to provide a functional mimic to human milk oligosaccharides (Boehm *et al.*, 2002). Galactooligosaccharide is slightly sweet without aftertaste, resistant to heat, and can retain moisture. It is also not digested or absorbed by the human small intestine, therefore it can be used as a sugar substitute that has reduced calories. Examples of GOS application in dairy products are fermented milk, lactic acid bacteria beverages,

ice-cream and milk beverages. Apart from dairy products, GOS can be used in beverages, sweets, dessert, bakery, jams and other food products.

1.1.5 3 *The prebiotic effect of galactooligosaccharide*

The non-digestibility of GOS was demonstrated by van Loo *et al*. (1999) and they concluded that more than 90% of GOS can be recovered in the colon. However, there is no *in vivo* human study showing non-digestibility, which is normally done by analysing digesta from ileostomy volunteers after feeding the prebiotic.

Tanaka *et al.* (1983) carried out a human study to observe the changes in faecal microbiota composition by associated with GOS supplementation: *Bifidobacterium* spp. increased while *Bacteroidaceae* spp. decreased. Other human studies have shown similar results, that GOS can modulate human colonic bacteria by increasing bifidobacteria (Ito, 1993, Bouhnik *et al.*, 1997). One study that recruited 37 volunteers (who were more than 50 years old) revealed an increase in faecal *Bifidobacterium* spp. after a 3-week intervention, of the ten bacteria groups that were monitored (Walton *et al.*, 2012). A study with elderly people and another with overweight volunteers both found an increase of bifidobacteria, and decrease of *Bacteroides* spp., *Clostridium histolyticium*, and *Desulfovibrio* spp. (Vulevic *et al.*, 2008, Vulevic *et al.*, 2013). Consuming 2.5 g d^{-1} of GOS can affect microbiota changes within one week. A parallel study with 8 volunteers in each group that tested interventions with 2.5 g, 5 g, 7.5 g and 10 g d^{-1} of GOS did not show any dose-dependent effect (Bouhnik *et al.*, 2004). However, another study with 18 volunteers consuming different doses of GOS for 3 weeks, showed a dose-dependent change of faecal bifidobacteria (Davis *et al.*, 2010). Galactooligosaccharides produced from various enzymatic sources differ in their bifidogenic effect. A cross-over study with 59 healthy human subjects found intake of

GOS produced from *Bifidobacterium* spp. was more effective for enhancing bifidobacteria growth (Depeint *et al.*, 2008).

1.1.5.4 *Infant nutrition and galactooligosaccharides*

Infants are free of bacteria before delivery. The infant gut is first inoculated with microbes resulting from contact with the mother during delivery. The maternal microbiota is transferred to the infant during natural delivery, therefore modulation of the mother's microbiota while pregnant should help infant health by passing on a healthier microbiota. Sixteen expectant mothers received prebiotic treatment with 9 g d^{-1} of 9:1 of GOS/FOS for 15 weeks before their delivery; these women showed higher number of faecal bifidobacteria compared to the placebo group (n=17). However, bifidobacteria of the neonates did not differ significantly between the two groups (Shadid *et al.*, 2007).

Breast and formula feeding shape the infant microbiome in different ways: bifidobacteria tend to be higher in breastfed infants compared to formula-fed infants. After the introduction of solid food, children acquire more microbes and the microbial ecology of the gut starts to become similar to that of adults. Before this, bifidobacteria are the dominant bacterial group in breastfed infants. It is believed that human milk oligosaccharides play an important role in bifidobacterial colonisation in the infant colon. Researchers have investigated the addition of prebiotics to infant formula with the aim of boosting bifidobacteria and exerting health benefits to the infants. The most widely studied prebiotic combination for infant formula is a mixture of 90% GOS and 10% FOS. This combination has both low molecular weight GOS and inulin, which is believed to have a similar health benefit as human milk oligosaccharides (Boehm *et al.*, 2002).

As many as 1032 infants have been enrolled in various studies investigating infant tolerance of GOS with diverse targeted groups, such as premature infants, full term infants and infants born from human immunodeficiency virus positive mothers. Safety and tolerance were evaluated in terms of weight gain, length gain, digestive tolerance and adverse events, and no safety issues were found with GOS or GOS/FOS combination as supplement in infant formula (Boehm *et al.*, 2002, Chouraqui *et al.*, 2008, Fanaro *et al.*, 2009, Holscher *et al.*, 2012, Ribeiro *et al.*, 2012, da Costa Ribeiro *et al.*, 2015, Lee le *et al.*, 2015).

Studies investigating infant formula, prebiotic supplemented formula, and human milk showed a higher number of bifidobacteria, improved stool consistency and lower faecal pH in the infants fed the prebiotic formula or human milk compared to the infants given non-supplemented formula. The organic acid profile of the infant's faecal water was also different with higher concentrations of acetate and lactate with the prebiotic formula and human milk fed infants (Boehm *et al.*, 2002, Bakker-Zierikzee *et al.*, 2005, Knol *et al.*, 2005, Ben *et al.*, 2008).

Apart from gastrointestinal function, other studies have focused on other health benefits of prebiotics given to infants, such as improvement of lipid profile. Alliet *et al.* (2007) investigated lipid profile differences among breastfed infants, prebiotic supplemented formula and non-supplemented formula; they found that total cholesterol and LDL were higher in breastfed infants compared to the formula fed infants. There was no difference between two different formulae in terms of the infants' lipid profile.

Many studies have looked at various parameters regarding the prebiotic effects on immune function in infants. Faecal secretory IgA levels were higher in 19 infants who had 6 g L^{-1} 9:1 GOS/FOS supplemented formula for 16 weeks compared to 19

infants given control formula, indicating a better host defence against pathogens (Bakker-Zierikzee *et al.*, 2006). The feeding of 8 g L^{-1} 9:1 GOS/FOS formula to infants for 6 months did not affect their diphtheria, tetanus, polio (DTP) vaccination response; however, a significant reduction of immunoglobulins relating to cow's milk allergy were observed with prebiotic formula (van Hoffen *et al.*, 2009). Fifty-five new-born premature infants, who consumed prebiotic supplement for 30 d, had the same level of proinflammatory cytokines as the control group (Westerbeek *et al.*, 2011). Without a control formula group, Bocquet *et al.* (2013) failed to find any significant difference between a prebiotic-fed group and a probiotic-fed group (*Bifidobacterium animalis* subsp. *lactis*) in terms of infection incidence. Another recent study did not find any significant difference in IgA secretion, infection incidence and allergic incidence between infants who had GOS until they were 1-year-old, when compared to a control group (Sierra *et al.*, 2015). A summary of human studies of infant health feed containing GOS is shown in Table 1.5.

Table 1.5 Some reported human studies of infant health with galactooligosaccharides (GOS) supplementation

Treatments and dosage	Formula and subjects number	Targeted group	Duration	Results	References
10 g L^{-1} 9:1 GOS/fructooligosaccharides (FOS)	Prebiotic formula (n=15) Control formula (n-15) Human milk (n-12)	Premature infants	28 d	Higher number of bifidobacteria; improved stool consistency	(Boehm *et al.*, 2002)
6 g L^{-1} 9:1 GOS/FOS or *Bifidobacterium animalis* BB-12[1] (6 x 10^{10} colony forming units (cfu) L^{-1})	Prebiotic formula (n=19) Probiotic formula (n=19) Control formula (n=19) Human milk (n=63)	New born infants starting the study at day 5 after delivery	16 weeks	Higher acetate and lactate; lower pH	(Bakker-Zierikzee *et al.*, 2005)
8 g L^{-1} 9:1 GOS/FOS	Prebiotic formula (n=15) Control formula (n=19) Human milk (n=19)	Infants with average age of 7.7 weeks at enrolment	6 weeks	Higher number of bifidobacteria; higher acetate and lactate; lower pH	(Knol *et al.*, 2005)
4.5 g d^{-1} 9:1 GOS/FOS	Prebiotic formula (n=11) Placebo (n=9)	Weaning infants aged 4-6 months	6 weeks	Higher number of bifidobacteria	(Scholtens *et al.*, 2006)
2.4 g L^{-1} GOS	Prebiotic formula (n=37) Prebiotic formula and human milk (n=58) Human milk (n=24) Control formula (n=45)	Term infants	3 months	Higher number of bifidobacteria and lactobacilli; higher acetate; lower pH	(Ben *et al.*, 2008)
5 g L^{-1} GOS	Prebiotic formula (n=77) Control formula (n=82)	Infants with age of 4-6 months	12 weeks	Higher number of bifidobacteria; improved stool consistency	(Fanaro *et al.*, 2009)
4 g L^{-1} 1:1 polydextrose (PDX)/GOS, 4 g L^{-1} 3:2:1 PDX/GOS/lactulose (LOS) and 8 g L^{-1} 3:2:1 PDX/GOS/LOS	Prebiotic formula (n=27) Prebiotic formula (n=27) Prebiotic formula (n=25) Human milk (n=30) Control formula (n=25)	Term infants	28 days	Human milk group had better stool consistency. No other significant difference was observed	(Nakamura *et al.*, 2009)
4 g L^{-1} 9:1 GOS/FOS	Prebiotic formula (n=53) Control formula (n=59) Human milk (n=57)	Infants aged no more than 30 d at enrolment	Until infants reach 4-month old	Higher number of bifidobacteria; improved stool consistency	(Vivatvakin *et al.*, 2010)
9:1 GOS/FOS	Prebiotic formula (n=36) Control formula (n=33) Human milk (n=33)	Term infants aged 2-8 weeks at enrolment	6 weeks	Higher number of bifidobacteria; lower pH; no difference with stool consistency	(Holscher *et al.*, 2012)
4 g L^{-1} 1:1 PDX/GOS	Prebiotic formula (n=78) Control formula (n=81) Human milk (n=71)	Infants aged 21-30 days at enrolment	60 days	Higher number of bifidobacteria;	(Scalabrin *et al.*, 2012)
4 g L^{-1} GOS	Prebiotic formula (n=83) Control formula (n=80) Human milk (n=199)	New born infants starting the study at day 15 after delivery	Before complementary feeding	Higher number of bifidobacteria and lower number of clostridia;	(Giovannini *et al.*, 2014)
3 g L^{-1} GOS	Prebiotic formula (n=9) Control formula (n=13)	Term infants	2 weeks	Higher number of bifidobacteria; no difference with SCFA and faecal pH	(Matsuki *et al.*, 2016)

[1] '*Bifidobacterium animalis* BB-12 (presumed to be *Bifidobacterium animalis* subsp. *lactis* BB-12 (Anonymous, 2013).

***1.1.5.5** Health benefit of galactooligosaccharides*

Rat studies on GOS found enhanced absorption of calcium, iron, magnesium and zinc; furthermore, two studies that monitored bone health parameters confirmed improvement of bone health with GOS supplementation (Chonan *et al.*, 1995, Chonan *et al.*, 1996, Chonan and Watanuki, 1996, Weaver *et al.*, 2011, Takasugi *et al.*, 2013). Unlike with inulin, the effect of GOS supplementation on mineral absorption is not well studied in human trials. Healthy males are not the main target group for mineral absorption improvement. Two studies with females of different age ranges found mineral absorption enhancement associated with GOS: ingesting 20 g d^{-1} for 9 days significantly increased true calcium absorption in 12 postmenopausal women in a crossover design study (van den Heuvel *et al.*, 2000). Another crossover study looked at how two different doses of GOS (5 g and 10 g d^{-1} for 3 weeks) affected calcium absorption in adolescent girls compared to the control, GOS improved calcium absorption significantly without any dose-dependent effect (Whisner *et al.*, 2013). Though mineral absorption was analysed in these studies, no further analysis on bone mass density and other bone health parameters was done to confirm the health benefit.

Lipid profile improvement associated with intervention with 5.5 g d^{-1} of GOS for 12 weeks was seen in overweight adult subjects, but not in generally healthy young adults who had 15 g d^{-1} of GOS (van Dokkum *et al.*, 1999, Vulevic *et al.*, 2008). Various studies with healthy subjects, overweight subjects, UC patients and elderly subjects have investigated the effect of GOS on immune function in terms of reduction of pro-inflammatory cytokines, improvement of host defence against pathogens and improvement in the wellbeing of chronic inflammatory disease patients (see Table 1.6).

Table 1.6 Human studies of adult health with galactooligosaccharides (GOS) supplementation					
Treatments and dosage	Study design and subjects number	Targeted group	Duration	Result	Reference
15 g d^{-1} Inulin 15 g d^{-1} fructooligosaccharide (FOS) 15 g d^{-1} GOS	Cross-over study (n=12)	Healthy male with an average age of 23	3 weeks	Inulin and GOS increased faecal acetate concentration; inulin increased faecal valerate concentration; inulin and FOS decreased secondary bile acids concentration; GOS and inulin decreased β-glucuronidase activity; no significant difference was found with lipid profile	(van Dokkum *et al.*, 1999)
5.6 g $d^{=1}$ GOS	Cross-over study (n=44)	Elderly subjects	10 weeks	Higher number of bifidobacteria; increased natural killer (NK) cell activity and anti-inflammatory cytokines; decreased proinflammatory cytokines; no difference of lipid profile	(Vulevic *et al.*, 2008)
Probiotic strains (*Lactobacillus rhamnosus* GG, *Lb. rhamnosus* LC705, *Propionibacterium freudenreichii* subsp. *shermanii* JS, and *Bifidobacterium breve* BB-99) (2×10^{10} colony forming units (cfu) d^{-1}) 3.8 g d^{-1} GOS 120 g d^{-1} Whole grain rye bread	Sequential intervention (n=18)	Healthy male	6 weeks	Probiotic lactobacilli, propionibacteria and GOS increased the counts of bifidobacteria and decreased β-glucuronidase activity	(Kekkonen *et al.*, 2011)
Bif. breve (3 × 10^9 cfu d^{-1}) and 5.5 g d^{-1} GOS	Parallel study (n=44)	Ulcerative colitis	1 year	Colonoscopy showed better condition; decreased myeloperoxidase indicating decreased severity of ulcerative colitis (UC); decreased Bacteroidaceae and faecal pH	(Ishikawa *et al.*, 2011)
8 g d^{-1} GOS	Cross-over study (n=39)	Healthy subjects aged more than 50	3 weeks	Increased counts of bifidobacteria; No difference of faecal water genotoxicity	(Walton *et al.*, 2012)
5.5 g d^{-1} GOS	Cross-over study (n=45)	Overweight adults	12 weeks	Increased number of bifidobacteria and decreased number of bacteroides and *Clostridium histolyticum*; no difference of blood cytokines; faecal calprotectin and plasma C-reactive protein decreased indicating decrease inflammation; Increased faecal secretory IgA; decreased plasma insulin; decreased total cholesterol, and total cholesterol/high density lipoprotein (HDL)-cholesterol ratio; triglyceride reduction was only significant in male	(Vulevic *et al.*, 2013)
7.5 g d^{-1} GOS Amoxicillin (1125 mg d^{-1}) for 5 days	Parallel study (n=12)	Healthy adults	12 days	Restored bifidobacteria from antibiotic treatment and increased butyrate concentration	(Ladirat *et al.*, 2014)
6, 12, 18 g d^{-1} α-GOS	Parallel study (n=88)	Overweight adults	14 days	Improvement of appetite with dose-dependent effect; reduced energy intake; lipopolysaccharides reduced dose-dependently; decreased plasma C-reactive protein	(Morel *et al.*, 2015)

1.1.6 Resistant starch and other glucose based non digestible carbohydrates

Resistant starch (RS) refers to those types of starch that are not hydrolysed and absorbed in the small intestine. Type 1 RS is physically surrounded by other material that makes digestion impossible; type 2 RS represents natural uncooked starches, such as potato starch, green banana starch and high amylose maize starch; type 3 RS is retrograded amylose and starch; type 4 resistant starches are chemically modified starches.

The non-digestibility of RS was confirmed by Englyst *et al.* (1996) with 9 ileostomy subjects: more than 90% of tested RS 2 and RS 3 were recovered in ileostomy effluent. Microbiota changes associated with RS have not been conclusive in human intervention studies, which may be due to the different resistant starch types having different physiological effects. Two recent studies using RS 4 as supplementary treatment did not find any bifidobacterial changes; one of them found significantly higher number of *Bacteroides* spp. and *Ruminococcus* spp. with RS 4 (Upadhyaya *et al.*, 2016, Dahl *et al.*, 2016). An increase of *Ruminococcus* spp. was also seen in another two human intervention studies with RS 2 (Abell *et al.*, 2008, Venkataraman *et al.*, 2016). Twenty healthy young adults who had RS 2 for 3 weeks, and 24 volunteers who had RS 3 had higher numbers of faecal bifidobacteria after these treatments (Venkataraman *et al.*, 2016, Costabile *et al.*, 2016). Resistant starch has been studied in numerous human intervention trials, with an emphasis on insulin sensitivity, glycaemic homeostasis, appetite, satiety and weight management; however, many of these studies did not investigate if health benefits correlated with any change in the gut microbiota.

1.1.7 Xylooligosaccharides (XOS)

Xylooligosaccharides and xylan are xylose based oligo- or poly-saccharides. They are produced by hydrolysis of hemicellulose following by purification. Nine species of

bifidobacteria were tested in pure culture fermentation with different carbon sources, and one species (*Bifidobacterium catenulatum*) preferred XOS over FOS (Palframan *et al.*, 2003). There are two *in vivo* studies on the effect of XOS in the human colon (Finegold *et al.*, 2014, Lecerf *et al.*, 2012a). Xylooligosacchardides (4.2 g d^{-1} for 4 weeks) reduced constipation severity in constipated pregnant women (Tateyama *et al.*, 2005).

1.1.8 Other potential prebiotics candidates and summary

There are many other emerging carbohydrates that may have prebiotic effect, such as human milk oligosaccharides, isomaltooligosaccharides, lactosucrose, lactulose, soya/soybean oligosaccharides, pyrodextrins, polydextrose, arabinogalactans, pectin/pectic oligosaccharides, and seaweeds/microalgae (Gibson *et al.*, 2004). Many of them are under investigation by scientists.

Gut bacteria are involved in various metabolic activities, and these activities affect human health in different ways. It is becoming apparent that it is not only gut health that is related to colonic bacteria activity; there is an increasingly interest in the gut-brain axis, gut-kidney axis, and gut-heart axis (Cryan and Dinan, 2012, Meijers and Evenepoel, 2011, Tang *et al.*, 2013). Serotonin is a key neurotransmitter, and a recent study found that gut bacteria regulate serotonin biosynthesis through signalling by host colon enterochromaffin cells (Yano *et al.*, 2015). Production of *p*-cresol and indole by gut bacteria from protein fermentation contributes to serum *p*-cresyl sulphate and indoxyl sulphate levels, which are risk factors for chronic kidney disease (Meijers *et al.*, 2010). Metabolism of L-carnitine and phosphatidylcholine by some gut bacteria produces trimethylamine, which can be further oxidised to trimethylamine-*N*-oxide; the latter is a promoter of cardiovascular disease, such as atherosclerosis (Koeth *et al.*, 2013, Wang *et al.*, 2011). With more host-microbiome

interactions to be elucidated, prebiotics may apply to more health areas by their modulation of the gut bacteria composition and associated health benefits.

1.1.9 References

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1.2 Proteolysis by gut bacteria

1.2.1 Protein consumption and human gut bacteria metabolism

Protein supply worldwide is increasing annually, and reached 81.23g/capita/d in 2013(FAO, 2017). After ingestion, proteins are partially hydrolysed by stomach acid, which involves cleavage of amino acids from the N terminal. This is then followed by enzymatic protease breakdown in the small intestine (Fuller and Tome, 2005). Proteins are not always fully digested and absorbed allowing colonic delivery. Moreover, endogenous proteins are excreted into the digestive tract, such as secretions from digestive organs, sloughed epithelial cells and debris, and mucosal content (Fuller and Tome, 2005). From various ileostomy studies, protein digestibility in the diet is influenced by protein source, quantity of the protein and other dietary components (Gibson *et al.*, 1976, Chacko and Cummings, 1988, McBurney and Thompson, 1989). Enhanced protein absorption by thermal treatment was observed in two studies using egg protein (Evenepoel *et al.*, 1999, Evenepoel *et al.*, 1998). Silvester and Cummings (1995) found similar nitrogen outputs in various protein diets in volunteers ileal efflux, and a strong correlation between ileal nitrogen and total dietary protein consumption. Two recent endogenous composition studies suggested that endogenous proteins are 69.2% bacterial proteins, 16.9% mucin, 7.65% enzymes, and 6.2% mucosal cells (Miner-Williams *et al.*, 2012, Miner-Williams *et al.*, 2014). Absorption of amino acids in the large intestine is limited comparing to the small intestine, with various bacteria being able to utilise them.

Metabolism of protein by gut bacteria firstly involves breakdown of peptides into amino acids, and then further metabolism of amino acids producing different metabolites. Smith and Macfarlane (1997) studied amino acid fermentation by colonic bacteria, and end products include CO_2, NH_3 and SCFA. Branched chain amino acid utilisation by bacteria generates branched chain fatty acids: valine is the main source of iso-butyrate production; leucine from iso-valerate production; catabolism of iso-leucine produces 2-methyl-butyrate and valine. Microbial degradation of aromatic amino acids (tyrosine, phenylalanine, and tryptophan) leads to the production of phenolic and indolic compounds: tyrosine is degraded by bacteria and generates phenol and p-cresol, phenylalanine triggers the production of phenylacetate, and main tryptophan end products are indole and skatole. Among amino acids, acetate, propionate and butyrate production rates differ: glutamate, histidine, cysteine, glycine, serine, and proline have acetate as the highest SCFA produced; aspartate, methionine, alanine, and threonine have

propionate as the highest SCFA; metabolism of lysine produces more butyrate, followed by acetate, and trace amount of propionate (Smith and Macfarlane, 1997).

In healthy adult humans, the proximal colon is approximately pH 5.5 which increases gradually to around pH 6.7 in the distal colon (Fallingborg, 1999). At different stages, microbes would have various substrates to ferment. Metabolites from protein fermentation are found more in the distal colon, where carbohydrates are limited and transit is slowest (Macfarlane *et al.*, 1992b).

1.2.2 Proteolysis metabolites in the gut and their possible health effects

1.2.2.1 Ammonia and amines

Deamination of amino acids in the gut generates ammonia, and alimentary protein consumption is correlated with its production (Geypens *et al.*, 1997). By investigating Caco-2 cell lines after treatment of ammonia, permeability was significantly increased without affecting occludin expression, suggesting change of barrier function (Hughes *et al.*, 2008). Lin and Visek (1991) found, in rats, that ammonia promoted cell proliferation and shortened the lifespan of mucosal cells. In another rat study using *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine as the carcinogen, ammonia treatment had more adenocarcinomas generated suggesting cancer promoting property (Clinton *et al.*, 1988).

Amines are produced from decarboxylation of amino acids, and gut mucosa detoxify amines by oxidation (Windey *et al.*, 2012). Dietary polyamines contribute more than those of bacterial origin from a rat study comparing conventional rats with antibiotic treated rats (Loser *et al.*, 1999). Main sources of carcinogenic *N*-nitrosamines are from diet, occupational exposure, cigarette smoking, and other minor sources, however, bacteria enzymes and amines can promote the formation of *N*-nitrosamines (Tricker, 1997). A human trial with 21 male subjects revealed that dietary haem from red meat, but not protein, promoted endogenous *N*-nitrosation(Cross *et al.*, 2003).

1.2.2.2 Hydrogen sulphide

Apart from dissimilatory sulphate reduction, bacterial degradation of sulphur containing amino acids such as cysteine and methionine can form hydrogen sulphide. With both rat epithelial cells and human cell lines, hydrogen sulphide inhibited the oxidation of butyrate which lead to colonocyte energy deficiency in a dose-dependent manner (Roediger *et al.*, 1993, Leschelle *et al.*, 2005). H_2S, which is a cellular toxin, was found to affect apoptosis of rat epithelium cells,

and dysregulation of cell cycles may trigger ulcerative colitis (Deplancke and Gaskins, 2003). Further studies evaluated the genotoxicity of H_2S on hamster ovary cells, nontransformed human intestinal epithelial cells, and HT29 cell lines: these studies confirmed DNA damage caused by H_2S, besides, accelerated DNA mutation and free radical DNA damage were found with the presence of H_2S (Attene-Ramos *et al.*, 2006, Attene-Ramos *et al.*, 2007, Attene-Ramos *et al.*, 2010). One animal study confirmed the inhibition of colonocyte energy intake, however there was no inflammatory effect (Moore *et al.*, 1997).

Compared to healthy human subjects, sulphate-reducing bacteria (SRB) isolated from ulcerative colitis patients tended to have higher H_2S production rates than conventional counterparts from the healthy gut (Gibson *et al.*, 1991). Another study showed that SRB viable counts were significantly correlated with ulcerative colitis disease severity (Pitcher *et al.*, 2000). This study also confirmed that consuming 5-aminosalicylic acid as a treatment for ulcerative colitis was related to reduced H_2S.

1.2.2.3 Phenolic and indolic compounds

Phenol and p-cresol can reduce intestinal epithelial barrier function *in vitro*, however, the carcinogenic effect on human subjects is difficult to resolve (Hughes *et al.*, 2008, McCall *et al.*, 2009, Cerini *et al.*, 2004). Indole is a quorum sensing molecule for bacteria, and recently, has been found to improve barrier function of epithelial cells and communicate with L cells to mediate hormone secretion in the colon (Chimerel *et al.*, 2014, Bansal *et al.*, 2010, Shimada *et al.*, 2013). After conjugation of indole and p-cresol, indoxyl sulphate and p-cresol sulphate are generated and may be disposed via urine. Incubation of human umbilical vein endothelial cells with indoxyl sulphate and p-cresol sulphate under concentrations found in uremic patients, revealed inhibitive effects of proliferation and wound repair by these compounds, and inductive effect of oxidative stress (Dou *et al.*, 2004, Dou *et al.*, 2007). Serum levels of indoxyl sulphate and p-cresol sulphate have been correlated with renal disease progression, and vascular dysfunction in chronic kidney diseases (Vanholder *et al.*, 2014, Barreto *et al.*, 2009). Phenol, indole and p-cresol are known for their gut bacterial origin from aromatic amino acids metabolism. In chronic kidney diseases, there is lack of proof to verify the causal relationship of disease progression and uremic toxin accumulation from bacteria (Wikoff *et al.*, 2009, Yap *et al.*, 2008).

1.2.3 Epidemiology studies on protein and host health

The International Agency for Research on Cancer (IARC), an agency under World Health Organization (WHO) published a press release in October 2015, where it classified red meat

as “probably carcinogenic to humans”, and processed meat as “carcinogenic to humans”, with concerns over colorectal cancer (IARC, 2015). This was supported by various epidemiology studies that red meat and processed meat were epidemiologically correlated with colorectal cancer (CRC) (Willett *et al.*, 1990, Giovannucci *et al.*, 1994, Norat *et al.*, 2005, Kirkegaard *et al.*, 2010). Whether the risk is correlated with protein consumption or other dietary components in the meat is unclear. Kato *et al.* (1997) discovered a correlation between total protein intake and CRC risk in New York female populations, meanwhile, an European cohort study revealed that heterocyclic amines were related to CRC risk (Rohrmann *et al.*, 2009).

Bacterial proteolysis may contribute to other colonic inflammatory diseases. Both Japanese and French studies concluded that animal protein was associated with increased risk of Inflammatory Bowel Disease (IBD) (Shoda *et al.*, 1996, Jantchou *et al.*, 2010). A recent systematic review of 19 studies, found that meat consumption, together with total fat, polyunsaturated fatty acids, and omega-6 fatty acids were positively related with Crohn’s disease and ulcerative colitis(Hou *et al.*, 2011).

1.2.4 Intervention studies on proteolysis of gut bacteria

Rats were fed with different amount of beef to determine colon DNA damage after 4 weeks treatment, and elevated level of DNA damage was seen in high meat consumption, especially red meat (Toden *et al.*, 2007b). High protein consumption was correlated with decreased colonocyte membrane height, increased protease activity in the lumen and higher ammonia concentration in rats(Andriamihaja *et al.*, 2010). One rat study did not find any difference between high protein and high carbohydrate diets, but a lower genotoxicity was observed at the end of both treatments. However, baseline dietary protein and carbohydrate were not concluded to be the cause of this change (Benassi-Evans *et al.*, 2010). One human intervention study fed seven healthy subjects twelve days of high fat, meat, with a low fibre diet, and faecal water genotoxicity increased with the intervention(Rieger *et al.*, 1999).

1.2.5 Possible approaches to prevent the negative effect of the proteolysis by prebiotics

Prebiotics are “a substrate that is selectively utilised by host microorganisms conferring a health benefit” (Gibson *et al.*, 2017). They can modulate the microbiota composition, therefore, possibly increasing saccharolytic bacterial populations possibly at the expense of proteolysis. Moreover, some prebiotics, such as fructans can reduce colonic transit time which results in less protein being catabolised. A placebo controlled crossover study was conducted to study a prebiotic FOS enriched inulin, two probiotic strains, and a synbiotic mixture impact on nitrogen

metabolism in the gut: with the prebiotic treatment, reduced urinary N excretion occurred, together with higher faecal bacterial N fractions (De Preter *et al.*, 2007a).

Resistant starch (RS) has been studied in various animal trials using rats or piglets to investigate the potential of prebiotics on colonic protein fermentation. In both rats and piglets, RS was found to decrease urinary excretion of nitrogen, and increase faecal excretion instead (Younes *et al.*, 1995, Heijnen and Beynen, 1997). Nitrogen disposal was not sufficient to support the protective effects of RS on microbial protein degradation. Three studies looked at different dietary protein sources and compared the effect of RS on protein fermentation in rats: red meat, casein, white meat, soya protein, and whey protein (Toden *et al.*, 2006, Toden *et al.*, 2007b, Toden *et al.*, 2007a). These studies again confirmed that high consumption of dietary protein induced colonocyte DNA damage and reduced colonic mucus layer thickness. Addition of RS was able to abolish these unfavourable changes.

Human studies have focused on protein fermentation metabolites. Table 1.7 is a summary of prebiotic human studies with protein metabolite analysis.

Table 1.7 Human studies of colonic protein metabolism with prebiotic supplementation				
Prebiotics treatment	Duration and study design	Targeted group and number of subjects	Gut proteolysis related outcomes	Reference
GOS 3 g d-1/10g d-1	1 week—parallel study	Healthy male adults (n=16)	Reduction of faecal ammonia concentration and urine indican secretion	(Tanaka *et al.*, 1983)
FOS 8 g d-1	1 month—parallel study	Hypercholesterolemia adults (n=6)	Decreased concentration of faecal BCFA and phenolic and indolic compounds	(Hidaka, 1991)
GOS 2.5 g d-1	3 weeks—one arm study	Healthy male adults (n=12)	Significantly lower amount of nitroreductase and indole in faeces. Ammonia and p-cresol did not differ before and after treatment	(Ito, 1993)
FOS 15 g d-1	15 days—one arm study	Healthy adults (n=8)	Increased faecal nitrogen secretion	(Gibson *et al.*, 1995)
FOS	60 days—parallel study	Cirrhotic patients (n=125)	Reduction in blood ammonia	(Malaguarnera *et al.*, 2010)
Resistant starch 30g d-1	10 days—crossover study	Healthy adults (n=14)	Decreased gut ammonia production and urinary ammonia excretion	(Wutzke *et al.*, 2010)
XOS 5g d-1 XOS and inulin mixture (3g inulin+1g XOS per day)	4 weeks—parallel study	Healthy adults (n=60)	Lower concentration of *p*-cresol was observed in XOS group, but not in XOS and inulin group	(Lecerf *et al.*, 2012b)
XOS 5g d-1	3 weeks—crossover study	Healthy children aged 8-12 years (n=29)	Significant reduction of BCFA in faeces	(Francois *et al.*, 2014)
A synbiotic combination (1.3×1010 d-1 of *Lactobacillus casei* Shirota cells+ 20g d-1 of oligofructose-enriched inulin)	4 weeks—one arm study	Healthy adults (n=9)	Dimethyl trisulfide and ethyl benzene were significantly lower after the synbiotic treatment.	(De Preter *et al.*, 2011)
Oligofructose enriched inulin 20g d-1	4 weeks—cross over study	Healthy adults (n=20)	Reduction of urinary N content and p-cresol excretion with significant changes	(De Preter *et al.*, 2007a)

1.2.6 References

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Chapter 2 *In vitro* faecal batch culture fermentation of different dietary protein sources and influences of prebiotic supplementation

Abstract

Dietary protein levels are high in Western populations. Metabolism of protein can be potentially detrimental due to the generation of various toxic metabolites, such as ammonia, *p*-cresol, and indole. Increased consumption of prebiotic carbohydrates allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health. Few studies, however, have focused on the impact of prebiotics on proteolysis within the gut.

pH controlled anaerobic stirred batch culture systems were inoculated with omnivore and vegetarian volunteers' faecal samples. Ten treatments were used in the systems: four different protein sources (casein, meat, mycoprotein and soy protein) with and without supplementation of inulin/oligofructose mixture (Synergy1), positive control with oligofructose enriched inulin, and a negative control without substrate. Bacterial changes during fermentation were monitored over 48h by fluorescence in situ hybridisation with flow cytometry. Concentration of various fermentation products such as short chain fatty acid (SCFA), ammonia, phenol, indole, and *p*-cresol were monitored. A significant increase of bifidobacteria was observed with the addition of the fructan prebiotic Synergy1 ($p < 0.05$). Branched chain fatty acids (BCFA) were lower in fermenters with vegetarians' faeces, with reduced production by prebiotic treatment in donors with high BCFA production on protein substrates. Furthermore, ammonia production was lower on Synergy1. Bacterial adaptation to different diets was also observed through different patterns of ammonia production between vegetarians and omnivores. In volunteer samples with high baseline levels of phenol, indole, *p*-cresol and skatole, Synergy1 fermentation led to a complete elimination of these compounds.

Conclusion: Supplementation with a prebiotic resulted in a change in human gut microbiota and metabolism, reducing the production of some proteolytic metabolites. In addition, a difference was seen in BCFA between omnivores and vegetarians gut microbiota.

2.1 Introduction

Dietary protein levels are relatively high in western European populations, as high as 105g/d according to the Food and Agriculture Organization (FAO, 2017). However, the recommended

dietary allowance (RDA) is 56g/d for men and 46g/d for women (Institute of Medicine (U.S.). Committee on Use of Dietary Reference Intakes in Nutrition Labeling., 2005). This may result in high residual colonic nitrogen, with dietary protein having escaped digestion in the upper intestine entering the large gut where it can become a substrate for the colonic microbiota. Approximately 16g of protein will reach the colon following ingestion of 105g protein/day of which 8g are endogenous and 8g are exogenous (Gibson *et al.*, 1976, Moughan *et al.*, 2005). Among the endogenous proteins, there are 69.2% bacterial proteins, 16.9% mucin, 7.65% enzymes, and 6.2% mucosal cells (Miner-Williams *et al.*, 2014, Miner-Williams *et al.*, 2012).

Metabolism of carbohydrate by gut bacteria produces short chain fatty acids (SCFA), and gases from different pathways. Production of SCFA, mainly acetate, propionate, and butyrate, in the lumen is generally believed to mediate health benefits (Russell *et al.*, 2013). Microbial breakdown of protein not only generates SCFA and gases, however, but also ammonia, amines, indolic and phenolic compounds, and branched chain fatty acids (BCFA) through the deamination and decarboxylation of amino acids (Smith and Macfarlane, 1997). In rat and *ex vivo* studies, ammonia at a physiologically relevant dose can harm colon barrier function, shorten colonocyte lifespan, and is co-carcinogenic for rats (Clinton *et al.*, 1988, Hughes *et al.*, 2008, Lin and Visek, 1991). Hydrogen sulphide can be produced from sulphur containing amino acids and is toxic to colonocytes by damaging their DNA and blocking utilisation of butyrate as an energy source (Attene-Ramos *et al.*, 2010, Attene-Ramos *et al.*, 2007, Attene-Ramos *et al.*, 2006, Roediger *et al.*, 1993). Metabolism of tyrosine, phenylalanine and tryptophan produces phenol, indole, *p*-cresol and skatole; phenol and *p*-cresol can reduce intestinal epithelial barrier function *in vitro* (Hughes *et al.*, 2008, McCall *et al.*, 2009, Cerini *et al.*, 2004). BCFAs are generated from branched chain amino acids such as valine, leucine, and iso-leucine and no physiological role for BCFA is known so far (Verbeke *et al.*, 2015).

Thus, foods entering the colon can have a health impact on the host, possibly by changing gut microbiota composition and activity. The International Agency for Research on Cancer (IARC), an agency under the World Health Organization (WHO) published a press release in October 2015: where it classified red meat as “probably carcinogenic to humans”, and processed meat as “carcinogenic to humans”, with concerns over colorectal cancer (IARC, 2015). Some epidemiological studies found reduced risk of colorectal cancer (CRC) with high consumption of dietary fibre, while red meat and processed meat had a positive correlation with CRC (Young and Wolf, 1988, Cottet *et al.*, 2005, Willett *et al.*, 1990, Gonzalez and Riboli,

2010). Animal protein intake was associated with increased inflammatory bowel disease (IBD) risk in two Japanese and French studies (Shoda *et al.*, 1996, Jantchou *et al.*, 2010).

Increased consumption of prebiotics, which can reach the colon resulting in specific changes in the composition and/or activity in the gastrointestinal microflora, may counter the negative effects of gut microbial proteolysis in persons ingesting high protein diets (Gibson *et al.*, 2017). Inulin-type fructans can resist hydrolytic enzymes in the human GI tract and are resistant to small intestinal absorption, subsequently they become a food source for the microbiota within the large intestine. The impact of inulin on the gut microbiome has been studied using *in vitro* and *in vivo* approaches (Wang and Gibson, 1993, Kolida *et al.*, 2002).

2.2 Materials

2.2.1 Proteins

Protein substrates used were casein hydrolysate (Sigma-Aldrich, Poole, UK, meat extract for microbiology (Sigma-Aldrich, Poole, UK), soy protein acid hydrolysate powder (Sigma-Aldrich, Poole, UK), and mycoprotein which was extracted from a commercial product (Quorn^{TM}) purchased from a local supermarket.

2.2.2 Prebiotic

Inulin-type fructan was a mixture of oligofructose and inulin: 50%±10% DP (degree of polymerisation) 3-9 and 50%±10% DP≥10 (Orafti®Synergy 1, BENEO-Orafti, Tienen, Belgium).

2.3 Methods

2.3.1 Protein extraction

Mycoproteins were extracted from Quorn^{TM} based on the method described by Williams *et al.* (2001). 500g of Quorn^{TM} mince was mixed with 1200ml water and then homogenised in a blender. 60ml of formic acid was added after homogenisation and the pH lowered to 1.6. Afterwards, 5g pepsin was added and the solution incubated at 37°C for 48 hours. Samples were centrifuged at 3000g for 15 minutes and the supernatants freeze-dried for later use. After extraction, the nitrogen content of mycoproteins was quantified using the Kjeldahl method (Campden BRI, UK) and was found to be 10.3%. The remaining mycoprotein was stored at -20°C.

2.3.2 Protein dose determination

Based on previous validation work from *in vitro* batch culture experiments and in human trials,

the dose of 1 % of substrate (w/v) equates to 5g inulin reaching the colon (Wang and Gibson, 1993, Buddington *et al.*, 1996). 1% of Synergy1 was used in this study to investigate the prebiotic effect on bacterial proteolysis. As described before: 8g endogenous and 8g exogenous protein which is approximately 1.846g mucin and 0.612g enzymes apart from bacterial origin protein will reach the colon following ingestion of 105g protein/day. The approach used in a 150ml batch culture experiments is shown in Table 2.1. The amount of casein, meat extract, mycoprotein and soy protein was adjusted based on their true protein content which is shown in Table 2.2.

	In vitro fermentation dosage
Dietary protein	2.4g
Mucin	0.57g
Digestive enzymes	0.18g
Note: digestive enzyme is a mixture of 0.107g pepsin, 0.022g pancreatin, and 0.00079g α-amylase based on an *in vitro* digestion simulation paper (Mills *et al.*, 2008)	

Table 2.1 Endogenous and exogenous protein dosage to simulate the *in vivo* effect of 105g dietary protein per day consumption for 150ml batch culture experiment.

Protein	**Protein content**	**Protein dose**
Casein	68.75%	3.5g
Soy protein	75%	3.2g
Meat extract	76%	3.2g
Mycoprotein	64.2%	3.7g

Table 2.2 Protein dose that is equivalent to 2.4g dietary protein responding with protein content

2.3.3 *In vitro* batch culture fermentation

2.3.3.1 Faecal Sample Preparation

Faecal samples were obtained from three healthy meat eating individuals and three healthy vegetarian volunteers between the ages of 18 and 60, who had not taken antibiotics for at least six months prior to the experiment and had no history of gastrointestinal disorders.

Faecal samples were diluted 1 in 10 (w/v) using 1M, pH7.4, anaerobically prepared phosphate buffered saline (PBS, Oxoid, Hampshire, UK). This solution was homogenised in a stomacher (Seward, stomacher 80, Biomaster) for 120 seconds at normal speed. 15mL of this was then immediately used to inoculate batch culture vessels.

2.3.3.2 Batch Culture Basal Nutrient Medium.

Basal nutrient medium was prepared with chemicals obtained from Sigma-Aldrich, Poole, UK unless otherwise stated. In one litre: 2g peptone water, 2g yeast extract (Oxoid, Hampshire, UK), 0.1g NaCl, 0.04g K_2HPO_4 (BDH, Poole, UK), 0.04g KH_2PO_4 (BDH), 0.01g $MgSO_3.7H_2O$ (Fischer scientific, Loughborough, UK), 0.01g $CaCl_2.6H_2O$, 2g $NaHCO_3$ (Fischer), 0.5g L–cystine HCl, 2mL Tween 80, 10µL vitamin K1, 0.05g haemin, 0.05g bile salts (Oxoid), 4ml resazarin (pH7).

2.3.3.3 pH controlled, stirred batch culture fermentation

Vessels with an operating volume of 300mL were set up. 135mL of basal nutrient medium was autoclaved (121°C for 15 minutes) and aseptically poured into sterile vessels. This system was left overnight with oxygen free nitrogen pumping the medium at a rate of 15mL/min. After 4 hours fermentation, nitrogen flow was stopped and gas outlets were clamped to trap gas. pH meters (Electrolab pH controller, Tewksbury, UK) were connected to each vessel to regulate pH 6.7 to 6.9 with the aid of 0.5M HCl or NaOH.

Each vessel was also temperature controlled at 37°C and stirred using a magnetic stirrer. 1% w/v of prebiotic and relative protein treatment were added to the vessels prior to inoculation with 15mL of faecal inoculum. For each donor, 10 vessels were prepared for 10 treatments: casein, meat extract, Quorn, soy protein, casein+Synergy1, meat extract+Synergy1, Quorn+Synergy1, soy protein+Synergy1, Synergy1, and negative control.

Samples were removed from the fermenters after 0, 6, 10, 24 and 48 hours incubation.

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

A 750µl sample of batch culture fluid was centrifuged at 11337 × g for 5 minutes and the supernatant discarded. The pellet was then suspended in 375µl filtered 1M PBS solution. 1125µl filtered cold (4°C) 4% paraformaldehyde (PFA) was added and samples were stored at 4°C for 4 hours. These were then washed thoroughly with PBS to remove PFA and re-suspended in a mixture containing 300 µl PBS and 300 µl 99% ethanol. Samples were then stored at -20°C prior to FISH analysis by flow cytometry. FISH by flow cytometry was carried out as described by Grimaldi *et al.* (2017). The probes used in this study are listed below:

Table 2.3 Name, sequence, and target group of oligonucleotide probes used in this study for FISH of bacterial enumeration

Probe name	Sequence (5' to 3')	Target groups	References
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	(Wallner *et al.*, 1993)
Eub338‡	GCTGCCTCCCGTAGGAGT	Most Bacteria	(Amann *et al.*, 1990)
Eub338II‡	GCAGCCACCCGTAGGTGT	Planctomycetales	(Daims *et al.*, 1999)
Eub338III‡	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	(Daims *et al.*, 1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	(Langendijk *et al.*, 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*	(Harmsen *et al.*, 1999)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz *et al.*, 1996)
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium* coccoides-*Eubacterium rectale* group (Clostridium cluster XIVa and XIVb)	(Franks *et al.*, 1998)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus	(Walker *et al.*, 2005)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(Harmsen *et al.*, 2000a)
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX	(Walker *et al.*, 2005)
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives	(Suau *et al.*, 2001)
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	Ramsing *et al.* (1996)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)	Franks *et al.* (1998)
EC 1531	CAC CGT AGT GCC TCG TCA TCA	*Escherichia coli* BJ4	(Poulsen *et al.*, 1994)

2.3.5 Short chain fatty acid (SCFA) analysis by gas chromatography

Samples were centrifuged at 11337 × g for 10 minutes to remove all particulate matter. Supernatants were then filtered through a 0.2 µm polycarbonate syringe filter (VWR, Farlington, UK). Extraction was done with some modifications of a method from Richardson *et al.* (1989). 500 µl of sample was transferred into a labelled 100 mm×16 mm glass tube (International Scientific Supplies Ltd, Bradford, England) with 25 µl of 2-ethylbutyric acid (0.1 M, internal standard) (Sigma, Poole, UK). 250 µl concentrated HCl and 1 ml diethyl ether were added to each glass tube and samples vortexed for 1 minute. Samples were then centrifuged at 2000 g for 10 minutes. The diethyl ether (upper) layer of each sample was transferred to a labelled clean glass tube. A second extraction was conducted by adding another 0.5 ml diethyl ether, followed by vortexing and centrifugation. The diethyl ether layers were pooled. 400 µl of pooled ether extract and 50 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) (Sigma-Aldrich, Poole, UK) were added into a GC screw-cap vial. Samples were left at room temperature for 72 hours to allow lactic acid in the samples to completely derivatise.

An Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS 30m×0.25mm column with a 0.25µm coating (Crosslinked (5%-Phenyl)-methylpolysiloxane, Hewlett Packard, UK) was used for analysis of SCFA. Temperatures of injector and detector were 275°C, with the column programmed from 63°C for 0 minutes to 190°C at 15°C min-1 and held at 190°C for 3 minutes. Helium was the carrier gas (flow rate 1.7 ml min-1; head pressure 133 KPa). A split ratio of 100:1 was used. Quantification of the samples was obtained through calibration curves of lactic acid and acetic, propionic, butyric, valeric and branched SCFA (iso-butyric and iso-valeric) in concentrations between 12.5 and 100 mM.

2.3.6 Volatile organic compounds analysis by GC-MS

2.3.6.1 Entrapment of volatile compounds

All fermentation samples were adjusted to a pH of 7.0 ± 0.3 using hydrochloric acid or sodium chloride prior to volatile entrapment. Each sample (1 g) was placed in a 250mL conical flask fitted with a Dreschel head. The flask was placed in a water bath maintained at a temperature of 30°C for 1 hour. The flask was attached to oxygen-free nitrogen (40mL/min) which swept volatile compounds from the headspace above the sample onto a glass trap (4 mm i.d., 6.35 mm o.d. x 90 mm long), containing 85mg of Tenax TA poly (a porous polymer absorbent based on 2,6-diphenylene-oxide) (Supelco, Poole, UK). Following volatile extraction, 1µL of 1, 2

dichlorobenzene in methanol (653ng/µL) was added to each trap as an internal standard and the trap was then flushed with oxygen free nitrogen to remove moisture (100mL/min) for 10 minutes.

2.3.6.2 Gas Chromatography and Mass spectrometry (GC-MS)

Volatile compounds collected on the Tenax adsorbent were analysed using a Perkin-Elmer Claris 500 GC-MS, attached to an automated thermal desorber (Turbomatrix ATD, Perkin Elmer, Beaconsfield, UK). Tenax traps were desorbed at 300°C for 10 min and the volatiles cryofocused on the internal cold trap held at -30°C. After desorption, the cold trap was heated to 300 °C at 40°C per second to release volatile material onto the GC column. GC separation was carried out on a DB-5 non-polar column (60m x 0.32mm id, 1µm film thickness, J&W Scientific from Agilent). Helium at 145 kPa was used as the carrier gas. The GC oven temperature program was 2min at 40°C followed by an increase at 4°C/min up to 260°C, where it was held for 10 min. All data were collected and stored using Turbomax software (version 3.5, Perkin Elmer). Compounds were identified from their mass spectra and identities confirmed by comparison of retention time (linear retention index, LRI) and mass spectra with those of authentic compounds analysed in online library database. Analyses were carried out using Agilent 6890/5975 GC-MS system (Agilent Technologies, Palo Alto, CA, USA) fitted with a Turbomatrix ATD.

Indole, *p*-cresol and phenol (Sigma-Aldrich, Poole, UK) were diluted using the same internal standard. Quantification of the samples was obtained through calibration curves of phenol, *p*-cresol, indole and skatole in concentrations between 25 and 100 µg/ml

2.3.7 Ammonia Analysis

Samples at 0, 10 and 24 hours were diluted 1 in 50 v/v prior to analysis. Ammonia concentration of diluted fermentation samples was analysed using a FluoroSELECT™ ammonia kit (Sigma-Aldrich, Poole, UK). Enough reagent was prepared by combining 100 µL assay buffer, 4 µL reagent A and 4 µL reagent B in the kit. 10 µL H_2O (blank) and 10 µL sample was added to each glass vial. Afterwards, 100 µL reagent was added to each tube. Samples were kept in the dark for 15 minutes at room temperature before they were read in the fluorometer. Ammonia standards were prepared by diluting 20 mmol/L NH_4Cl in distilled water and the concentration range was 0.25-1 mmol/L).

2.4 Results

2.4.1 Bacterial Enumeration

In order to determine bacterial populations, twelve 16S-rRNA fluorescent *in situ* hybridisation probes were used to follow the changes in population of total bacteria and 11 functionally significant faecal microbial groups.

Negative control with omnivore microbiota did not change throughout the fermentation and bacteria from vegetarian donors were reduced throughout the whole fermentation from 8.25±0.38 to 7.67±0.38 $\log_{10}$ bacteria per ml ($p=0.04$ at 48 hours comparing with 0 hour; Figure 2.1). Total bacterial counts of omnivore microbiota on 1.5g Synergy1 were higher than counts on 2.4g protein, though this effect was not clear with vegetarian microbiota. The combination of protein+Synergy1 resulted in higher total bacterial counts than the corresponding protein substrate alone in all cases.

Bacterial populations from omnivores and vegetarians responded differently to the proteins: faecal bacteria from omnivores had higher counts on meat and casein than on soy protein and Quorn™ extract, while faecal bacteria from vegetarians had higher counts on soy protein and Quorn™ extract than meat and casein (Figure 2.1).

One way ANOVA was performed to compare differences between negative controls and various substrates and showed that Synergy1 significantly boosted the growth of bifidobacteria: count differences compared to the negative control were casein+Synergy1($p=0.003$; Figure 2.2), meat extract+Synergy1($p=0.005$), soy protein+Synergy1($p=0.001$), Quorn™ extract+Synergy1($p=0.001$) and Synergy1($p=0.01$).

With faecal samples from vegetarians, bifidobacteria did not increase on protein substrates but they decreased after 6 hours fermentation (Figure 2.2). Bifidobacteria did, however, increase on Synergy1, even in the presence of protein. After 24 hours, meat extract+Synergy1 and Quorn™+Synergy1 supported significant growth of bifidobacteria.

Lactobacilli from both omnivore and vegetarian donors increased on Synergy1 whether alone or with protein. With omnivore microbiota, lactobacilli increased significantly with Synergy1 (Figure 2.2): Quorn™+Synergy1 ($p=0.04$) and soy protein+Synergy1 ($p=0.03$) had significant growth at 6 hours; Synergy1 caused significant growth of lactobacilli at 10 hours ($p=0.004$); Casein+Synergy1 responded significantly after 24 hours ($p=0.01$). However, the increase of

lactobacilli with vegetarian donors was not statistically significant. In contrast with the growth of lactobacilli on Synergy1, those from negative controls with omnivore microbiota decreased after 10 hours fermentation, while the count of negative controls with vegetarian microbiota decreased from 0 hours of fermentation.

Stimulation of bifidobacteria and lactobacilli by Synergy1 was more significant with omnivore microbiota, however, the vegetarian microbiota had higher bifidobacteria and lactobacilli counts (around 7.5 and 5.7 $\log_{10}$ bacteria per ml respectively; Figure 2.2) at the beginning compared to omnivore microbiota (around 6.7 and 4.8 $\log_{10}$ bacteria per ml respectively).

Though there are studies confirming that many *Bacteroides* spp., are proteolytic (Macfarlane *et al.*, 1986), we found no significant changes in *Bacteroides.* spp. on protein substrates and Synergy1 (Figure 2.3). *Clostridium coccoides*, *Eubacterium rectale* and *Clostridium cluster* XIVa and XIVb responded to some protein substrates: these bacteria from omnivore donors increased on casein and meat while vegetarian microbiota increased with soy protein and Quorn^{TM} extract. *Roseburia* did not respond to protein or Synergy1. *Atopobium* cluster from all donors grew on protein substrates but this was only statistically significant on meat extract ($p=0.006$) at 48 hours using faecal inocula from omnivores and on Quorn^{TM} ($p=0.01$) and soy protein ($p=0.003$) at 10 hours using inocula from vegetarians (Figure 2.3). Clostridial cluster IX populations in cultures inoculated with samples from vegetarian donors increased on the protein substrates, but not significantly (Figure 2.4). Clostridial cluster IX populations in cultures inoculated with samples from omnivore donors significantly increased after 24h on meat extract, Quorn^{TM} extract, soy protein and protein+Synergy1 ($p<0.05$). Clostridial cluster IX populations dropped and then increased on all protein treatments (Figure 2.4). *Clostridium* clusters I and II also grew more on proteins compared to both the positive and negative controls, but only reached statistical significance on soy protein with inocula from omnivores at 24 hours ($p=0.03$; Figure 2.5).

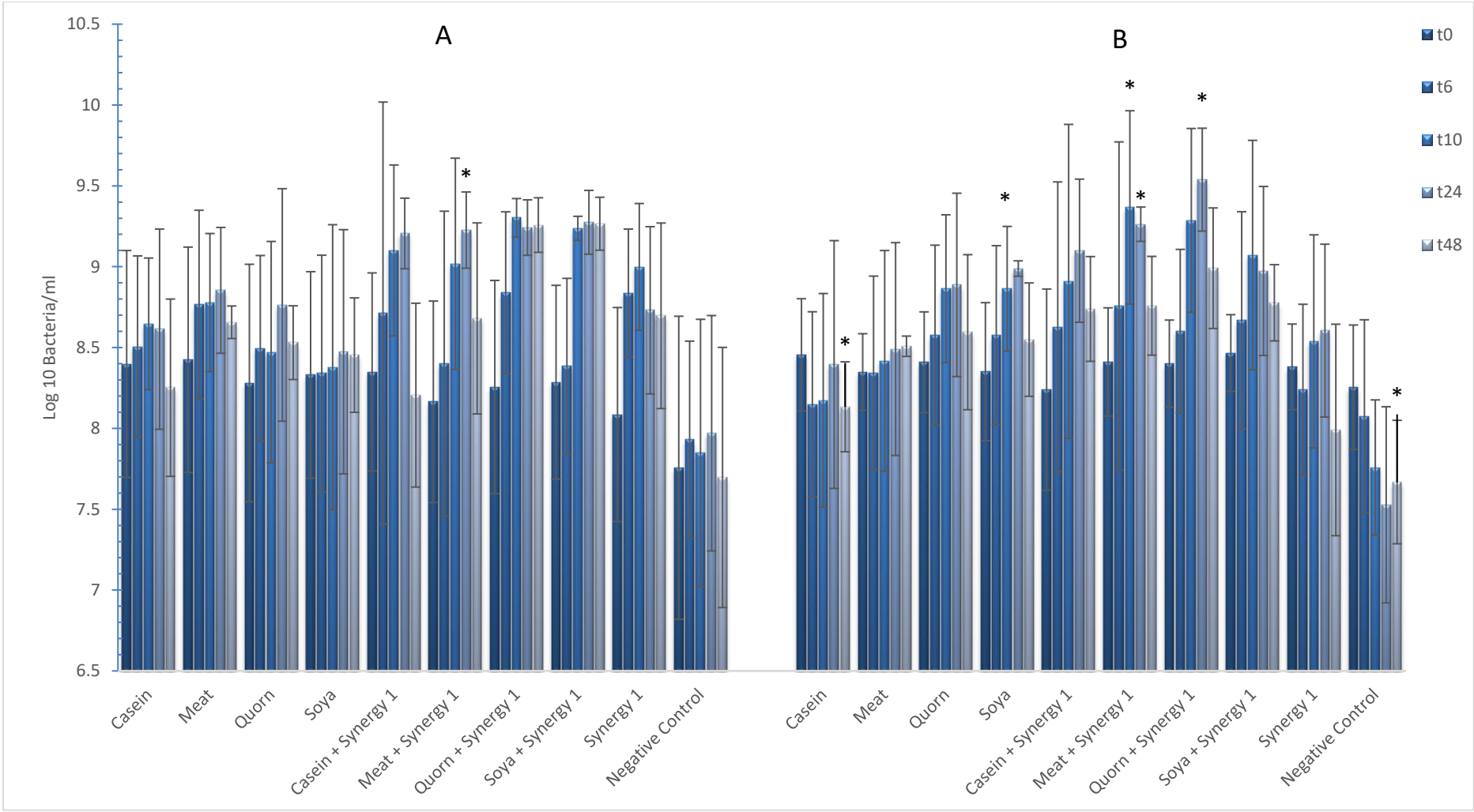

Figure 2.1 Total bacterial changes over time as $\log_{10}$ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at five time points from 3 omnivore faecal donors' (A) and 3 vegetarian faecal donors' (B) ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$).

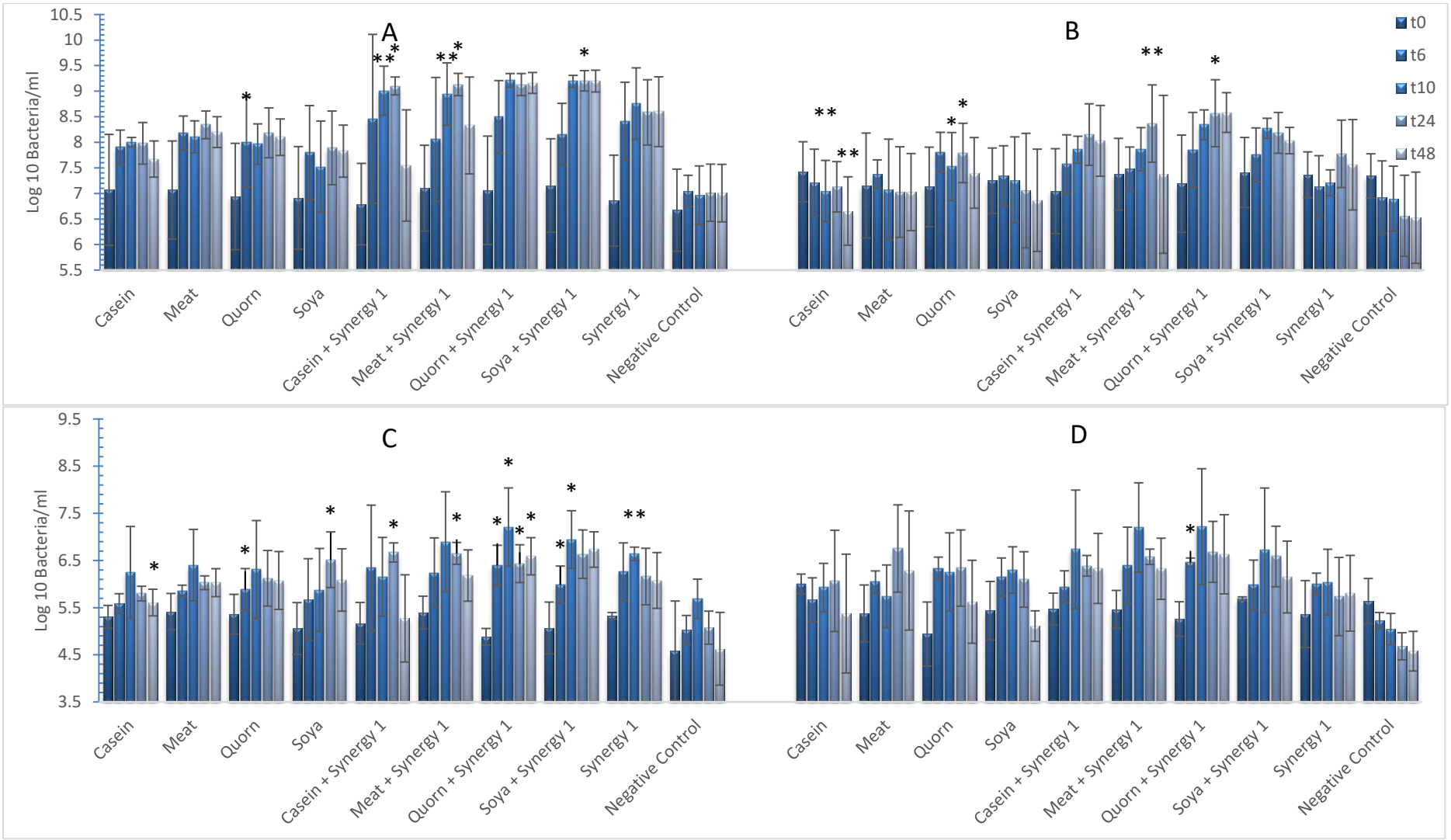

Figure 2.2 Bif164 (A and B) and Lab158 (C and D) changes over time as $\log_{10}$ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at five time points from 3 omnivore and 3 vegetarian faecal donors' ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$). ** Mean values were highly significantly different from 0h fermentation ($p<0.01$). A and C-Omnivores' microbiota; B and D-Vegetarians' microbiota

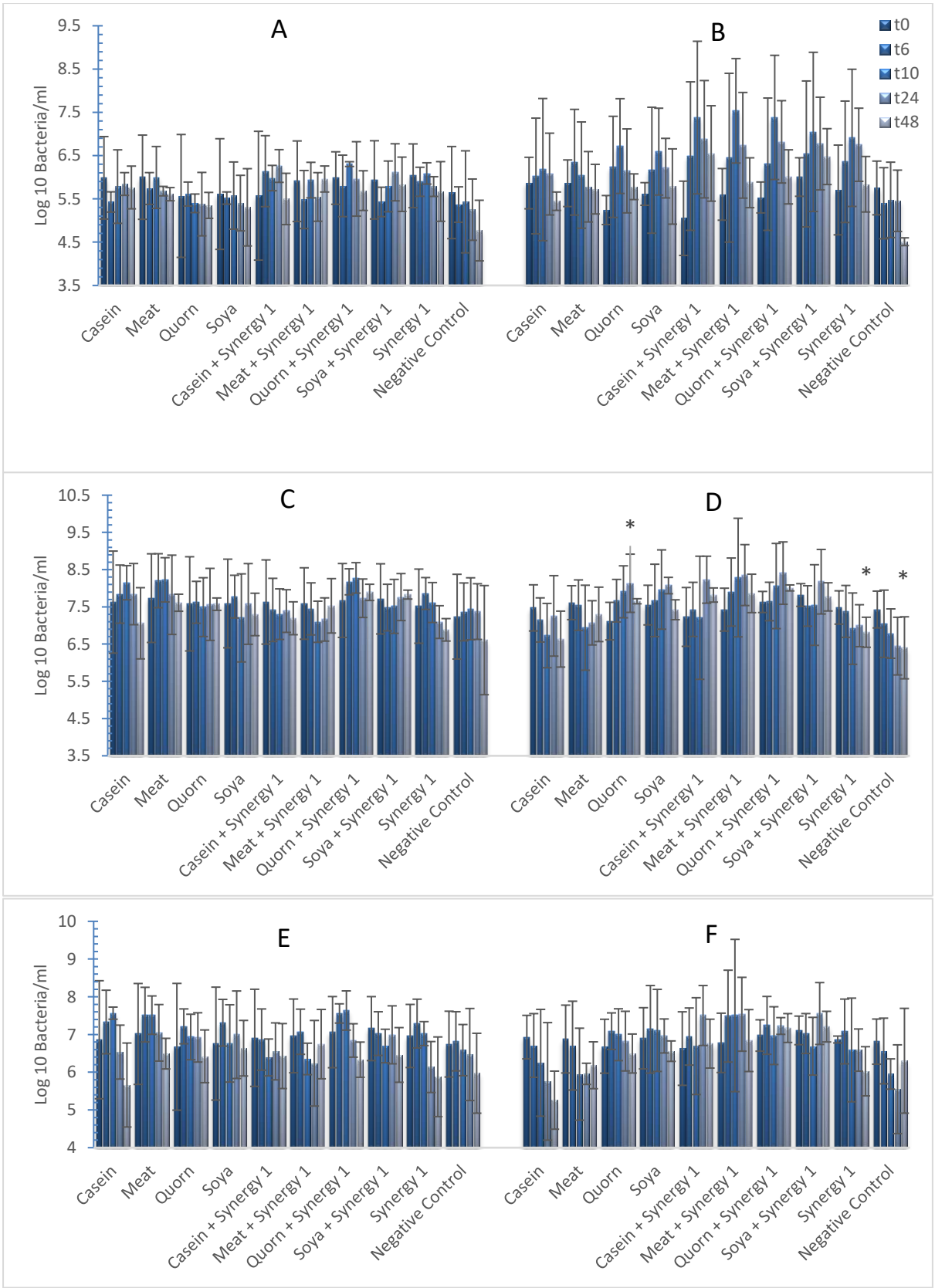

Figure 2.3 Bac303 (A and B), Erec482 (C and D) and Rrec584 (E and F) changes over time as $\log_{10}$ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at five time points from 3 omnivore and 3 vegetarian faecal donors ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$). A, C and E-Omnivores' microbiota; B, D and F-Vegetarians' microbiota

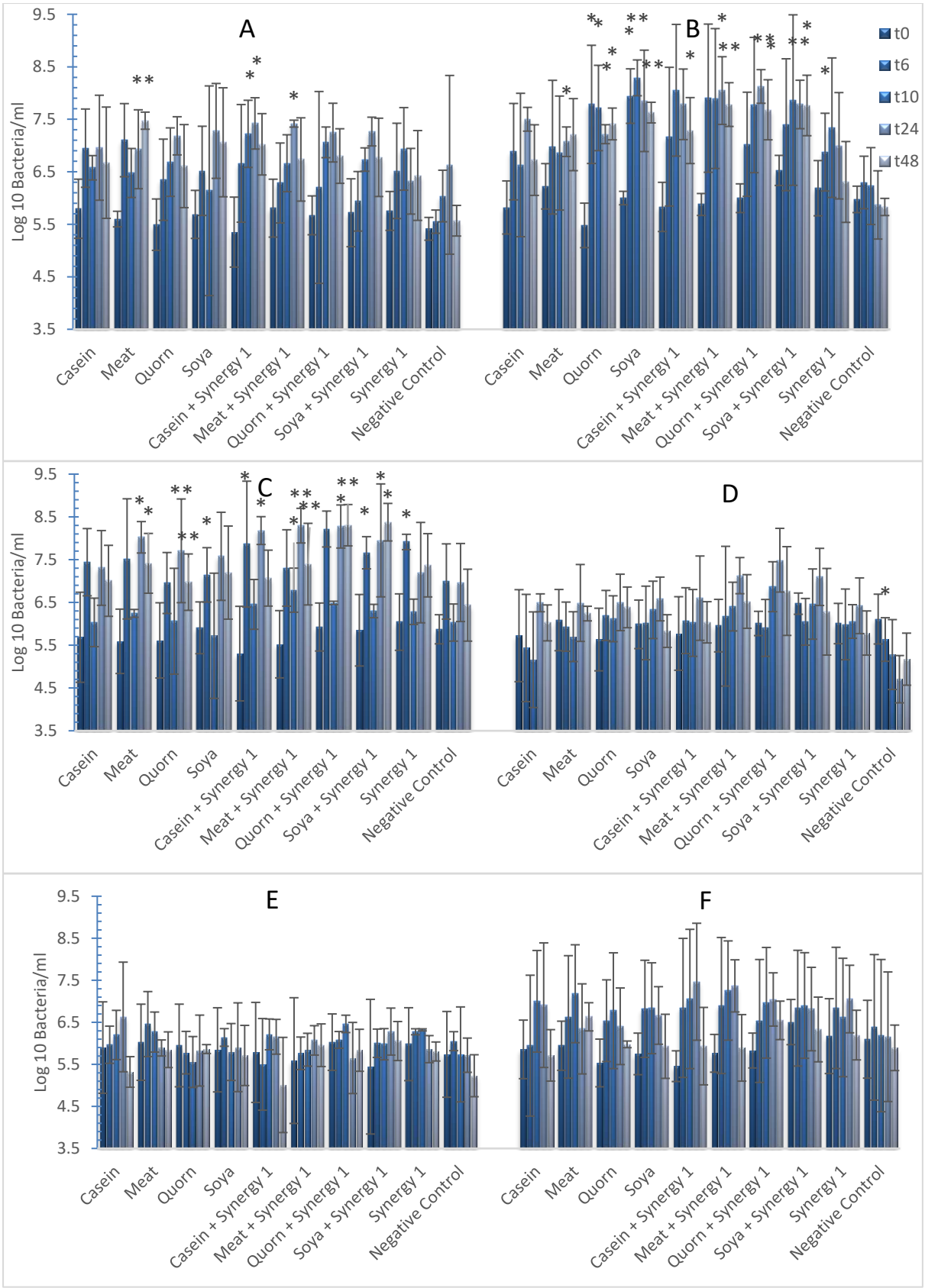

Figure 2.4 Ato291 (A and B), Prop853 (C and D) and Fprau655 (E and F) changes over time as $\log_{10}$ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at five time points from 3 omnivore and 3 vegetarian faecal donor's ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$). ** Mean values were highly significantly different from 0h fermentation ($p<0.01$).A, C and E-Omnivores' microbiota; B, D and F-Vegetarians' microbiota

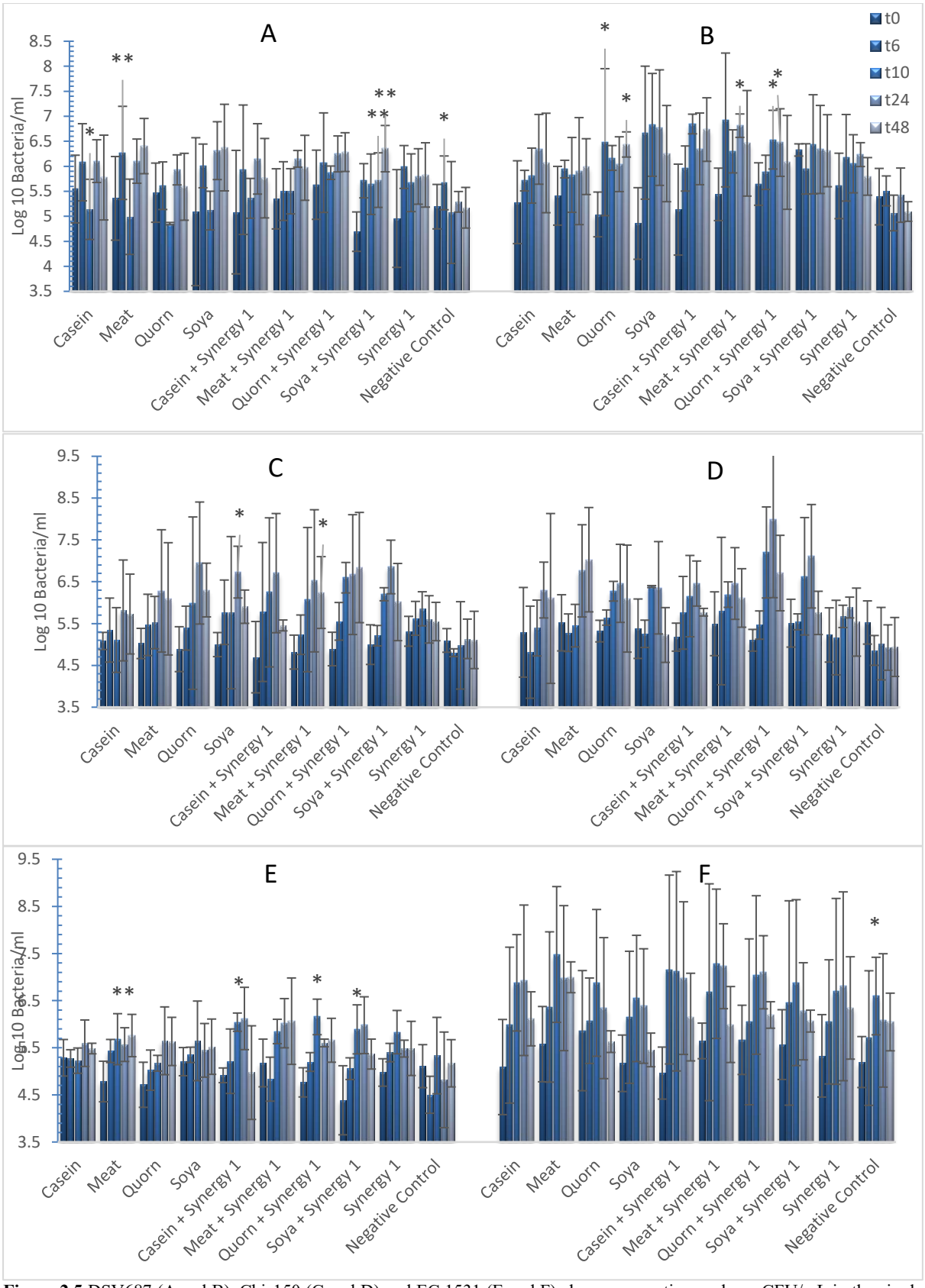

Figure 2.5 DSV687 (A and B), Chis150 (C and D) and EC 1531 (E and F) changes over time as $\log_{10}$ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at five time points from 3 omnivore and 3 vegetarian faecal donor's ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$). ** Mean values were highly significantly different from 0h fermentation ($p<0.01$). A, C and E-Omnivores' microbiota; B, D and F-Vegetarians' microbiota

2.4.2 Organic Acids

SCFAs such as acetic, propionic and butyric acid can affect health positively.
Acetate concentrations increased on Synergy1 (Figure 2.6). The production of acetate from omnivores' gut bacteria had to the highest production on meat extract, while vegetarians' gut bacteria produced the most with soy protein. Acetate is the main SCFA produced by bifidobacteria and there was a high concentration on Synergy1.

Propionic acid was also lower in those vessels without Synergy1 (Figure 2.7). Although large changes in *Bacteroides* populations were not seen, propionic acid could be produced by some species of *Clostridium* which increased in vessels containing Synergy1.
Butyric acid production was low in this study and no significant changes were found (Figure 2.7); this correlates with the lack of change in population of butyrate-producing bacteria (*Roseburia* and *Faecalibacterium prausnitzii*) throughout the fermentation.

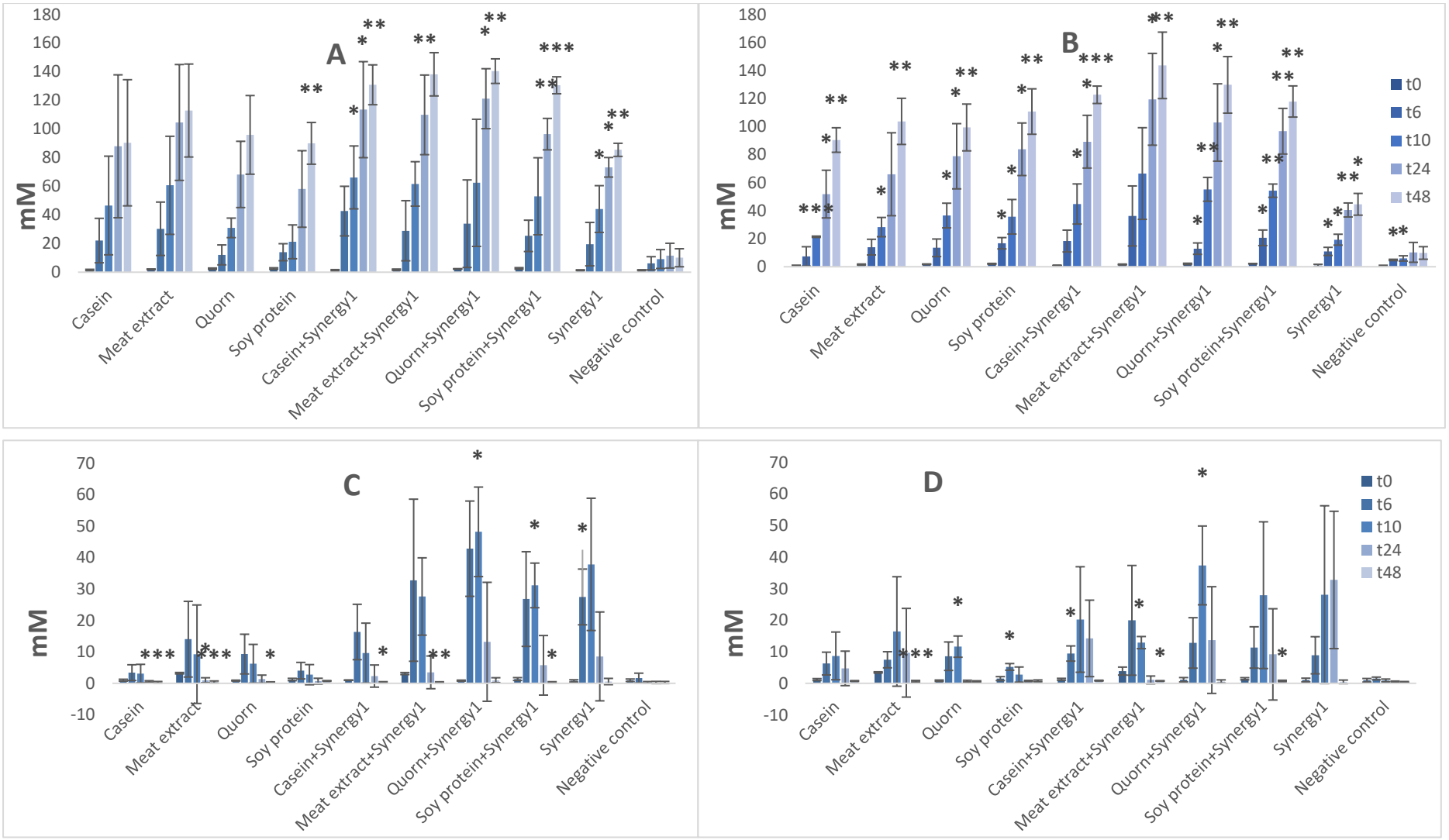

Figure 2.6 Changes in Acetate (A and B) and Lactate (C and D) (mM per mL) of batch culture sample concentration over time. Values are mean values at five time points from 3 omnivore and 3 vegetarian faecal donor's ± standard deviation. A and C-Acids production with omnivores' microbiota; B and D-Acids production with vegetarians' microbiota. * Mean values were significantly different from 0h fermentation ($p<0.05$). ** Mean values were highly significantly different from 0h fermentation ($p<0.01$). *** Mean values were highly significantly different from 0h fermentation ($p<0.001$).

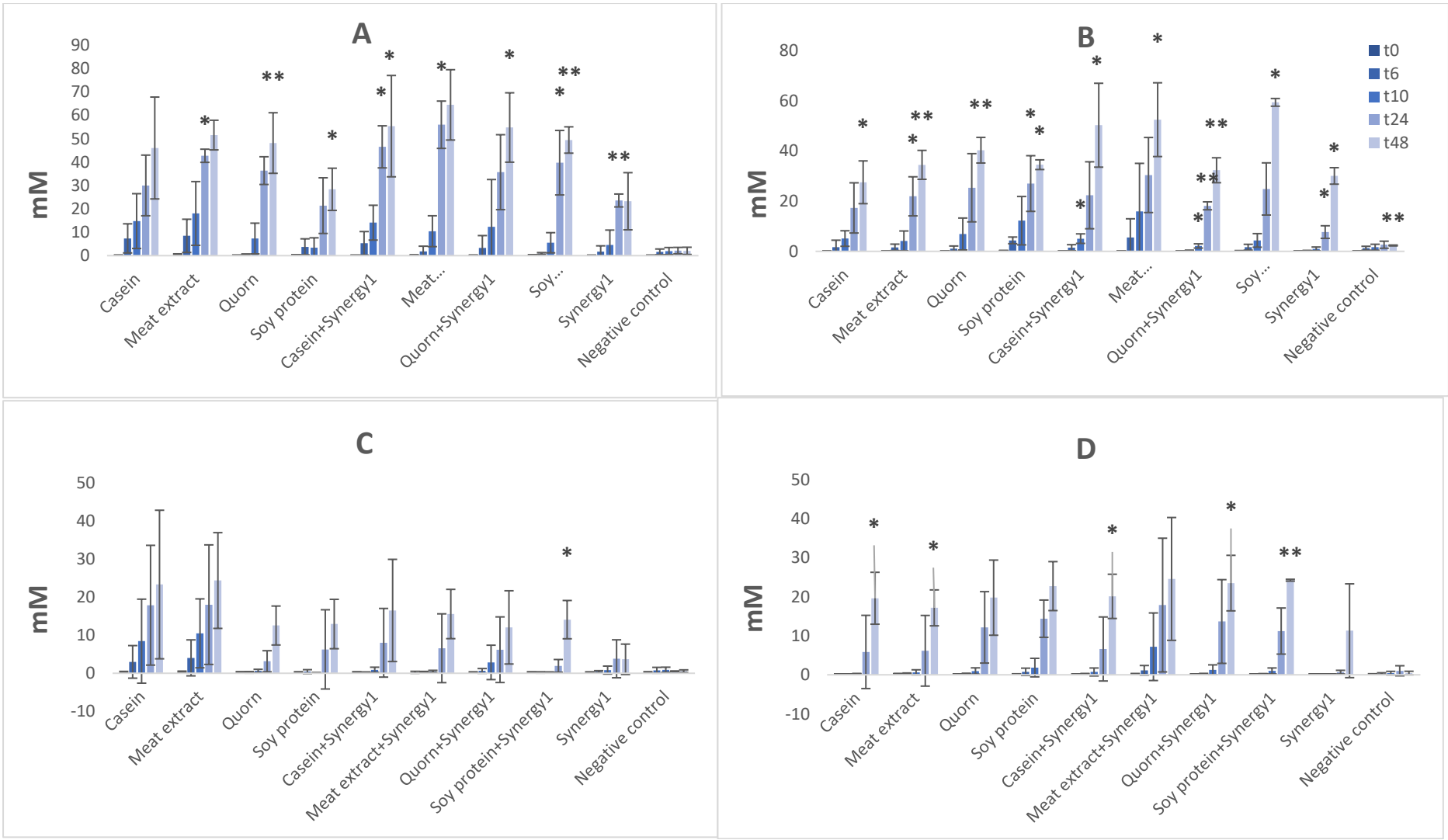

Figure 2.7 Changes in Propionate (A and B) and Butyrate (C and D) (mM per mL) of batch culture sample concentration over time. Values are mean values at five time points from 3 omnivore and 3 vegetarian faecal donor's ± standard deviation. A and C-Acids production with omnivores' microbiota; B and D-Acids production with vegetarians' microbiota. * Mean values were significantly different from 0h fermentation ($p<0.05$). ** Mean values were highly significantly different from 0h fermentation ($p<0.01$).

Branched amino acids such as leucine and isoleucine can be metabolised by faecal bacteria to produce branched chain fatty acids indicating proteolytic fermentation. Omnivores had much higher BCFA production while vegetarians had little production (Figure 2.8). Isovaleric acid production was higher than isobutyric acid. In vessels with omnivore microbiota, the presence of Synergy1 inhibited production of BCFA indicating that Synergy1 can reduce proteolysis by the gut microbiota.

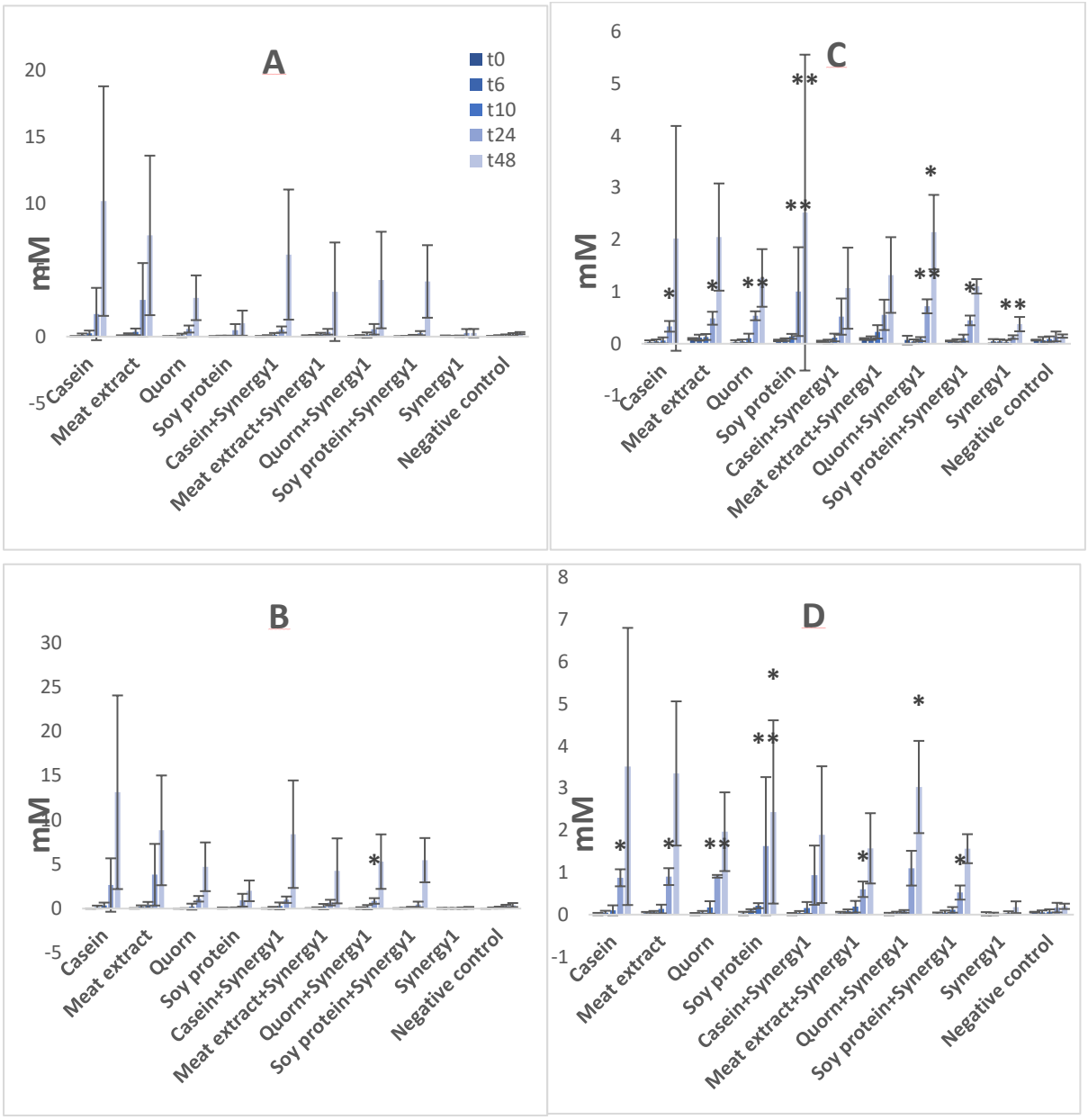

Figure 2.8 Changes in Isobutyrate (A and C) and Isovalerate (B and D) (mM per mL) of batch culture sample concentration over time. Values are mean values at five time points from 3 omnivore and 3 vegetarian faecal donor's ± standard deviation. A and B-Acids production with omnivores' microbiota; C and D-Acids production with vegetarians' microbiota. * Mean values were significantly different from 0h fermentation ($p<0.05$). ** Mean values were highly significantly different from 0h fermentation ($p<0.01$).

2.4.3 Volatile Organic Compounds

This study quantified four potentially detrimental volatile organic compounds (VOCs) which were indole, phenol, *p*-cresol and skatole. Production of these compounds varied individually and the effect of prebiotics on VOCs production also varied according to donor diet (Figure 2.9). The production of these volatile compounds from highest to lowest was indole, phenol, *p*-cresol and skatole in most cases, however with soy protein phenol production was higher than indole production. With all donors, comparing negative and positive controls, the production of volatile compounds was reduced by Synergy1. However, comparing with protein+Synergy1 and the corresponding protein, production of indole, phenol, *p*-cresol and skatole were inhibited by Synergy1 after 48 hours fermentation, only with faecal samples from omnivore donor 1, omnivore donor 2 and vegetarian donor 1. These three donors produced relatively high levels of VOCs compared with others: the concentration of VOCs was higher than 100mM. There is the possibility that Synergy1 could inhibit production of VOCs in the human gut.

Protein source affected the production of VOCs. According to this study, casein resulted in the highest concentration of VOCs in five donors, this was probably because casein is high in aromatic amino acids which are the main substrates for bacteria to produce phenolic and indolic compounds. Omnivore donor 3 had low phenolic production with casein correlating with this donor's low total bacterial count.

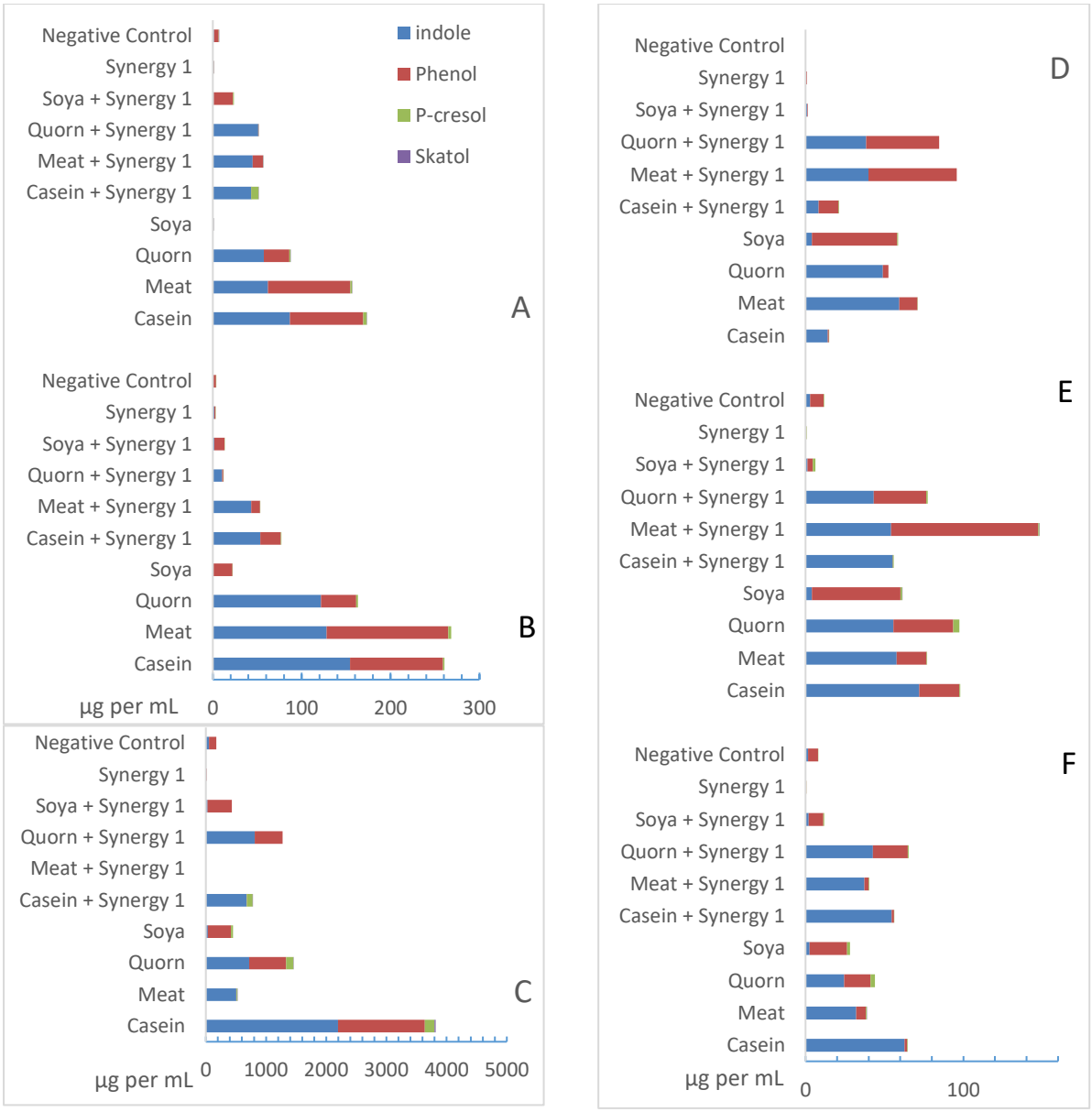

Figure 2.9 Changes in Phenolic compounds (µg per mL) of batch culture sample over time. Values are mean values at five time points from 3 omnivore and 3 vegetarian faecal donor's ± standard deviation. A-Omnivore donor1, B-Omnivore donor2, C-Vegetarian donor1, D-Omnivore donor3, E-Vegetarian donor2 and F-Vegetarian donor3.

2.4.4 Ammonia

Ammonia is considered a major metabolite of protein fermentation by faecal bacteria. Ammonia concentrations increased gradually during fermentation on all substrates together with the negative control when compared to the positive control (Figure 2.10). Ammonia concentrations of Synergy1 treatment, however, remained at low levels (17.55±4.53mM at 48 hours for omnivores and 25.47±4.55mM for vegetarians) compared with all protein treatments. Vessels with Synergy1 had less ammonia production compared to protein treatments, inferring that Synergy1 could inhibit the proteolytic activity of faecal bacteria. The host diet also had an effect on the selective fermentation of faecal substrates. With faecal samples from omnivores, fermentation had higher production of ammonia on casein and meat extract, however, with faecal samples from vegetarians, soy protein and Quorn extract produced more ammonia.

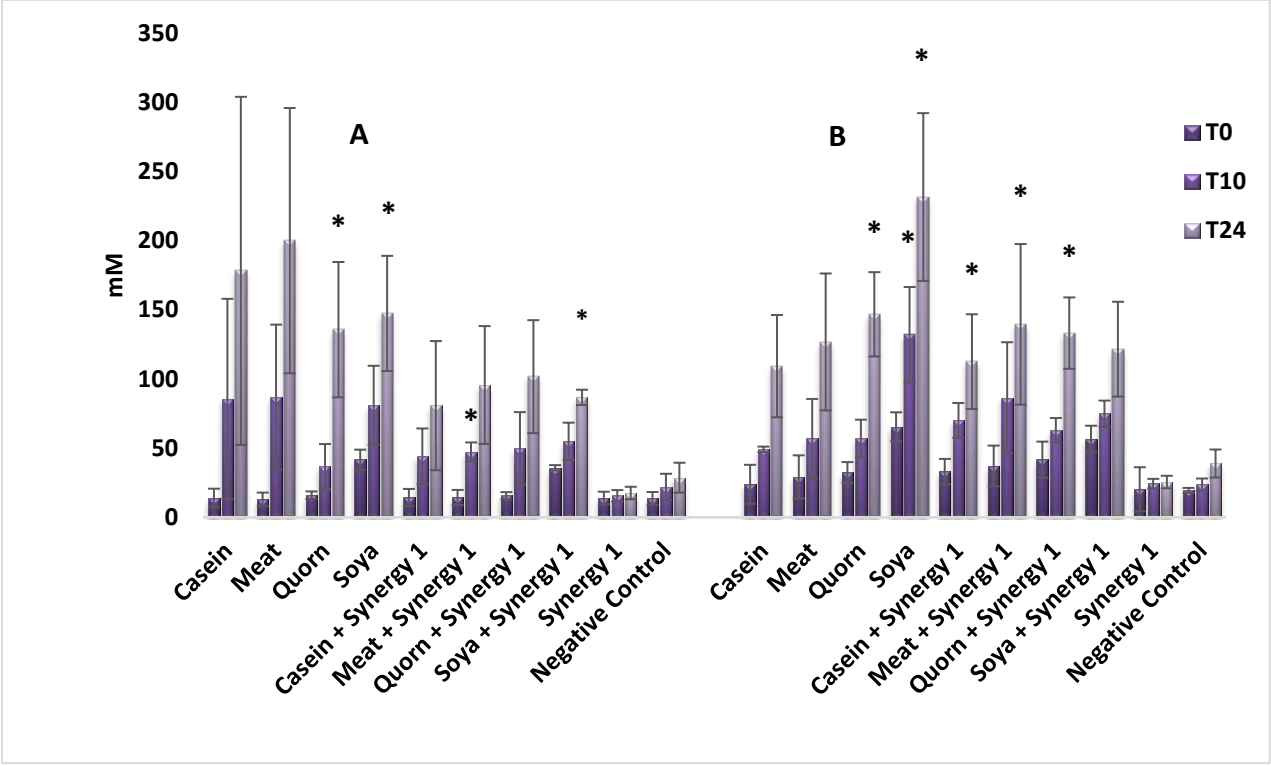

Figure 2.10 Changes in ammonia concentration (mM) of batch culture sample over time. Values are mean values at five time points from 3 omnivore and 3 vegetarian faecal donor's ± standard deviation. * Mean values were significantly different from 0h fermentation ($p<0.05$). A: omnivore donors. B: Vegetarian donors.

2.5 Discussion

Faecal bacteria responded differently on various substrates in pH controlled stirred batch cultures. Total bacterial populations were higher on protein+Synergy1 and Synergy1 solely

indicating a preference for polysaccharides by faecal bacteria. Proteins are less favoured than carbohydrates by the gut microbiota and this explains why proteolysis mainly happens in the distal colon where carbohydrates are depleted. One study testing different protein sources on the gut microbiota of weaning piglets revealed that total anaerobes increased on soy protein more so than casein and decreased with meat meal (Kellogg, 1964). However, in the present study, only a vegetarian microbiota had similar changes to that in this piglet study; the omnivore microbiotas were boosted by meat protein and casein rather than soy protein. In addition to differences between pigs and humans, host dietary habits may explain the preference of protein source. Growth of proteolytic bacteria within the human gut supported this: *Clostridium coccoides* and *Eubacterium rectale* from omnivore microbiota and vegetarian microbiota grew on meat/casein and soy/Quorn^{TM} respectively. Ammonia concentrations also indicate that an omnivore microbiota and a vegetarian microbiota favour different protein sources based on their host diet. A possible reason is the differences of amino acid composition among various proteins: bacteria that have adapted to the host diet can breakdown peptides, metabolise amino acids or utilise coupled Stickland amino acid fermentation.

By observing fermentation characteristics of the negative controls only: saccharolytic bifidobacterial growth at 6 hours with omnivore faeces occurred indicating that there was a small amount of undigested saccharides within the omnivore faecal sample; however, this was not seen from the vegetarian donors.

Even when total bacteria tend to be more saccharolytic, there were some proteolytic bacteria present in the gut microbiota. *Bacteroides* are deemed as having proteolytic activities; however, this study did not find any changes in this group of bacteria. Within the genus *Bacteroides*, many saccharolytic and proteolytic species are present. There is the possibility that donors in this study carried saccharolytic or weakly proteolytic *Bacteroides*, and the slight increase in *Bacteroides* from vegetarian microbiota on Synergy1 may prove this. The genus *Clostridium* contains more than 100 species and these bacteria can be saccharolytic, proteolytic, or both. Within Clostridial clusters I and II, there are saccharolytic species such as *C. butyricum* and *C. beijerinckii*, species that are both saccharolytic and proteolytic such as *C. sporogenes* and *C. acetobutylicum*, and proteolytic species such as *C. limosum* and *C. histolyticum*. This might explain why *Clostridium* numbers dropped and then increased at later times on those protein treatments with an omnivore microbiota: saccharolytic types from this genus were likely boosted by undigested saccharides within the fermenters at the beginning, and when carbohydrates became less abundant the number of saccharolytic clostridia dropped and

proteolytic types gradually increased. Vegetarian faeces may not contain as much undigested saccharides as omnivore faeces, therefore, the drop of *Clostridium* after 10 hours and increase after 24 hour occurred without any increase after 6 hours.

Vegetarian donor 1 had the highest production of phenolic and indolic compounds together with the highest *E. coli* population suggesting that *E. coli* contributes to the production of phenolic compounds as suggested by previous studies (Nicholson *et al.*, 2012). Indole and *p*-cresol are conjugated as indoxyl sulfate and *p*-cresol sulphate in the human body; before they are excreted via urine, they are toxic to human endothelial cells, can reflect the progression of chronic kidney diseases, and increase cardiovascular disease risk for these chronic kidney disease patients (Dou *et al.*, 2004, Dou *et al.*, 2007, Aronov *et al.*, 2011, Vanholder *et al.*, 2014). Therefore, reduced production of indole and *p*-cresol can benefit human health in many ways.

A few studies feeding rats with different protein sources did not find higher colonic toxicity of casein comparing with soybean, which is contrary to the phenol and *p*-cresol results in this study (Govers *et al.*, 1993, Toden *et al.*, 2007a). Feeding red meat gave higher DNA damage than casein in rats (Toden *et al.*, 2006). Similar effects were found in human epidemiological research: dairy products were inversely correlated with colorectal cancer in Finnish men and New York University women; they speculated that this protective effect may result from other nutrients in the dairy products but not from macronutrients such as protein (Pietinen *et al.*, 1999, Kato *et al.*, 1997). Mycoprotein is a relatively new protein source from the filamentous fungus *Fusarium venenatumsource* under the trade mark of Quorn™ (Wiebe, 2004). Quorn™ products contain all essential amino acids, is low in fat and high in dietary fibre. However, in terms of protein fermentation by gut microbiota, Quorn™ did not show any difference compared to other proteins.

The use of pH controlled stirred batch culture system allowed rapid analysis of different protein fermentations by gut microbiota and the impact of prebiotics. This fermentation system is limited: SCFAs would be absorbed in the human colon and digesta supply is continuous.

Some animal studies have revealed an inhibitory effect of proteolysis by resistant starch (Younes *et al.*, 1995, Heijnen and Beynen, 1997, Toden *et al.*, 2005). These were investigated by analysing indolic/phenolic compounds, or nitrogen secretion in the urine and faeces. One of these studies also compared DNA damage with and without resistant starch in rat colonic cells,

and found resistant starch protected cells from DNA damage (Toden *et al.*, 2005). One possible mechanism of decreased proteolytic fermentation by prebiotics is through growth of saccharolytic bacteria that would need more amino acids for reproduction, therefore amino acids would not be available for proteolytic bacteria to metabolise.

When concidering fermentation patterns of omnivores and vegetarians omnivores fermentation models had higher production of ammonia on casein when fermenting meat extract; on the otherhand vegetarians' models produced more ammonia from soy protein and Quorn extract. This could be due to diet protein sources difference: microbiota from vegetarian donors have adapted to vegetarian protein sources and can utilise these proteins more efficiently. Besides, in this study, lower BCFA production was found with vegetarians' gut bacteria; this could suggest that these vegetarian donors had lower branched chain amino acids in their diet.

Addition of Synergy1 to 48 hour batch culture fermentation changed the microbiota to a more saccharolytic nature by stimulation of bifidobacteria and lactobacilli without a significant change of *Bacteroides*, *Clostridium* and *E. coli*. Supplementation with Synergy1 also reduced the concentration of protein metabolites (ammonia and BCFA); in those donors with high production of VOCs, inhibition was also found with Synergy1. Therefore, adding fructan prebiotics could potentially reduce or eliminate the negative consequences of ingesting high protein diets.

2.6 References

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Chapter 3 The effect of prebiotic inulin type fructans on bacterial metabolism of high protein levels, an *in vitro* study

Abstract

Dietary protein levels are generally higher in Western populations than the world average, and this can result in microbial generation of various toxic metabolites in the gut. A prebiotic is "a substrate that is selectively utilised by host microorganisms conferring a health benefit". Prebiotics may have the potential to reverse the harmful effects of gut bacterial protein fermentation.

Three-stage continuous colonic model systems were inoculated with faecal samples from omnivore and vegetarian volunteers. Casein (equivalent to 105g protein consumption per day) was used within the systems as a fermentable protein source. Two different doses of inulin type fructans (Synergy1) were later added (equivalent to 10g per day *in vivo* and 15g per day) to assess whether this influenced proteolysis. Bacteria were enumerated by fluorescence *in situ* hybridisation-flow cytometry. Metabolites from bacterial fermentation (short chain fatty acid (SCFA), ammonia, phenol, indole and *p*-cresol) were monitored to further analyse proteolysis. A significant higher number of bifidobacteria was observed with the addition of Synergy1, together with reduction of *Bacteroides* and *Desulfovibrio* spp. Furthermore, metabolites from protein fermentation such as branched chain fatty acids (BCFA), ammonia production were significantly lowered with Synergy1. The higher dose of prebiotic had a more inhibitory effect on protein fermentation.

Conclusion: Faecal fermentations of proteins with a prebiotic supplementation resulted in a change in the human gut microbiota and inhibited the production of some proteolytic metabolites.

3.1 Introduction

Protein consumption is increasing annually worldwide and 2.5% of British adult males consume 136g protein per day according to a recent national dietary survey (FAO, 2017, Food Standards Agency, 2016). Most protein is metabolised in the small intestine, but residual dietary protein may enter the large intestine where colonic bacteria can utilise it and produce various metabolites. Proteolysis gradually increases throughout the colon, being highest in distal regions where the carbohydrate is depleted (Macfarlane *et al.*, 1988, Macfarlane *et al.*,

1992b). Bacterial degradation of amino acids produces ammonia, amines, CO_2, short chain fatty acids, branched chain fatty acids, occasionally H_2S and aromatic molecules such as indole, *p*-cresol and skatole (Smith and Macfarlane, 1997). These metabolites may interact with the host and affect human health in many ways. In cell culture experiments, ammonia at relevant lumenal concentrations was found to have a negative effect on barrier function with elevated permeability (Hughes *et al.*, 2008). Two animal studies illustrated how ammonia can shorten mucosal cell lifespan, and promote colon cancer together with other carcinogens *in vivo* (Clinton *et al.*, 1988, Lin and Visek, 1991). Hydrogen sulphide can be generated from sulphur containing amino acids together with reduction of dietary sulphates; both rat and human colonocyte cell lines showed that H_2S had a negative effect on butyrate and acetate oxidation by cells for energy uptake (Roediger *et al.*, 1993, Leschelle *et al.*, 2005). Bacterial H_2S production rates are higher in ulcerative colitis patients compared to healthy adults which may contribute to the pathogenesis of some inflammatory bowel diseases (Gibson *et al.*, 1991). Aromatic amino acids such as tyrosine, phenylalanine and tryptophan can be degraded by colonic bacteria producing phenol, indole, *p*-cresol, skatole and other aromatic metabolites. Phenol and *p*-cresol have been shown to damage epithelial barrier function *in vitro* (Cerini *et al.*, 2004, Hughes *et al.*, 2008, McCall *et al.*, 2009). Indole and *p*-cresol would be transformed to indoxyl sulphate and *p*-cresol sulphate after conjugation in the human body and indoxyl sulphate and p-cresol sulphate levels in blood are correlated with renal disease progression and vascular dysfunction in chronic kidney disease (Barreto *et al.*, 2009, Vanholder *et al.*, 2014).

A prebiotic is defined as "a substrate that is selectively utilised by host microorganisms conferring a health benefit" by Gibson *et al.* (2017). One confirmed type of prebiotic is inulin type fructans which are carbohydrates comprised of fructose residues, sometimes with a glucose at the terminal. The D-fructose molecules are linked by β (2→1) linkages and when there is a glucose, the chain is terminated by a D-glucose molecule bonded to fructose by an α(1↔2) linkage. A randomised, double-blind, placebo-controlled, cross-over human study recently analysed bacterial compositional changes after inulin type fructan intervention by 16S rDNA sequencing: bifidobacteria and *Anaerostipes* increased while *Bilophila* decreased after prebiotic supplementation for 4 weeks, together with an improvement in constipation (Vandeputte *et al.*, 2017). Other human intervention studies have investigated various health benefits of inulin type fructans such as mineral absorption, energy regulation and lipid lowering properties (Holloway *et al.*, 2007, Russo *et al.*, 2011b, Jackson *et al.*, 1999). Metabolism of inulin type fructans not only promotes specific bacterial changes in the colon, but also generates

short chain fatty acids (SCFA). Bifidobacteria can produce acetate and lactate from carbohydrates by the bifid shunt and these two acids are substrates for butyrate producers such as *Eubacterium rectale* and *Anaerostipes caccae* resulting in enhanced butyrate production in the gut (Riviere *et al.*, 2015, Moens *et al.*, 2017). SCFA, especially butyrate, can promote epithelial cell differentiation and apoptosis *in vitro*, therefore they may possibly reduce colorectal cancer risk in the human body (Hague *et al.*, 1995).

Here, prebiotic effects on the possible negative consequence of protein fermentation in the gut were studied using an *in vitro* multiple stage continuous culture system.

3.2 Materials

3.2.1 Proteins

Protein substrates used were casein hydrolysates (Sigma-Aldrich, Poole, UK)

3.2.2 Prebiotic

Inulin-type fructan was a mixture of oligofructose and inulin: 50% ± 10% degree of polymerisation (DP) of 3-9 and 50%±10% DP≥10 (Orafti®Synergy 1, BENEO-Orafti, Tienen, Belgium).

3.3 Methods

3.3.1 Protein and prebiotic dose determination

A miniature gut model system, 33% the size of the version described by Macfarlane *et al.* (1998) was used in this study. Approximate 16g proteins will reach the colon following ingestion of 105g protein/day of which 8g are endogenous and 8g are exogenous (Gibson *et al.*, 1976, Moughan *et al.*, 2005). The *in vitro* dose required to simulate prebiotic and protein effects *in vitro* was calculated as follows:

Table 3.1 Protein dosage calculation for continuous gut model fermentation.

Steady State	*In vivo*	*In vitro* fermentation
Steady state 2	8g dietary protein per day	2.67g per day
Steady state 3	8g dietary protein per day	2.67g per day
	10g prebiotic per day	3.33g per day
Steady state 4	8g dietary protein per day	2.67g per day
	15g prebiotic per day	5g per day

3.3.2 *In vitro* gut model fermentation

3.3.2.1 Faecal sample preparation

Faecal samples were obtained from three healthy meat eating individuals and three healthy vegetarian volunteers between the ages of 18 and 60, who had not taken antibiotics for at least six months prior to the experiment and had no history of gastrointestinal disorders.
Faecal samples were diluted 1 in 20 (w/v) using 1M phosphate buffered saline (PBS, Oxoid, Hampshire, UK), pH7.4. This suspension was homogenised in a stomacher (Seward, stomacher 80, Biomaster) for 120 seconds at normal speed.

3.3.2.2 Gut model medium.

Gut model medium was prepared with chemicals obtained from Sigma-Aldrich, Poole, UK unless otherwise stated. In one litre: 5g starch, 5g peptone water, 5g tryptone, 4.5g yeast extract (Oxoid, Hampshire, UK), 4.5g NaCl, 4.5g KCl, 4g mucin (porcine gastric type III), 3g casein, 2g pectin (citrus), 2g xylan from beech wood pure (SERVA, Heidelberg, Germany), 2g arabinogalactan (larch wood), 1.5g $NaHCO_3$ (Fischer scientific, Loughborough, UK), 1.25g $MgSO_4 \cdot 7H_2O$ (Fischer scientific, Loughborough, UK), 1g guar gum, 1g inulin (BENEO-Orafti, Tienen, Belgium)., 0.8g L–cystine HCl, 0.5g KH_2PO_4, 0.5g K_2HPO_4, 0.4g bile salts No. 3, 0.15g $CaCl_2 \cdot 6H_2O$, 0.005g $FeSO_4 \cdot 7H_2O$, 0.05g haemin, 10µl vitamin K, 1ml Tween 80 and 4ml resazarin (0.025g/100ml, pH7).

3.3.2.3 Three stage continuous pH controlled, gut model fermentation

A three stage continuous fermentation culture system was used to simulate the lumenal conditions in each of the three distinct regions of the human colon: proximal, transverse, and distal colon (V1, V2, and V3) (Macfarlane *et al.*, 1998). Vessels with an operating volume of 80mL, 100mL, and 120mL were set up in sequence. Autoclaved culture medium (51.43 ml (V1), 66.67 ml (V2), 82.5 ml (V3)) were aseptically poured into sterile vessels. This system was left overnight with oxygen free nitrogen pumping through the media at a rate of 15mL/min. Each vessel was temperature controlled at 37°C and stirred using a magnetic stirrer. Faecal slurry at 20% (w/v) was inoculated into the culture vessels (28.57 ml (V1), 33.33 ml (V2), 37.5 ml (V3)) and left to equilibrate for 24 h as a batch culture system prior to commencing the continuous medium flow. Control of pH was achieved by pH meters (Electrolab pH controller, Tewksbury, UK) connected to each vessel to regulate pH at 5.4 to 5.6 (V1), 6.1 to 6.3 (V2) and 6.7 to 6.9 (V3) with the aid of 0.5M HCl and NaOH. Oxygen free nitrogen flow and pH were

maintained throughout the whole experiment.

After 8 turnovers (16 days) of the operating volume (300ml in total) at a medium flow rate of 6.25ml/hour, SCFA were analysed for three consecutive days to confirm the establishment of steady state. Samples were taken for three consecutive days after confirmation of the equilibrium for analysis of bacterial populations and metabolite concentrations.

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

A 750µl sample of fermentation fluid was centrifuged at 11337 × g for 5 minutes to remove supernatant. The pellet was then suspended in 375µl filtered 0.1M PBS solution. Filtered 4% paraformaldehyde (PFA) at 4°C (1125µl) were added and samples were stored at 4°C for 4 hours. Samples were then washed thoroughly with PBS to remove PFA and re-suspended in a mixture containing 300 µl PBS and 300 µl 99% ethanol. Samples were then stored at -20°C, prior to FISH analysis by flow cytometry. FISH by flow cytometry was carried out as described by Grimaldi *et al.* (2017). The probes used in this study are listed below:

Table 3.2 Name, sequence, and target group of oligonucleotide probes used in this study for FISH of bacterial enumeration

Probe name	Sequence (5' to 3')	Targeted groups	Reference
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	(Wallner *et al.*, 1993)
Eub338‡	GCTGCCTCCCGTAGGAGT	Most bacteria	(Amann *et al.*, 1990)
Eub338II‡	GCAGCCACCCGTAGGTGT	Planctomycetales	(Daims *et al.*, 1999)
Eub338III‡	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	(Daims *et al.*, 1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	(Langendijk *et al.*, 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*	(Harmsen *et al.*, 1999)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz *et al.*, 1996)
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium* coccoides-*Eubacterium rectale* group (Clostridium cluster XIVa and XIVb)	(Franks *et al.*, 1998)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus	(Walker *et al.*, 2005)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(Harmsen *et al.*, 2000a)
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX	(Walker *et al.*, 2005)
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and relatives	(Suau *et al.*, 2001)
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	Ramsing *et al.* (1996)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)	Franks *et al.* (1998)
EC 1531	CAC CGT AGT GCC TCG TCA TCA	*Escherichia coli* BJ4	(Poulsen *et al.*, 1994)

3.3.4 Short chain fatty acid (SCFA) and volatile organic compounds analysis by GC

Samples (1ml) were collected and extractions performed with some modifications of a method from Richardson *et al.* (1989). Sample (1ml) was transferred into a labelled 100 mm×16 mm glass tube (International Scientific Supplies Ltd, Bradford, England) with 50 µl of 2-ethylbutyric acid (0.1 M, internal standard) (Sigma, Poole, UK). Concentrated HCl (500 µl) and 1 ml diethyl ether were added to each glass tube and samples vortexed for 1 minute. Samples were centrifuged at 2000 g for 10 minutes. The diethyl ether (upper) layer of each sample was transferred to a labelled clean glass tube. Ether extract (400 µl) and 50 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA, Sigma-Aldrich, Poole, UK) were added into a GC screw-cap vial. Samples were left at room temperature for 72 hours to allow lactic acid in the samples to completely derivatise.

An Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS 30m×0.25mm column with a 0.25µm coating of crosslinked (5%-Phenyl)-methylpolysiloxane, (Hewlett Packard, UK) was used for analysis of SCFA. Temperatures of injector and detector were 275°C, with the column temperature programmed from 63°C to 190°C at 15°C min^{-1} followed by 190°C for 3 minutes. Helium was the carrier gas (flow rate 1.7 ml min^{-1}; head pressure 133 KPa). A split ratio of 100:1 was used. Quantification of the samples was achieved by calibration with lactic, acetic, formic, propionic, butyric, valeric acids and branched SCFA (iso-butyric and iso-valeric) in concentrations between 12.5 and 100 mM.

3.3.5 Ammonia Analysis

Samples were diluted 1 in 50 v/v prior to analysis. Ammonia concentrations in diluted fermentation samples were analysed by a Sigma-Aldrich FluoroSELECT™ ammonia kit (Sigma-Aldrich, Poole, UK). Reagent was prepared by combining 100 µL assay buffer, 4 µL reagent A and 4 µL reagent B provided in the kit. H_2O (10 µL) served as a blank and 10 µL of each sample were added to glass vials. Reagent (100 µL) was then added to each tube. Samples were kept in the dark for 15 minutes at room temperature before they were read in a fluorimeter. Ammonia standards were prepared by diluting 20 mmol/L NH_4Cl into distilled water and the concentration range was 0.25-1 mmol/L).

3.4 Results

3.4.1 Bacterial Enumeration

High protein feeding did not affect bacterial numbers in the proximal colon simulation,

however the simulated transverse colon of both omnivore and vegetarian models and the simulated distal colon of vegetarian models had significantly higher bacterial populations after protein feeding (Figure 3.1). After prebiotic feeding total bacterial populations in gut models from both omnivore and vegetarian donors were slightly higher, especially at the higher dose in the models inoculated with omnivore donors ($p<0.001$ on all three regions). Meanwhile populations of bifidobacteria and lactobacilli were increased by growth on Synergy1 in all models (Figure 3.2). *Bacteroides.* spp, populations displayed trend to increase when grown on the protein and decreased significantly when grown on Synergy1 (Figure 3.3). *Bacteroides* spp. are significant propionate producers and a potential proteolytic species (Macfarlane *et al.*, 1986).

Within *Clostridium* cluster XIVa and XIVb there are some proteolytic bacteria and this group responded to the high protein dose (Figure 3.4): these bacteria from omnivore donors increased in all vessels while vegetarians' microbiota increased in transverse and distal colon simulations only. The number of facultatively anaerobic *Atopobium* cluster from all donors grew through all steady states.

Desulfovibrio can reduce dietary sulphate and produce H_2S (Gibson *et al.*, 1990). These organisms were not affected by the protein containing medium, however, both doses of prebiotics decreased numbers of *Desulfovibrio* (Figure 3.5). In omnivore-inoculated models, reduction was significant in the proximal and transverse colon vessels while in vegetarian-inoculated models the change was only significant in the proximal region with the lower dosage.

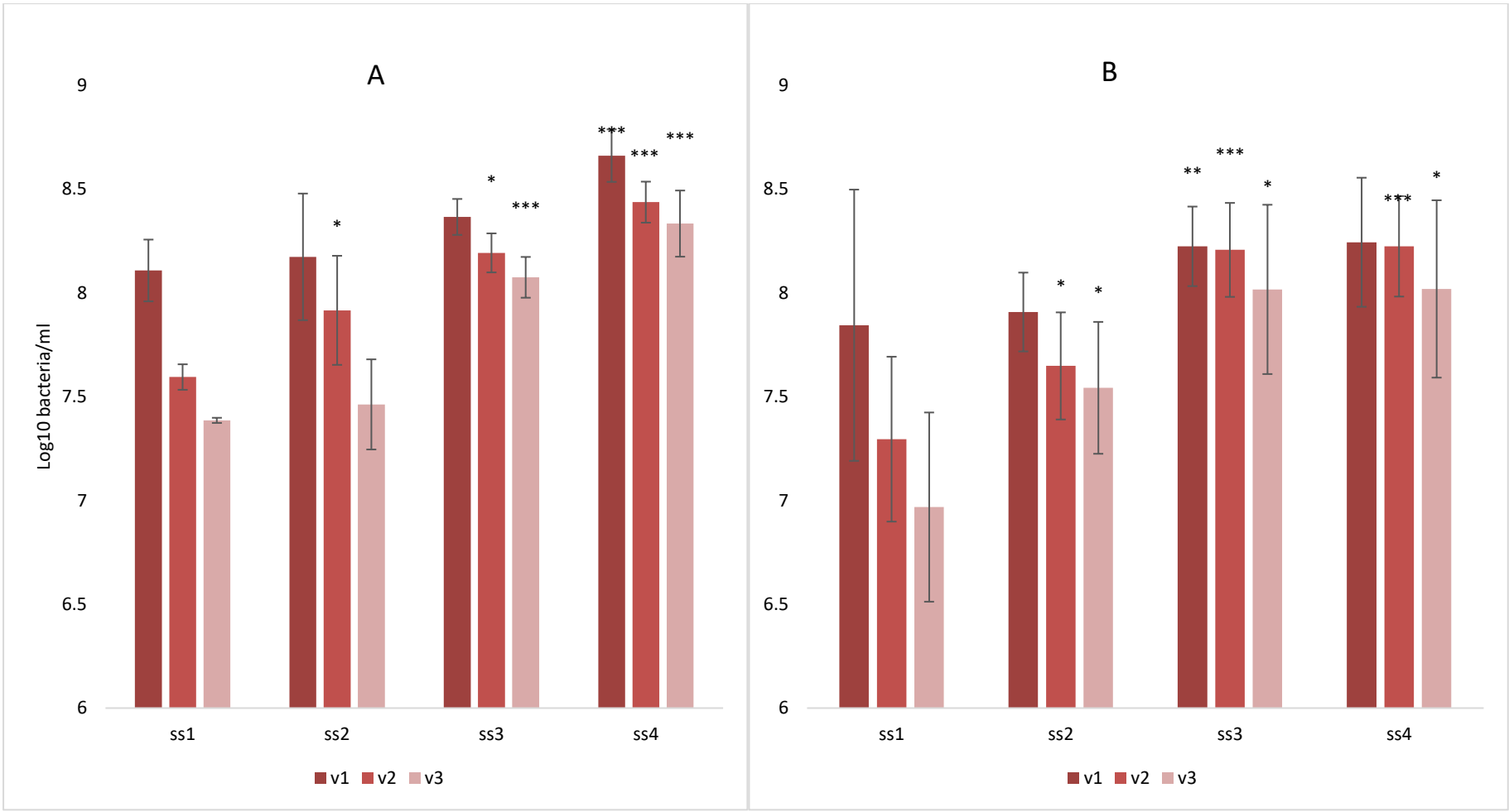

Figure 3.1 Total bacterial changes with different steady states as log10 CFU/mL in three different vessels as analysed by FISH. Values are mean values over consecutive three days from 3 omnivore microbiota (A) and 3 vegetarian microbiota (B) ± standard deviation. * Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.05$). ** Mean values were highly significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.01$). *** Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.001$).

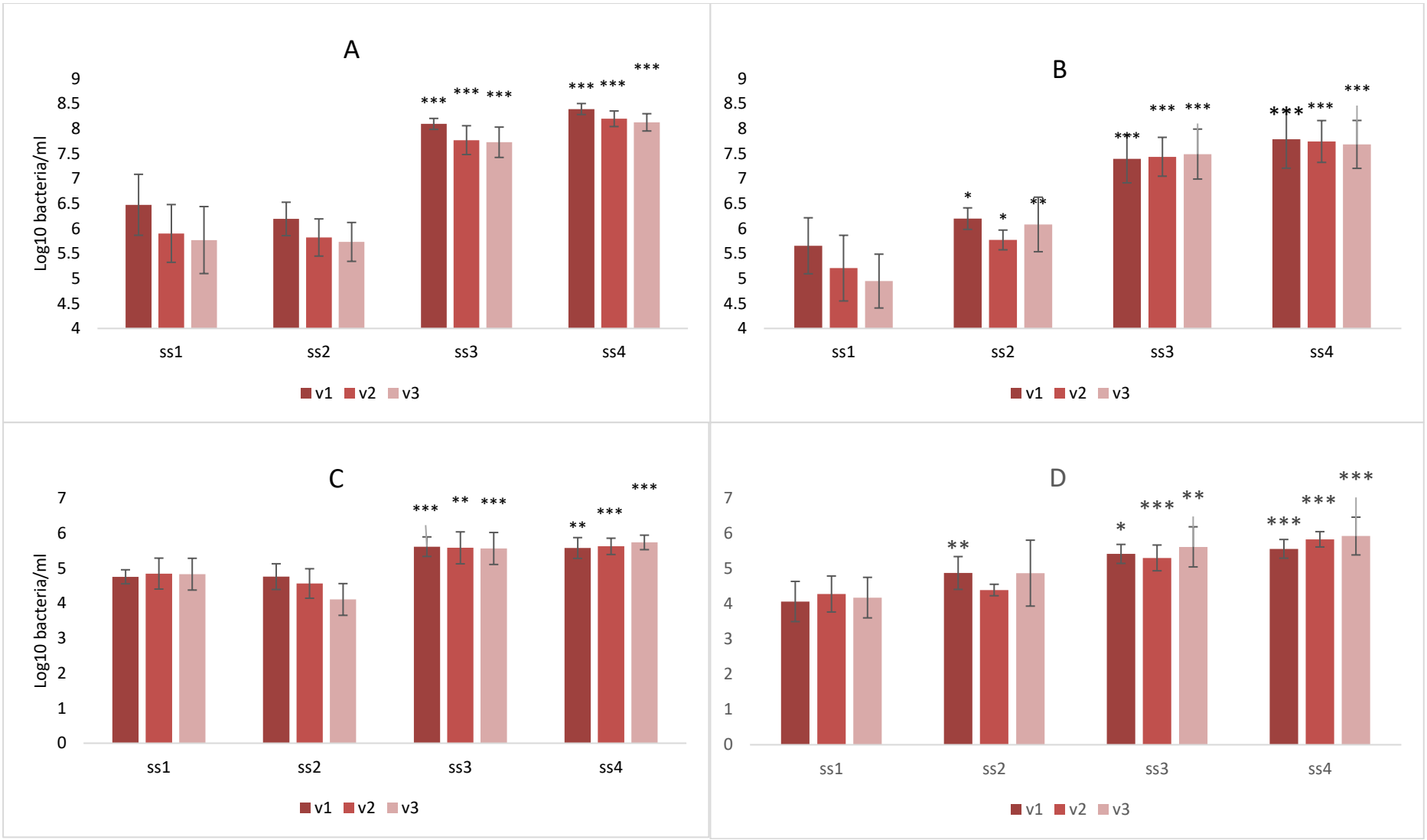

Figure 3.2 Bif164 (A and B) and Lab158 (C and D) changes with different steady states as log10 CFU/mL in three different vessels as analysed by FISH. Values are mean values over consecutive three days from 3 omnivore microbiota (A and C) and 3 vegetarian microbiota (B and D) ± standard deviation. * Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.05$). ** Mean values were highly significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.01$). *** Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.001$).

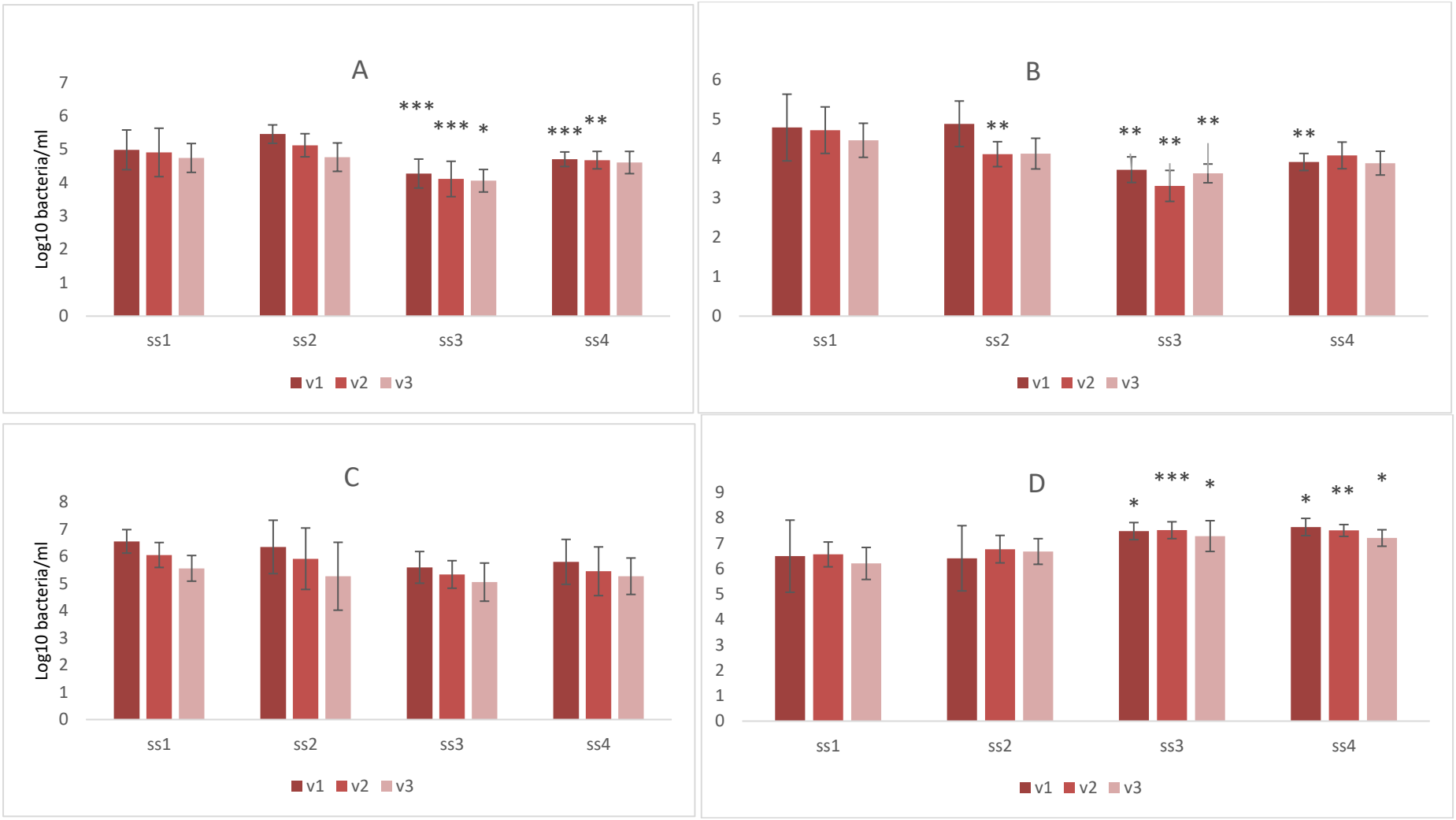

Figure 3.3 Bac303 (A and B) and Prop853 (C and D) changes with different steady states as log10 CFU/mL in three different vessels as analysed by FISH. Values are mean values over consecutive three days from 3 omnivore microbiota (A and C) and 3 vegetarian microbiota (B and D) ± standard deviation. * Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.05$). ** Mean values were highly significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.01$). *** Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.001$).

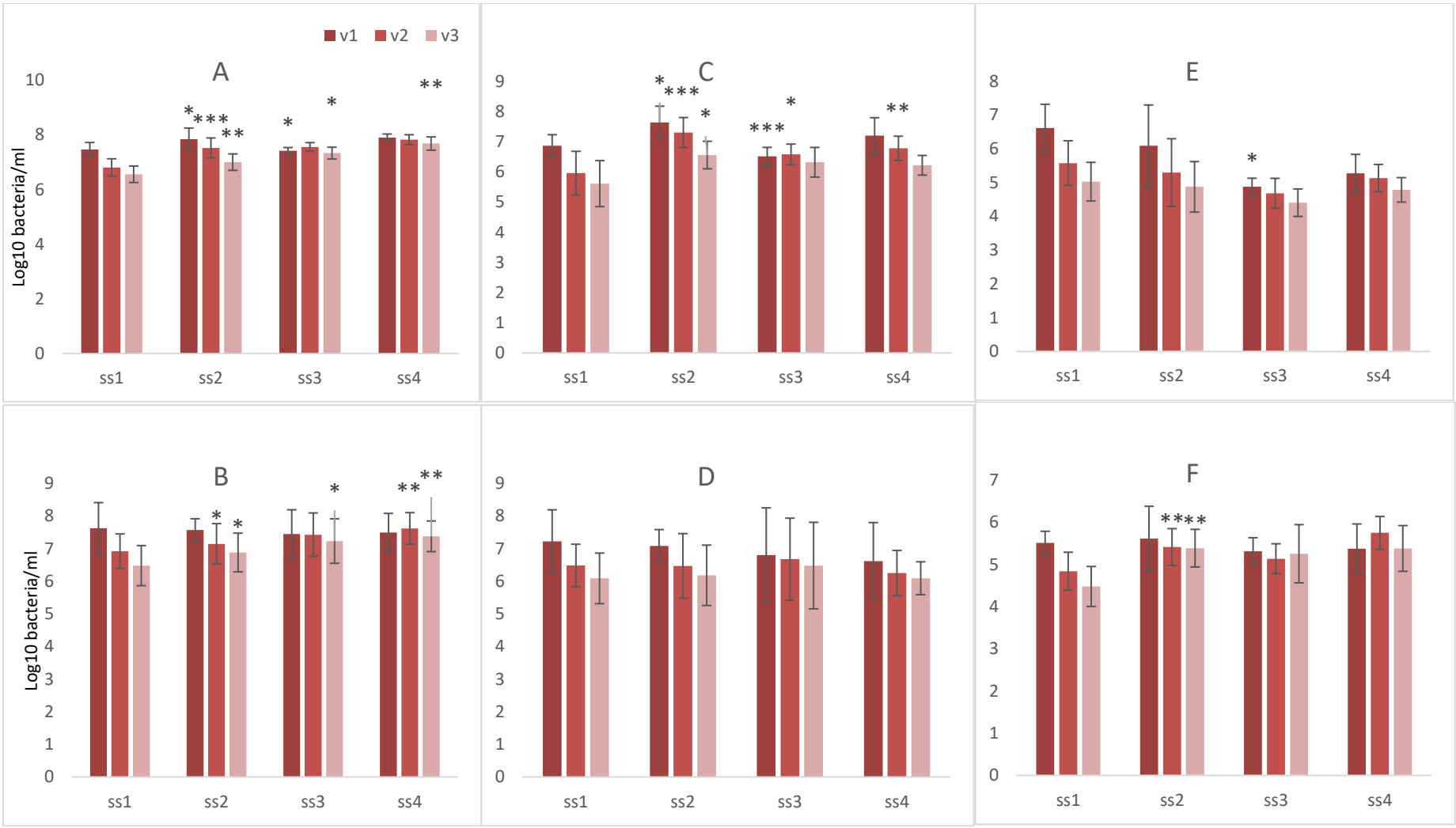

Figure 3.4 Erec482 (A and B) Rrec584 (C and D) and Fprau655 (E and F) changes with different steady states as log10 CFU/mL in three different vessels as analysed by FISH. Values are mean values over consecutive three days from 3 omnivore microbiota (A C and E) and 3 vegetarian microbiota (B D and F) ± standard deviation. * Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.05$). ** Mean values were highly significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.01$). *** Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.001$).

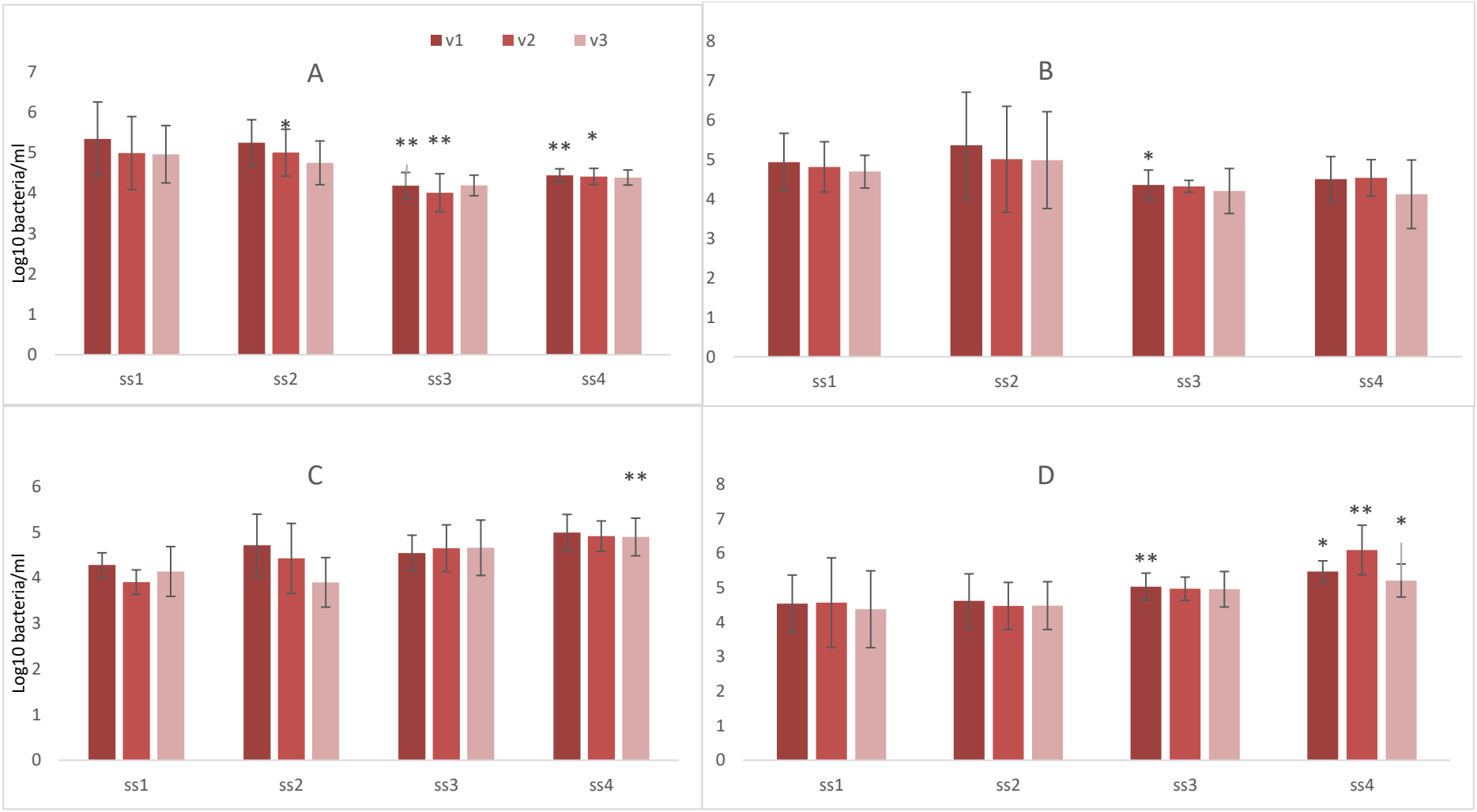

Figure 3.5 DSV687 (A and B) and Chis150 (C and D) changes with different steady states as log10 CFU/mL in three different vessels as analysed by FISH. Values are mean values over consecutive three days from 3 omnivore microbiota (A and C) and 3 vegetarian microbiota (B and D) ± standard deviation. * Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.05$). ** Mean values were highly significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.01$). *** Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.001$).

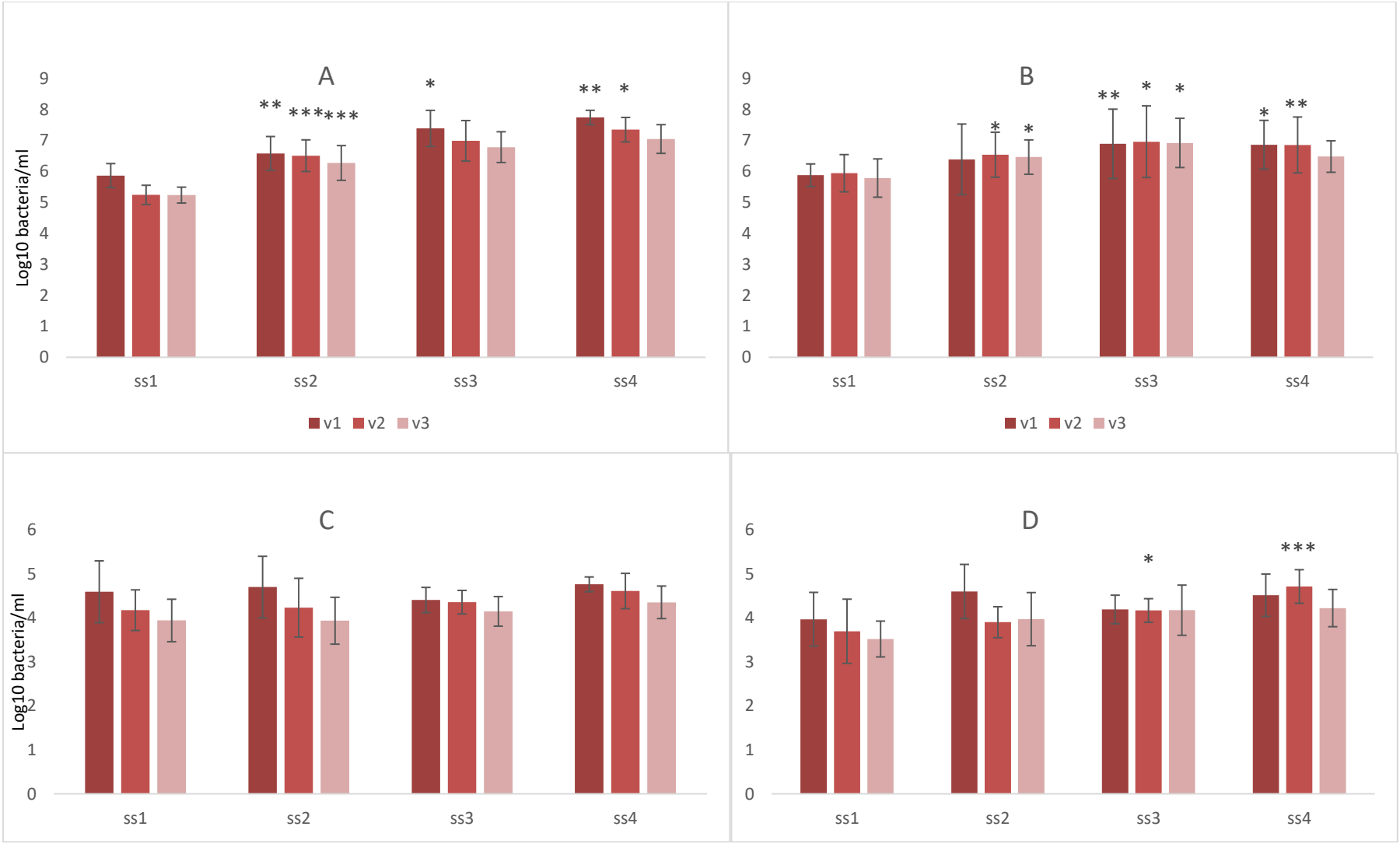

Figure 3.6 Ato291 (A and B) and EC1531 (C and D) changes with different steady states as log10 CFU/mL in three different vessels as analysed by FISH. Values are mean values over consecutive three days from 3 omnivore microbiota (A and C) and 3 vegetarian microbiota (B and D) ± standard deviation. * Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.05$). ** Mean values were highly significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.01$). *** Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.001$).

3.4.2 Organic Acids

Acetate concentrations decreased with protein treatment and increased significantly in all three regions after prebiotic steady state, which can be correlated with the higher total bacteria numbers in both prebiotic treatments (Figure 3.7). Specifically, a higher number of bifidobacteria would be expected to promote acetate production.

Concentrations of propionic acid were reduced by protein supplementation and further reduced by prebiotic supplementation (Figure 3.7). This can be explained by a decreased population of *Bacteroides* in the gut models.

Butyric acid was significantly increased in the later steady states (SS3 and SS4) when grown on Synergy1, which could be explained by increases in butyrate producing *Clostridium coccoides-Eubacterium rectale* group (Figure 3.7).

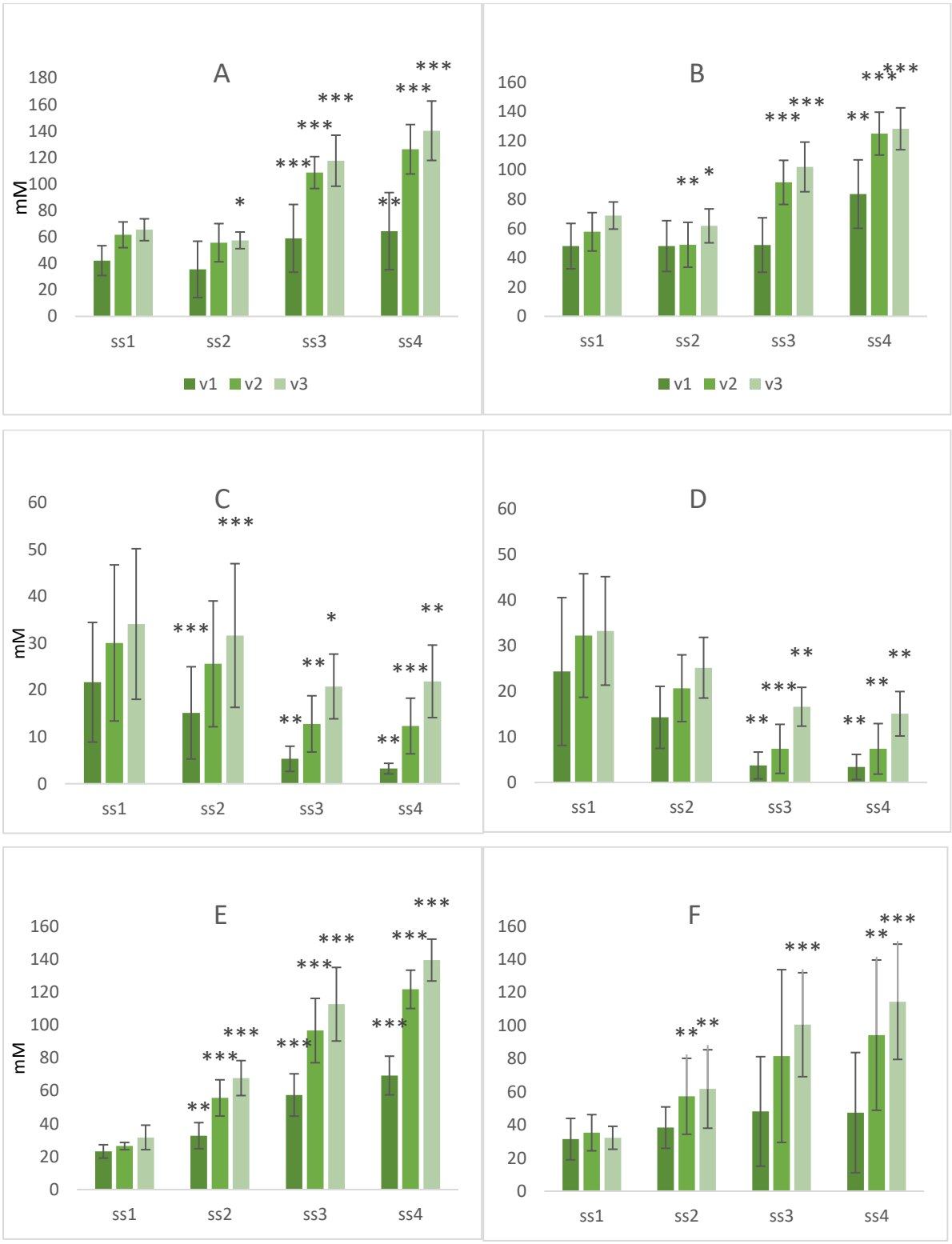

Figure 3.7 Acetate (A and B) propionate (C and D) and butyrate (E and F) (mM) with different steady states in three different vessels as analysed by GC. Values are mean values over consecutive three days from 3 omnivore microbiota (A C and E) and 3 vegetarian microbiota (B D and F) ± standard deviation. * Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.05$). ** Mean values were highly significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.01$). *** Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.001$).

Bacterial metabolism of valine and leucine produces iso-butyrate and iso-valerate respectively and these two metabolites indicate gut bacterial proteolysis (Macfarlane *et al.*, 1992a). Both isovaleric acid and isobutyric acid concentrations increased after extra protein was added to all three vessels (Figure 3.8). This increased production on the protein was inhibited by prebiotic supplementation, especially in vegetarian-inoculated models in the proximal colon simulation ($p<0.01$), where the BCFA concentration was lower than the baseline steady state 1.

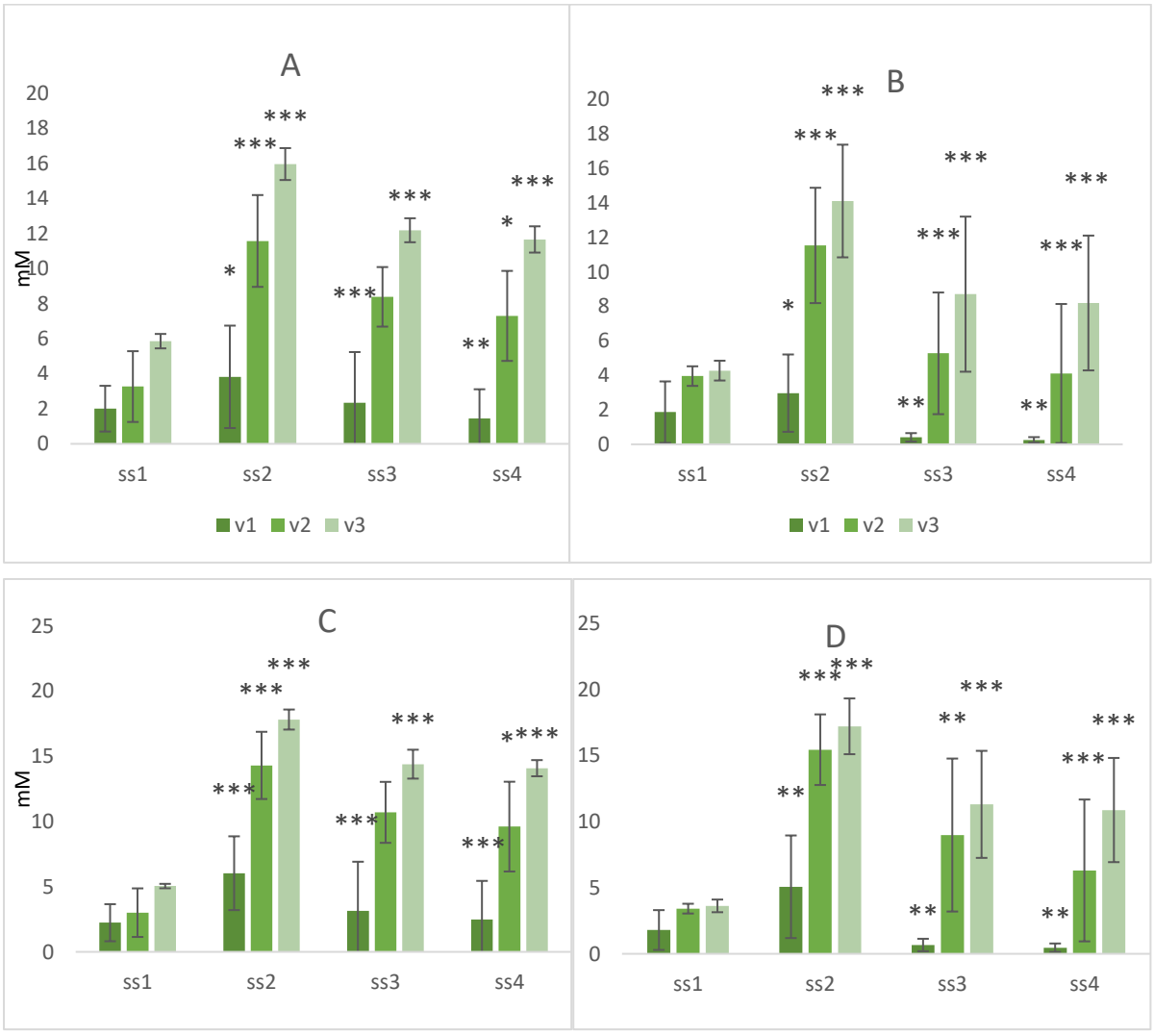

Figure 3.8 Changes in Isobutyrate (A and B) and Isovalerate (C and D) (mM) with different steady states in three different vessels as analysed by GC. Values are mean values over consecutive three days from 3 omnivore microbiota (A and C) and 3 vegetarian microbiota (B and D) ± standard deviation. * Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.05$). ** Mean values were highly significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.01$). *** Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.001$).

3.4.3 Volatile Organic Compounds

Three potentially detrimental volatile organic compounds (indole, p-cresol and skatole) were quantified (Figure 3.9). Concentrations of skatole were under the detection limit. Production of these compounds displayed high individual variation, as evidenced by high standard deviations these data. Indole was produced in low amounts and not decreased after Synergy1 addition. *p*-cresol concentrations were significantly higher in the transverse and distal vessels after steady state 2, however the inhibitory effect of prebiotics was only observed in vegetarian-inoculated models in distal colon simulations ($p<0.05$).

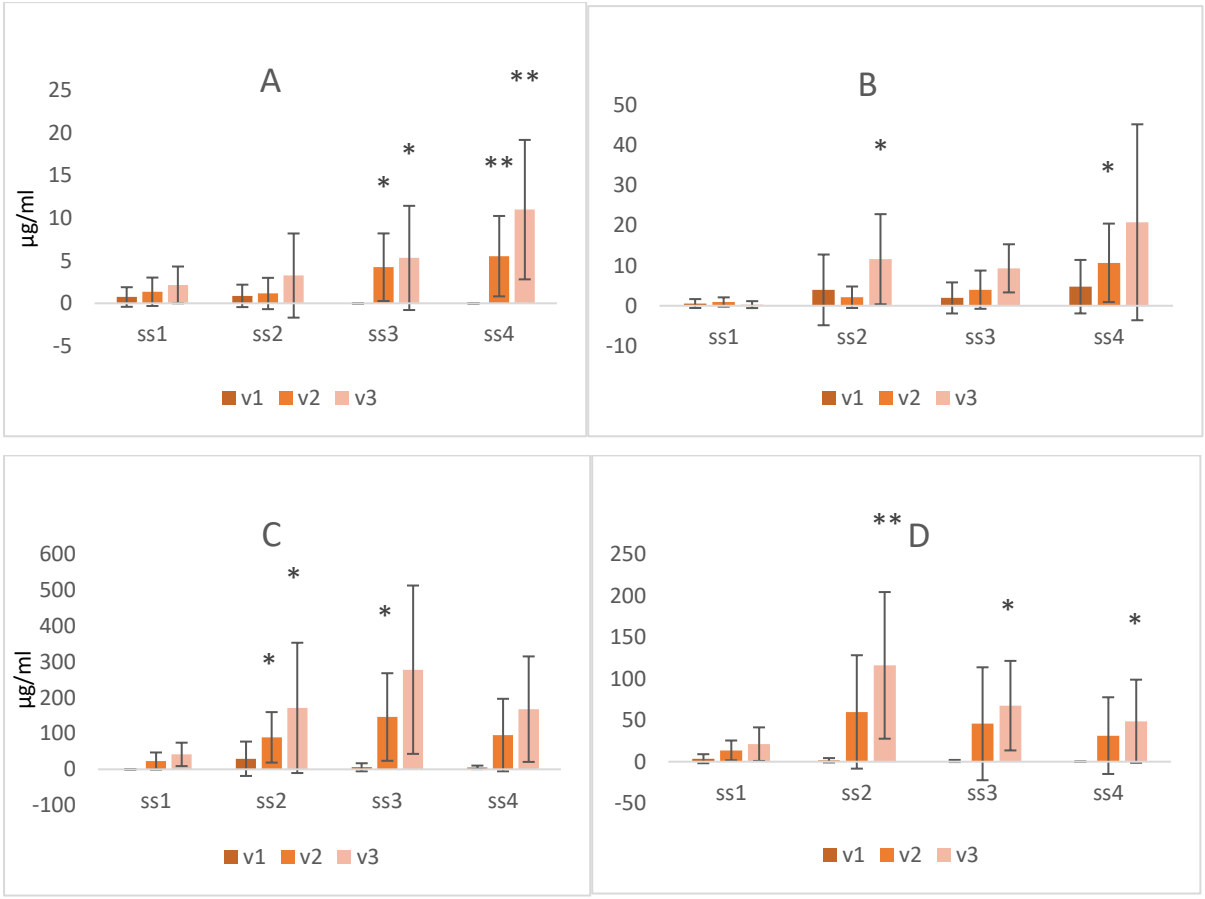

Figure 3.9 Changes in indole (A and B) and p-cresol (C and D) (µg/ml) with different steady states in three different vessels as analysed by GC. Values are mean values over consecutive three days from 3 omnivore microbiota (A and C) and 3 vegetarian microbiota (B and D) ± standard deviation. * Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.05$). ** Mean values were highly significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.01$).

3.4.4 Ammonia

Ammonia is considered a major metabolite of the fermentation of protein by faecal bacteria as a result of deamination of amino acids. Addition of protein to the colonic model significantly increased the production of ammonia in all three regions, especially in the

transverse and distal vessels (Figure 3.10). The higher prebiotic supplementation dose suppressed ammonia production to a greater degree, with significant differences in the transverse regions of all six models, and distal colon in omnivore-inoculated models.

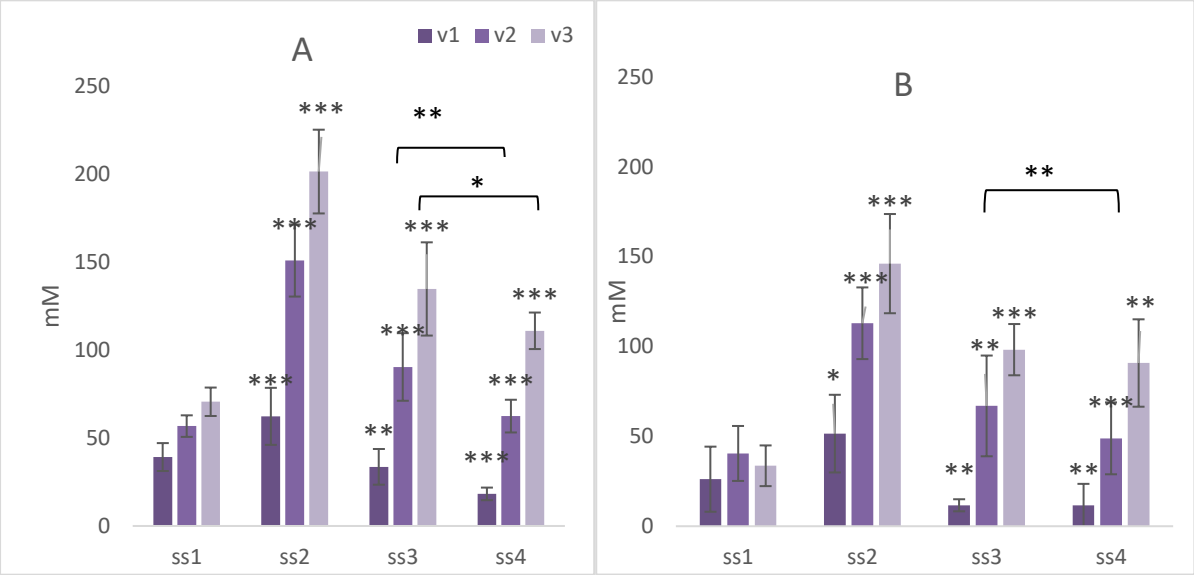

Figure 3.10 Changes in ammonia concentration (mM) with different steady states in three different vessels. Values are mean values over consecutive three days from 3 omnivore microbiota (A) and 3 vegetarian microbiota (B) ± standard deviation. * Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.05$). ** Mean values were highly significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.01$). *** Mean values were significantly different from SS1 or SS2 (for SS3 and SS4) ($p<0.001$).

3.5 Discussion

Three subjects following omnivore diets and three following vegetarian diets were recruited to donate their faecal samples for inoculation of the colonic models. Among these six donors, some different patterns of microbiota were observed. At the genus level, faecal bacterial composition differences between omnivores and vegetarians was not clearly seen, however metabolic activities of their microbiota may differ. After feeding standardised medium until the fermentation equilibrated, total bacteria counts were lower in vegetarian models compared to omnivore models, and this difference persisted in all steady states. A possible explanation for *in vitro* culture differences is that this three-stage continuous culture system has been previously validated with six sudden death subjects which most probably were not vegetarians (Macfarlane *et al.*, 1998). Therefore, carbohydrate and protein content in vegetarians' digesta can be different from the validated medium used by Macfarlane et al., (1998) resulting in differing bacteria growth in the gut model. In the present study, some bacterial groups from omnivore- and vegetarian-inoculated models displayed different responses on protein and

prebiotic addition indicating that, at genus level at least, bacteria have different metabolic activities. With various food sources entering the digestive tract, bacteria from different individuals adapt and may possess different metabolic activities (Giraud *et al.*, 2001).

According to Gibson (1988), SRB population are not always present in human populations because some individuals possess methanogens to dispose of hydrogen in the gut instead of SRB which may explain the high variation of SRB count in this study. Hydrogen sulphide possibly contributes to the pathogenesis of ulcerative colitis, potentially offering a role for prebiotics in management of this disease (Roediger *et al.*, 1997). In this study, SRB counts were slightly higher in the proximal region, which is the opposite of that described in another three stage continuous fermentation study looking at mucin metabolism, sulphate reduction and methanogenesis (Gibson *et al.*, 1988). By analysing colonic contents from three sudden death subjects, it was found in subjects without methanogenic activity that sulphate reduction rates decreased gradually from the proximal to the distal colon (Macfarlane *et al.*, 1992b).

VOCs had similar pattern as SRB: concentrations were variable in different models, therefore changes in VOCs production were not significant. When comparing changes at individual response levels, prebiotic based inhibition was seen in two out of three omnivore models. Indole production was not as high as *p*-cresol, and addition of Synergy1 did not affect production. Indole is a quorum sensing molecule for many biological organisms including bacteria, and recent studies investigated the positive role of this signalling molecule on host epithelial cell barrier function and hormone secretion modulation (Bansal *et al.*, 2010, Shimada *et al.*, 2013, Chimerel *et al.*, 2014).

The current study confirmed that the main proteolysis regions in the colon are the transverse and distal regions (Macfarlane *et al.*, 1988, Macfarlane *et al.*, 1992b). Both BCFA and ammonia were detected at the highest concentration in the distal colon simulation. This can result from accumulation of metabolites due to a lack of absorption in this *in vitro* continuous culture system. When comparing concentration differences, the transverse colon had the highest production of BCFA and ammonia. The simulated transverse vessel has a pH of 6.2 which is close to the optimum pH of proteolytic enzymes (Macfarlane *et al.*, 1992b).

One study on rats revealed that protein dose was correlated with negative consequences of colonic DNA damage, but DNA damage was not seen with whey protein (Toden *et al.*, 2007a).

A human intervention study with 3g or 10g of prebiotic galactooligosaccharides (GOS) fed to 5 healthy subjects with a sequential design: proteolysis inhibitory effects of GOS were seen, however, there was no difference between 3g and 10g of GOS per day (Tanaka *et al.*, 1983). This may due to huge variation among the volunteers, small sample size (n=5) and short time of intervention (1 week). A better proteolysis inhibitory effect of higher dose ITF was seen in this *in vitro* study, which may result from elimination of variation by standardised medium flow.

Vegetarian models had lower production of ammonia and *p*-cresol than omnivore models. Potentially, vegetarian diet has less protein content and vegetarians' gut bacteria had adapted to relatively low protein diet. Therefore it is likely that there was less potential for proteolytic metabolism within the microbiota. In terms of gut health, this may indicate vegetarian diet is beneficial.

In conclusion, supplementation of prebiotic Synergy1 to three stage, continuous, pH controlled, colonic fermentation models shifted the microbiota to a more favourable profile by stimulating bifidobacteria and lactobacilli while repressing bacteroides, and *Desulfovibrio* spp. The prebiotic addition also significantly decreased the concentration of protein metabolites (ammonia and BCFA), however, inhibitive effect of Synergy1 on VOCs production was only seen with vegetarians.

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Chapter 4 The effect of prebiotic oligofructose enriched inulin supplementation on microbiota, protein metabolism and gastrointestinal (GI) symptoms in people consuming high protein diets

Abstract

Dietary protein levels are increasing worldwide and high protein consumption can be detrimental due to the generation of various toxic metabolites from gut bacterial fermentation. On the contrary, consumption of prebiotic carbohydrates allows specific microbiota changes, which may confer benefits upon host wellbeing and health. A double-blind, crossover, placebo controlled, randomised study in healthy individuals aged 18-60 years old was performed to evaluate the effects of prebiotic use (oligofructose enriched inulin) on gut bacterial proteolysis.

Volunteers were recruited from the Reading local community and 43 people completed the trial. Fasting blood, 24 hour urine and fresh faecal samples were collected at the University of Reading. Gastrointestinal symptoms and defaecation records were taken throughout the trial.

Bacteria were enumerated by fluorescence *in situ* hybridisation-flow cytometry. A significant increase in bifidobacteria was observed with the addition of prebiotic treatment ($p<0.0001$). Urine, blood plasma and faecal water metabolite changes were monitored by ^{1}H-NMR. There were lower concentrations of aromatic metabolites in urine and lower concentrations of lipids in blood plasma with prebiotic treatment, however, differences were not significant between the two interventions. Stool frequency was significantly ($p<0.01$) higher with prebiotic supplementation comparing to the placebo group and stool consistency had a trend towards softness as based on the Bristol scale ($p=0.06$). Mild symptoms of bloating and flatulence were reported with the prebiotic, however these were tolerable. Total bacteria and bifidobacteria changes during interventions were significantly correlated with stool frequency ($p\leq0.05$).

4.1 Introduction

Western European populations typically ingest 105g/d protein, according to the Food and Agriculture Organization (FAO, 2017). A proportion of dietary protein enters the large intestine which, together with endogenous sources, may be metabolised by colonic bacteria. Deamination and decarboxylation of amino acids generates ammonia and amines, which are toxic by either affecting barrier function or enhancing *N*-nitrosamine formation (Hughes *et al.*, 2008, Tricker, 1997). Utilisation of aromatic amino acids by gut bacteria can form phenol,

indole and *p*-cresol. In healthy adults, these metabolites are readily absorbed by the colonocyte and conjugated mainly by sulphation in either the gastrointestinal tract or the liver (Teubner *et al.*, 2007, Riches *et al.*, 2009). Sulphated phenols and indoles are then excreted in urine, therefore, urinary indoxyl sulphate and *p*-cresol sulphate levels reflect these aromatic metabolites production in the colon (Verbeke *et al.*, 2015). Phenol and *p*-cresol can damage epithelial cell barrier function *in vitro* at physiologically relevant concentrations (Hughes *et al.*, 2008, Cerini *et al.*, 2004). Indoxyl sulphate and *p*-cresol sulphate can inhibit human cell proliferation and wound repair, and induce oxidative stress *in vitro* (Dou *et al.*, 2004, Dou *et al.*, 2007). Their effect on the healthy population is yet to be confirmed, but this may explain the correlation between urinary indoxyl sulphate and *p*-cresol sulphate levels and renal disease progression (Vanholder *et al.*, 2014).

The International Agency for Research on Cancer (IARC), an agency under the World Health Organization (WHO), published a press release in October 2015: where it classified red meat as "probably carcinogenic to humans", and processed meat as "carcinogenic to humans", with concerns over colorectal cancer (IARC, 2015). It is not clear if the risk was connected with protein consumption or other dietary components in the meat, however Kato *et al.* (1997) found a correlation between total protein intake and colo-rectal cancer (CRC) risk in a New York female population, whereas a European cohort study revealed that heterocyclic amines were related to CRC risk (Rohrmann *et al.*, 2009). Protein consumption may be associated with other colonic diseases: e.g. animal protein has been associated with increased risk of Inflammatory Bowel Disease (IBD) in Japanese and French studies (Shoda *et al.*, 1996, Jantchou *et al.*, 2010).

A prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson *et al.*, 2017). Inulin type fructans (ITF) are naturally present in many plant based foods, such as onion, Jerusalem artichoke, chicory, banana, wheat, and asparagus. ITF has been tested in humans for its effect on gut microbiota modulation with favourable bacteria changes, such as increased growth of bifidobacteria, being reported (Gibson *et al.*, 1995, Vandeputte *et al.*, 2017). ITF was found to improve mineral absorption in human subjects, particularly calcium, (Coudray *et al.*, 1997, van den Heuvel *et al.*, 1999a, van den Heuvel *et al.*, 2009). Satiety can be positively affected by ITF supplementation in both healthy and obese volunteers, possibly by modulating the secretion of certain gut hormones, for instance, increasing levels of glucagon-like peptide 1(GLP-1) and peptide YY (PYY), and decreasing levels of ghrelin (Piche *et al.*, 2003, Archer *et al.*, 2004, Cani *et al.*, 2006, Antal *et al.*, 2008, Parnell and Reimer, 2009). ITF can promote short chain fatty acid (SCFA) production, and SCFA receptors GPR41 and GPR43, also known as free fatty acid receptors (FFA3 and

FFA2), affect human energy intake and adipogenesis (Xiong *et al.*, 2004, Karaki *et al.*, 2008). According to a recent national dietary survey, British adults consume 14g dietary fibre daily, which is below the UK Dietary Reference Value (DRV) of 30g per day (Food Standards Agency, 2016, The Scientific Advisory Committee on Nutrition, 2015). EFSA recommended consumption of 25g of dietary fibre to maintain regular defaecation (EFSA Panel on Dietetic Products and Allergies, 2010a). Native chicory inulin has been recognised as having health benefits through "maintenance of normal defecation by increasing stool frequency" by EFSA, and SACN in the UK claimed that FOS and inulin have bulking capacity of "1 to 1.5g increase in faecal wet weight per 1g intake" (EFSA Panel on Dietetic Products and Allergies, 2015, The Scientific Advisory Committee on Nutrition, 2015). ITF were seen to be positive regarding increasing faecal weight, colon motility and decreasing colonic transit time (Roman *et al.*, 2008, Waitzberg *et al.*, 2013, Vandeputte *et al.*, 2017, Micka *et al.*, 2017). Short chain fatty acids (SCFA) produced by prebiotic fermentation affect colon motility: propionate increased muscle contraction frequency in rats, possibly linked to FFA2 and FFA3 (Tazoe *et al.*, 2008).

The aim of the present study was to investigate the effect of inulin-type fructans (ITF) on the negative consequences of colonic fermentation in individuals consuming high protein diets. The hypothesis to be tested is that their action promotes bacterial carbohydrate metabolism at the expense of bacterial protein metabolism. Three biofluids (urine, blood, and faecal water) were monitored to evaluate the effects on bacterial composition and mechanism. In addition, bowel function was assessed.

4.2 Subjects and methods

4.2.1 Subjects

Healthy adults, both men and women, were recruited from the Reading area. Inclusion criteria were subjects aged 18-60, no evidence of lactose intolerance or gluten allergy, without history or evidence of intestinal disease within the previous 5 years and good general health. Exclusion criteria were antibiotic treatment in the 6 months preceding the study, anaemia, chronic or acute diseases, smoking and a history of alcohol or drug misuse. Volunteers were also excluded if pregnant, lactating or planning pregnancy. Eligible volunteers were asked to fill food frequency questionnaire to calculate their protein intake; subjects with lower than 75g /d protein intake were excluded. Any intake of drugs active on gastrointestinal motility, probiotic/prebiotic supplements or any class of laxative was not permitted. Volunteers were instructed not to alter their usual diet or fluid intake during the trial. The study was a double-blinded, randomised, placebo controlled, crossover trial registered at Clinicaltrials.gov as NCT02827760. The study

was given a favourable ethical opinion by the University of Reading's Research Ethics Committee (16/17). All participants gave written informed consent prior to study entry.

The primary outcome measure was bifidobacterial population as $\log_{10}$ CFU/g wet faecal sample by fluorescence *in situ* hybridisation. It was calculated that a total of 43 patients were required to enrol this placebo controlled crossover study. This allows an 80% probability that the study could detect 0.5 $\log_{10}$ CFU/g wet faecal sample difference in colonic bifidobacterial population at a two-sided 0.05 significance level based on the assumption of a 0.8 $\log_{10}$ CFU/g wet faecal sample bificobacteria within subject standard deviation (Kruse *et al.*, 1999). Thus, fifty volunteers were recruited to allow for drop out.

43 volunteers completed the trial (23 females and 20 males, BMI 24.24 ± 3.58 kg/m^2), aged between 18 to 60, and the average age was 33.82±10.91 years old.

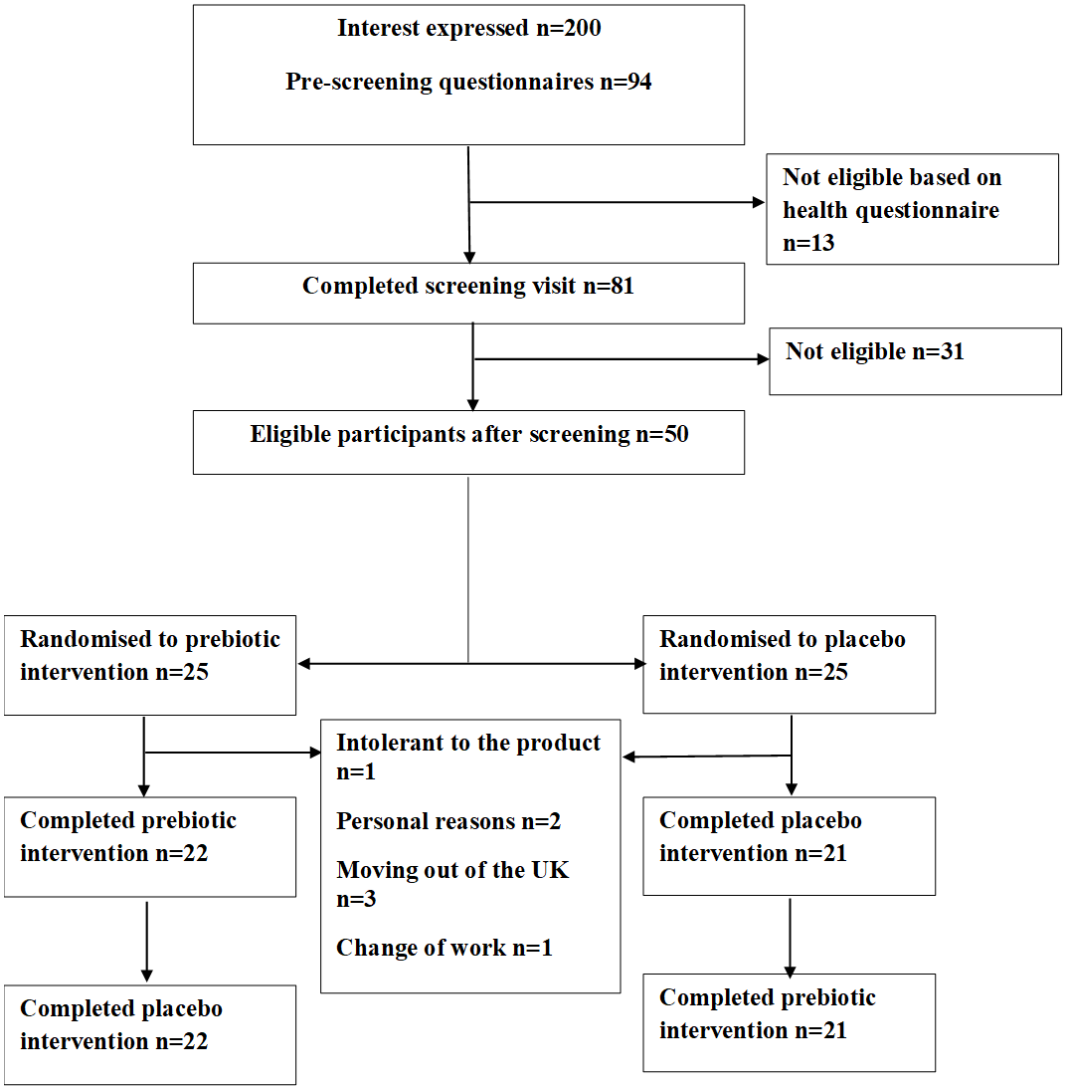

Figure 4.1 Participant flow diagram of the study

4.2.2 Study design

Enrolled volunteers were asked to undergo a two week run-in period before the study commenced. During this period, they were required to start restricting prebiotics probiotics or synbiotics (in food products or as supplements) if there were any in their diet (see Appendix 4.7). Randomisation was stratified by sex, and allocation occurred to one of the two treatment groups using a 1:1 ratio. Fifty volunteers were divided into group A (female; n=26) and B (male; n=24) and each group randomly distributed between groups I and II (A-I: n=13, A-II: n= 13 and B-I: n=12, B-II: n=12). Three female and four male volunteers dropped out before the end of the study. In total, 43 volunteers completed the study (A-I: n=11, A-II: n= 12 and B-I: n=10, B-II: n=10. Subjects started with the first intervention with either prebiotic (n=22) or placebo (n=21), followed by a further 3 week washout period, followed by crossover to the alternative intervention of placebo (n=22) or prebiotic (n=21), and then another 3 week washout period.

The IFT food supplement (supplied by the Beneo company) is a chicory inulin powder produced in Belgium. Prebiotic treatment (inulin-type fructan) was a mixture of oligofructose and inulin: 50%±10% DP (degree of polymerisation) 3-9 and 50%±10% DP≥10 (Orafti®Synergy 1, BENEO-Orafti, Tienen, Belgium). The placebo control food supplement was maltodextrin (Maltodextrin DE 19, Agrana Starch, Austria). The products were provided as a powder (served in sachets) and identical in texture, appearance and taste. The Beneo company provided barcoded sachets, blinded to investigators and volunteers. After all volunteers had completed their study visits and all samples had been analysed by researchers, Beneo company provided information about barcoded sachets. Participants were asked to take 2 sachets of 7.5g supplement daily, and were encouraged to take the sachet at the same time each day. Compliance to food supplement (prebiotic or placebo) was assessed by recording intake in GI diaries and participants were also asked to return any unused sachets to the researchers at the end of the each 6-week intervention period. Participants considered compliant and therefore included in the study, were required to take the food supplement or placebo at least 6 out of every 7 days of the trial for the 6 week period. 84 sachets were given to each volunteer, and volunteers returning less than 14 sachets at the end of the intervention were considered as compliant.

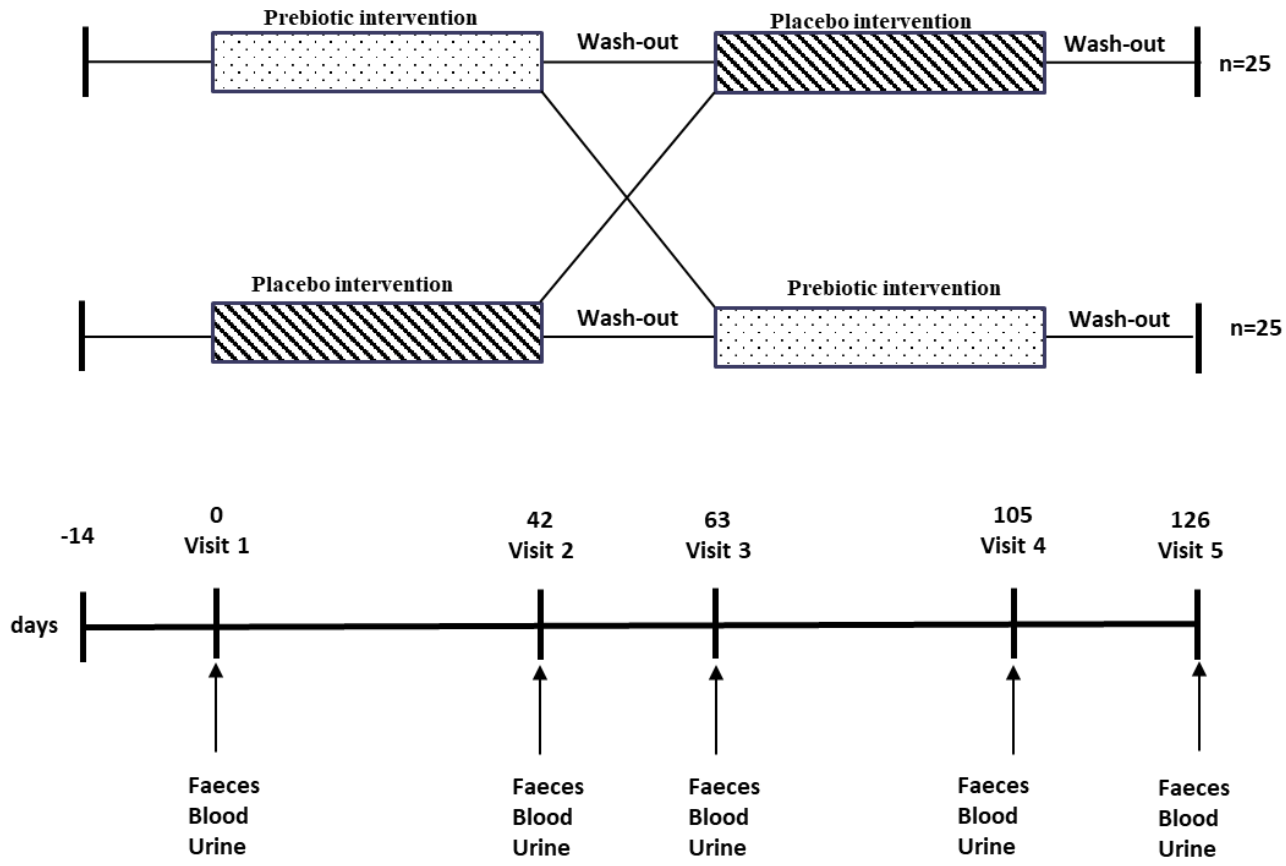

Figure 4.2 Study design of a randomised, controlled, crossover study in which 50 healthy volunteers received 15g ITF or maltodextrin daily for a period of 6 weeks each, following a 3-week washout period after each intervention. Fasting blood, 24 hour urine, and fresh stool sample were collected at each visit.

Volunteers were asked to keep diaries throughout the study to record stool frequency, consistency according with the Bristol chart (see Appendix 4.7) (constipation, hard, formed, or soft stool or diarrhoea), abdominal pain (none, mild, moderate or severe), intestinal bloating (none, mild, moderate or severe) and flatulence (none, mild, moderate or severe) daily. Any medication or adverse events were also recorded.

4.2.3 Sample collection and preparation

On the visit day, fasting blood samples were taken through venepuncture by trained phlebotomists. Blood samples were extracted into sodium heparin tubes (kept on ice). Samples were then centrifuged at 1700 × g for 10 min at 4°C to obtain plasma. 24 hour urine samples were collected 24 hours before the visit day in containers with frozen cool packs. Urine samples were then transferred into 15ml tubes on ice and centrifuged at 1700 × g for 10 min at 4°C to remove any particulate material. Blood plasma and urine samples were then stored at −80 °C for subsequent metabolic profiling.

Volunteers were instructed on how to collect fresh faecal samples in sterile plastic containers, kept in 2.5L Oxoid™ AnaeroJar™ (Oxoid, Hampshire, United Kingdom) with Oxoid™ AnaeroGen™ 2.5L Sachet (O_2 <0.1%; CO_2: 7-15%). These were brought to the university within 1 hour of voiding. Samples were diluted 1:10 (w:w) in anaerobic phosphate-buffered saline (PBS, 0.1 M; pH 7.4), then homogenised using a stomacher (460 paddle beats/min) for 2 min. Samples were vortexed with 3 mm diameter glass beads for 30 s before being centrifuged at 400 x g for 2 min at room temperature. Supernatant (1ml) was centrifuged at 11337 × g for 10 min and the aqueous layer stored at −80 °C for metabolic profiling. Another supernatant sample (375 µl) was fixed in 4% (w:v) paraformaldehyde (1125 µl) for 4 h at 4 °C. To wash the cells of paraformaldehyde, samples were centrifuged at 11337 × g in 1 ml PBS for 5 min at room temperature and this centrifugation and washing was repeated two more times. Samples were re-suspended in 150 µl PBS and stored in ethanol (1:1, v:v) at −20 °C for fluorescence *in situ* hybridisation (FISH).

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

FISH by flow cytometry was carried out as described by Grimaldi *et al.* (2017). Probes used in this study are listed in Table 1. The number of cells is presented as per gram of wet fresh faeces.

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

Probe name	Sequence (5' to 3')	Target groups	Reference
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	(Wallner *et al.*, 1993)
Eub338	GCTGCCTCCCGTAGGAGT	Most Bacteria	(Amann *et al.*, 1990)
Eub338II	GCAGCCACCCGTAGGTGT	Planctomycetales	(Daims *et al.*, 1999)
Eub338III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	(Daims *et al.*, 1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	(Langendijk *et al.*, 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*	(Harmsen *et al.*, 1999)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz *et al.*, 1996)
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb)	(Franks *et al.*, 1998)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus	(Walker *et al.*, 2005)

Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(Harmsen *et al.*, 2000a)
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX	(Walker *et al.*, 2005)
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium* prausnitzii and relatives	(Suau *et al.*, 2001)
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	Ramsing *et al.* (1996)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)	Franks *et al.* (1998)
CFB286	TCCTCTCAGAACCCCTAC	*Prevotella* group and *Bacteroides splanchnicus*	(Weller *et al.*, 2000)

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

A phosphate buffer (pH 7·4 sodium phosphate with 0.2M Na_2HPO_4, 0.04M NaH_2PO_4 in deuterium oxide 99·9 %) was prepared, with 1mM 3-(trimethylsilyl)propionic acid-d_4 sodium salt (TSP) and 3mM sodium azide in the solution. Urine and faecal water samples were thawed at 0-4°C, and 400 µl of each sample were mixed with 200 µl buffer. Samples were then centrifuged at 11337 × g for 10 min, and 550 µl aliquots of supernatant were collected to fill 5 mm NMR tubes.

A 0.9% w/v saline solution containing 20% deuterium oxide (v:v) and 3mM sodium azide was prepared for blood plasma samples. Samples (200 µl) were mixed with 400 µl buffer before centrifugation at 11337 × g for 10 min. Aliquots (500 µl) of supernatant were collected to fill 5 mm NMR tubes.

All ^{1}H-NMR spectroscopy data were acquired on a Bruker Avance DRX 500 MHz NMR spectrometer (Bruker Biopsin, Germany) operating at 500.13 MHz. Urine and faecal water spectra were acquired using a standard 1D pulse sequence [recycle delay (RD)-90°-t1-90°-Tm-90°-acquire free induction decay (FID)] with water suppression applied during RD of 2 s, a mixing time Tm of 100ms and a 90° pulse set at 7.70 µs. For each spectrum, a total of 128 scans were carried out with a spectral width of 14.0019 ppm. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening.

Spectra of blood plasma samples were acquired by using Carr-Purcell-Meiboom-Gill (CPMG) spin echo ^{1}H-NMR spectroscopy, which allows reduction of broad high molecular weight protein signals and generates spectra on smaller molecular metabolites. For each spectrum, a total of 128 scans were carried out with a spectral width of 14.0019 ppm. The FIDs were

multiplied by an exponential function corresponding to 0.3 Hz line broadening.

All spectra were manually phased, baseline corrected and calibrated to the chemical shift of TSP or glucose using TopSpin (Bruker Biopsin, Germany). NMR spectroscopy data were digitalised by Matlab R2017b, and normalised by using Matlab R2017b normalisation scripts by total area normalisation. Supervised OPLS-DA (orthogonal projections to latent structures discriminant analysis) modelling of ^{1}H-NMR spectroscopy data was performed by SIMCA 14.1 to compare metabolic changes in different experimental groups.

4.2.6 Correlation of bacterial population and bowel movement

Bacterial $\log_{10}$ populations and stool frequency results from visits 2, 3, 4 and 5 were extracted. Correlations between total bacteria, specific bacterial groups and stool frequency were analysed by using SPSS version 24 (SPSS Inc, Chicago, IL). Bacterial changes before and after each intervention were calculated. A bivariate Pearson correlation coefficient was calculated for all attribute combinations and two-tailed probability was assessed to determine the significance. In correlations summary tables, correlation coefficients significant at the 0.05 level are identified with *, and those significant at the 0.01 level are identified with **.

4.3 Results

4.3.1 Bacterial Enumeration

Changes in populations of total bacteria and 11 specific faecal microbial groups in the human GI tract were monitored in this study by FISH. Each intervention response was compared at its start and end. Placebo supplementation for six weeks did not change total bacterial counts significantly and all the bacteria groups that were analysed in this study were unaffected by six weeks of maltodextrin treatment (Table 4.2). Compared to baseline, after prebiotic intervention, total bacteria numbers were slightly elevated, bifidobacterial counts were significantly higher, while sulphate-reducing bacteria (*Desulfovibrio*) numbers were reduced significantly. No other significant changes were recorded.

	Eub338	Bif164	Lab158	Bac303	Erec482	Rrec584	Ato291	Prop853	Fprau655	DSV687	Chis150	CFB286
Pre-placebo intervention	9.12 ± 0.51	7.69 ± 0.92	6.70 ± 0.77	6.38 ± 0.69	8.33± 0.56	7.59± 0.60	7.17± 0.79	7.30 ± 0.69	7.37 ± 0.86	6.05 ± 1.02	6.39 ± 0.70	7.00 ± 0.58
After-placebo intervention	9.15 ± 0.36	7.84 ± 0.77	6.72 ± 0.65	6.14 ± 0.78	8.32 ± 0.45	7.43 ± 0.56	7.26± 0.68	7.20 ± 0.62	7.31± 0.76	5.96 ± 1.04	6.26± 0.81	6.94± 0.55
Pre-prebiotic intervention	9.08 ± 0.53	7.73 ± 0.88	6.60 ± 0.78	6.24± 0.80	8.30 ± 0.57	7.55 ± 0.78	7.08 ± 0.82	7.30 ± 0.82	7.41 ± 0.66	5.86 ± 0.97	6.18 ± 0.92	6.91 ± 0.56
After-prebiotic intervention	9.27 ± 0.36*	8.25 ± 0.80***	6.69 ± 0.68	6.04 ± 0.94	8.20 ± 0.84	7.55± 0.71	7.30 ± 0.83	7.22 ± 0.91	7.23 ± 0.76	5.42 ± 1.29*	6.01± 1.04	6.99 ± 0.46

Table 4.2 Total bacteria and 11 specific gut bacteria group counts as log10 CFU/g wet faeces in subjects' faeces as determined by FISH, before, and after each intervention. Values are mean values ± standard deviation. * Mean values were significantly different from pre intervention ($p<0.05$). *** Mean values were highly significantly different from pre intervention ($p<0.001$).

4.3.2 Metabolic profiling of biofluids

SCFA are the main metabolites that can be identified in faecal water by ^{1}H-NMR in this study. Aromatic metabolites including indole and *p*-cresol had very weak signals in the spectra, and BCFA signals were absent, see Figure 4.3. Faecal water metabolite differences between the placebo and prebiotic interventions were mainly SCFA, such as acetate, propionate and butyrate (Figure 4.4). Concentrations of SCFA were non-significantly higher in the faecal samples after placebo intervention compared to samples after prebiotic intervention, however, this does not indicate the production of SCFA in the lumen since faecal sample are excreted after nutrients absorption in the large intestine. These 24 hours one pot samples do not indicate daily output.

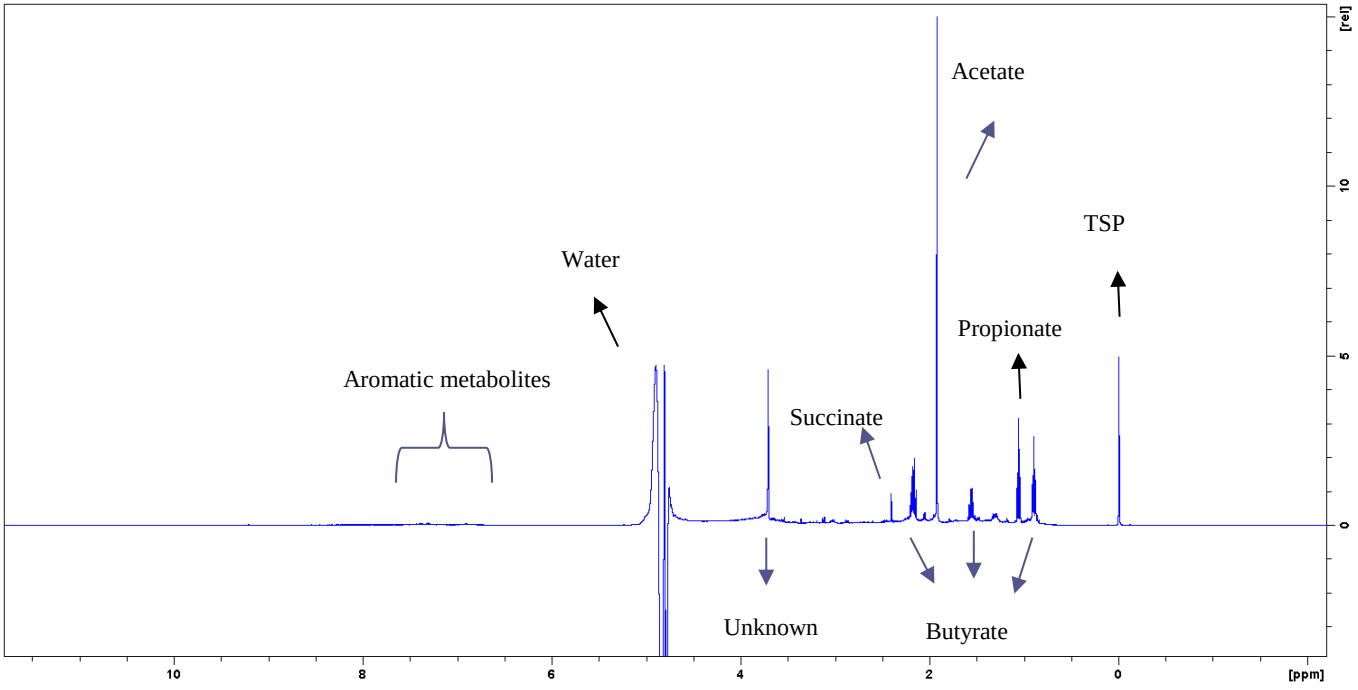

Figure 4.3 ^{1}H-NMR spectrum from a pooled faecal water sample of all visits of 43 subjects.

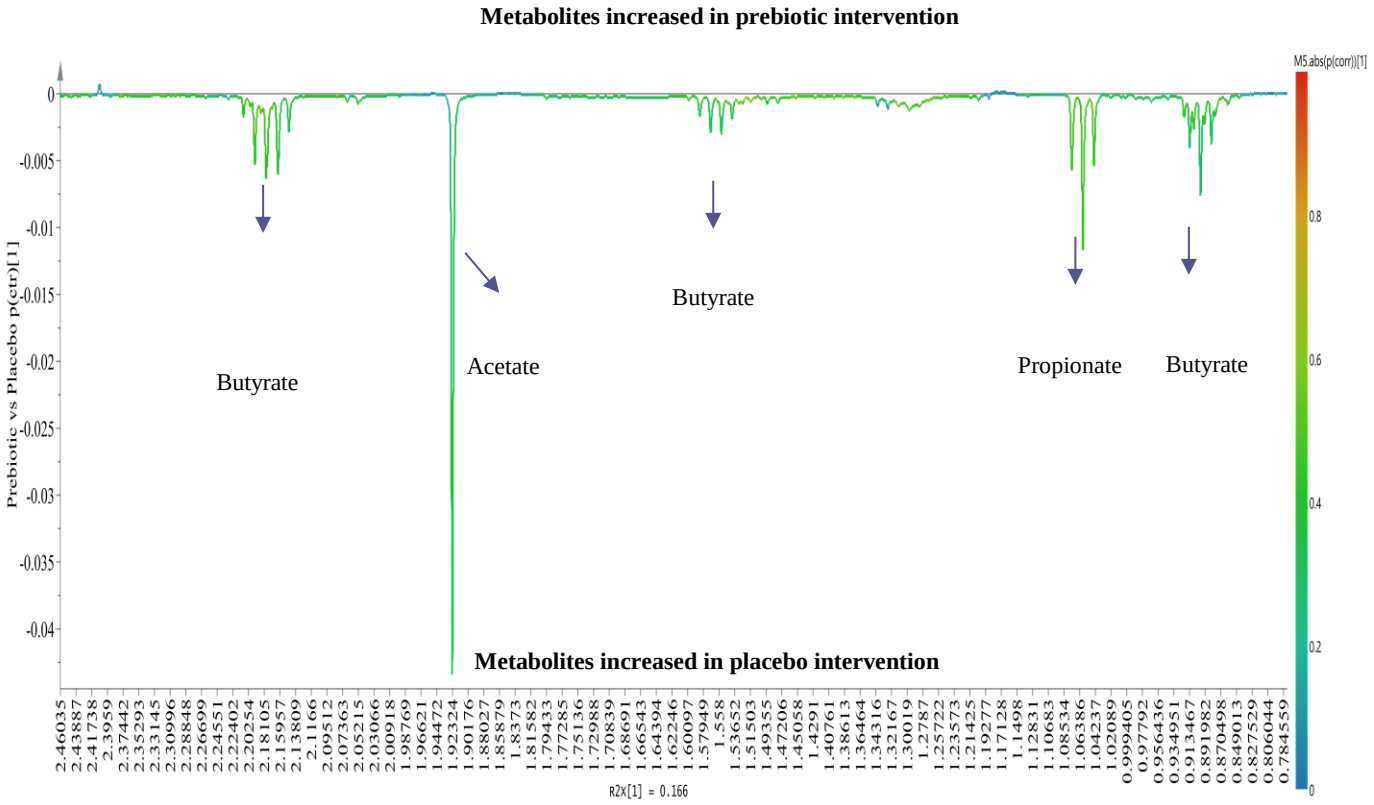

Figure 4.4 OPLS-DA model of ^{1}H-NMR spectra data from all faecal water samples comparing SCFA concentrations between prebiotic intervention and placebo intervention. Peaks pointing up are metabolites that were with high concentration in samples after the prebiotic intervention, and peaks pointing down are metabolites that were with high concentrations in samples after the placebo intervention. X axis is chemical shifts in ppm. Y axis is covariance of two intervention groups. Axis on the right shows the discriminatory strength: red is the strongest and blue is the weakest.

Comparison of spectra from blood plasma samples did not show any significant differences in the general metabolism post prebiotic and post placebo (Q^2= -0.44). The OPLA-DA model (Figure 4.5) showed partial separation between two groups; and it revealed some trending differences of blood lipid profiles. Between the placebo and prebiotic groups: after prebiotic treatment, volunteers had lower concentrations of lipids in their blood, especially, VLDL and LDL. Besides, higher concentration of blood plasma lactate was found in the prebiotic group (Figure 4.5 and 4.6).

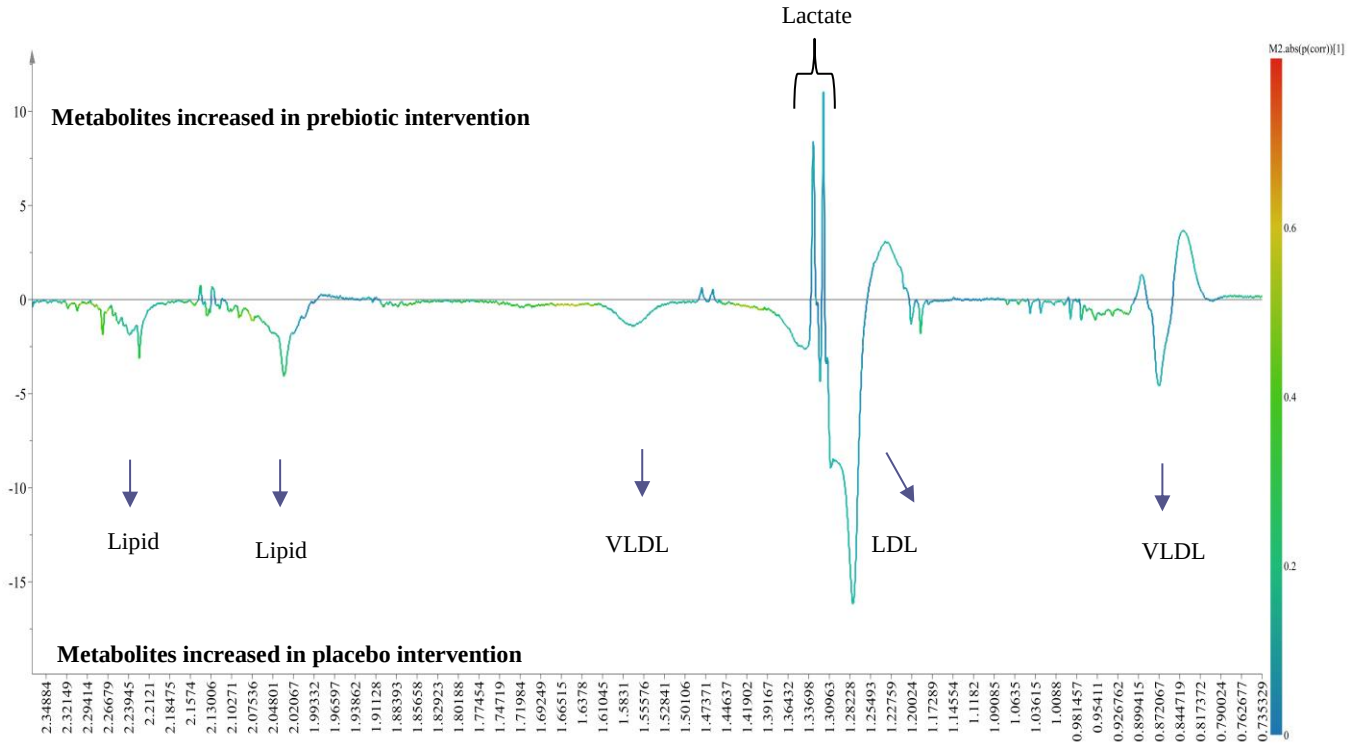

Figure 4.5 OPLS-DA model of ^{1}H-NMR spectroscopy data from blood plasma samples comparing lipids concentrations between prebiotic intervention and placebo intervention. Peaks pointing up are metabolites that were with high concentrations in samples after the prebiotic intervention, and peaks pointing down are metabolites that were with high concentrations in samples after the placebo intervention. X axis is chemical shifts in ppm. Y axis is covariance of two intervention groups. Axis on the right shows the discriminatory strength: red is the strongest and blue is the weakest.

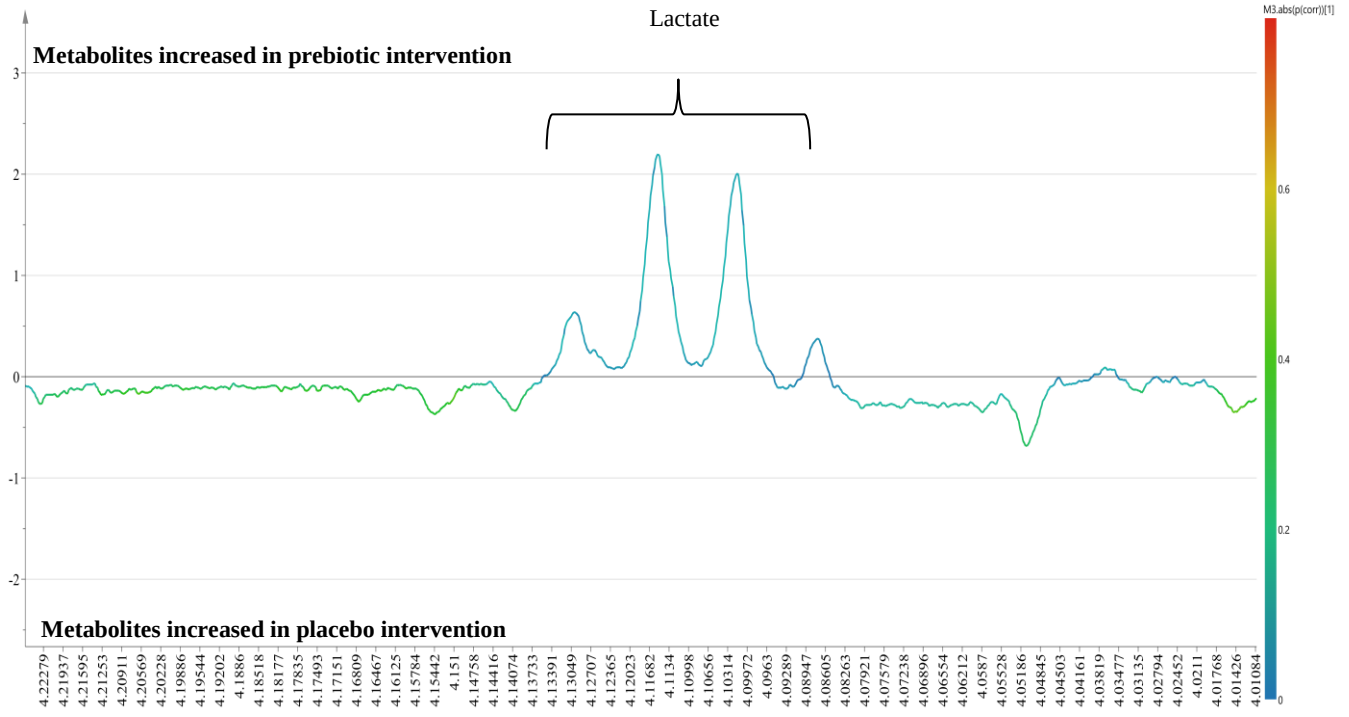

Figure 4.6 OPLS-DA model of ^{1}H-NMR spectra from blood plasma samples comparing lactate concentration between prebiotic intervention and placebo intervention. Peaks pointing up are metabolites that were with high concentrations in samples after the prebiotic intervention, and peaks pointing down are metabolites that were with high concentrations in samples after the placebo intervention. X axis is chemical shifts in ppm. Y axis is covariance of two intervention groups. Axis on the right shows the discriminatory strength: red is the strongest and blue is the weakest.

Spectra from 24 hour urine samples did not identify any significant differences between post prebiotic and post placebo groups (Q^2= -0.28). The OPLA-DA model (Figure 4.7) showed partial separation between two groups; and with OPLA-DA model of spectra some trending differences were seen in urine samples. Colonic bacterial fermentation-driven changes were evident in urine samples post prebiotic treatment. Urinary aromatic metabolites, such as *p*-cresol and its conjugated form, are gut bacteria derived metabolites from aromatic amino acids. Concentrations of these metabolites reflect bacterial aromatic amino acid metabolism in the gut. Aromatic amino acid derived metabolites were lower in the prebiotic group and hippurate was in lower concentration after prebiotic intervention.

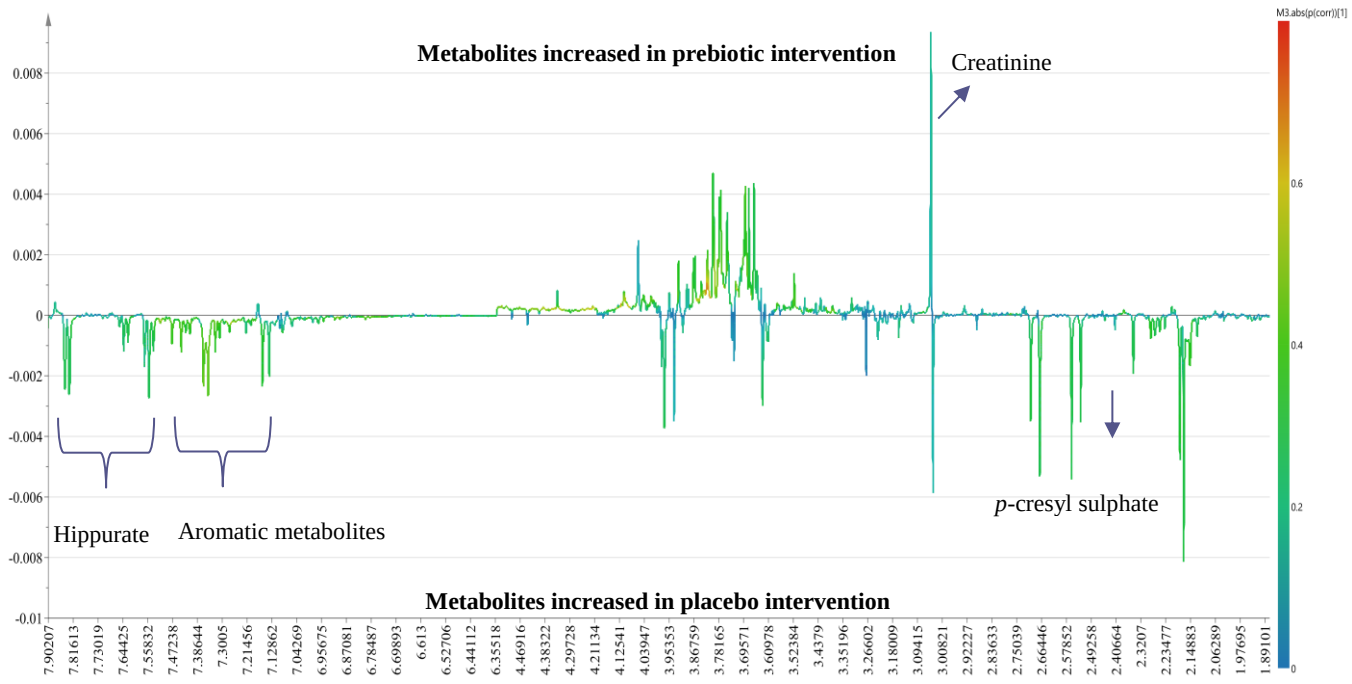

Figure 4.7 OPLS-DA model of ^{1}H-NMR spectra from 24 hour urine samples comparing urinary aromatic metabolites concentrations between prebiotic intervention and placebo intervention. Peaks pointing up are metabolites that were with high concentrations in samples after the prebiotic intervention, and peaks pointing down are metabolites that were with high concentrations in samples after the placebo intervention. X axis is chemical shifts in ppm. Y axis is covariance of two intervention groups. Axis on the right shows the discriminatory strength: red is the strongest and blue is the weakest.

4.3.3 Bowel habit and function

Subjects' faecal voiding frequencies, measured as number per day and stool consistency measured as Bristol score, are listed in Table 4.3. Self-reports were collected during intervention and wash-out periods and data were compared between prebiotic and placebo groups. Compared to the placebo supplementation period, prebiotic treatment significantly increased stool frequency. Consistency of volunteers' faeces did not differ significantly however, but there was an increasing trend of softer stools during the prebiotic intervention.

Treatment	Placebo intervention	Post placebo	Prebiotic intervention	Post prebiotic
Stool frequency	1.26+0.56	1.27+0.53	1.42+0.63**	1.32+0.61
Bristol score	3.67+1.10	3.59+1.28	3.99+0.99 (p=0.06)	3.50+1.128

Table 4.3 Stool frequency per day and stool consistency score based on Bristol scale reported by volunteers during the trial. Values are mean values ± standard deviation. ** Mean values were significantly different from placebo intervention ($p<0.01$).

Gastrointestinal symptoms were recorded daily during the trial to track bowel function changes (Table 4.4). 0, 1, 2, and 3 refer to none, mild, moderate or severe symptoms and each was averaged. During the placebo intervention slight symptoms were seen, but abdominal pain, bloating and flatulence became more noticeable in the prebiotic intervention. However, average severity of these symptoms was below mild, which was tolerable.

	Abdominal Pain	Stomach or intestinal bloating	Flatulence
Placebo intervention	0.07±0.10	0.12±0.20	0.34±0.39
Prebiotic intervention	0.31±0.59**	0.40±0.71*	0.76±0.68***

Table 4.4 Gastrointestinal symptoms reported by volunteers during interventions. Values are mean values ± standard deviation. * Mean values were significantly different from placebo intervention ($p<0.05$). ** Mean values were significantly different from placebo intervention ($p<0.01$). *** Mean values were significantly different from placebo intervention ($p<0.001$).

Table 4.5 summarises significant correlations between bacterial numbers in faeces and stool frequency at visits 2, 3, 4, and 5. Clostridial cluster XIVa and XIVb, and *Roseburia* numbers were positively correlated with stool frequency with high significance ($p< 0.001$). *Faecalibacterium prausnitzii* numbers were also significantly associated with stool frequency albeit at lower p-value ($p=0.0207$). These are the three butyrate producers that were monitored in this study, and this may indicate butyrate's role in bowel movement.

Table 4.6 summarises significant correlation between bacterial changes before and after the intervention in faeces and stool frequency after interventions of all volunteers. Total bacteria and bifidobacteria changes were positively correlated with stool frequency with significance ($p= 0.05$ and 0.031 respectively). Changes of three butyrate producers Clostridial cluster XIVa and XIVb, *Roseburia*, and *Faecalibacterium prausnitzii* had a trending positive correlation ($p=$ 0.059, 0.059, and 0.055 respectively).

Correlations of bacterial numbers at each visit and bowel movement													
		Eub338	Bif164	Lab158	Bac303	Erec482	Rrec584	Ato291	Prop853	Fprau655	DSV687	Chis150	CFB286
Stool frequency	Pearson Correlation	0.07121981	-0.056375893	0.047943077	0.017498493	0.262**	0.278**	-0.11783068	0.067213431	0.197*	0.115853373	0.017744085	-2.56465E-05
	Significance	0.408226089	0.512894383	0.58369004	0.845186925	0.002001688	0.001007836	0.173487015	0.436874867	0.020726344	0.234704593	0.842420974	0.999769848
**. Correlation is significant at the 0.01 level (2-tailed).													
*. Correlation is significant at the 0.05 level (2-tailed).													

Table 4.5 Correlation between bacterial numbers in faecal samples and stool frequency of all volunteers at visit 2, 3, 4, and 5. Pearson correlation coefficient gives strength of the correlation, and significance values were marked with two levels.

Correlations of bacterial numbers at each visit and bowel movement													
		Eub338	Bif164	Lab158	Bac303	Erec482	Rrec584	Ato291	Prop853	Fprau655	DSV687	Chis150	CFB286
Stool frequency	Pearson Correlation	0.234*	0.256*	0.113	-0.084	0.225	0.209	0.225	0.19	0.228	0.025	0.163	0.042699
	Significance	0.05	0.031	0.349	0.486	0.059	0.08	0.059	0.113	0.055	0.839	0.175	0.617709
**. Correlation is significant at the 0.01 level (2-tailed).													
*. Correlation is significant at the 0.05 level (2-tailed).													

Table 4.6 Correlation between bacterial changes in faecal samples during both interventions and stool frequency of all volunteers. Pearson correlation coefficient gives strength of the correlation, and significance values were marked with two levels.

4.4 Discussion

Gut microbiota analyses are achieved by molecular technologies that no longer require culturing viable bacteria. The basis for many of these techniques is the use of the 16S ribosomal RNA gene (16S rRNA) which can be used to specifically identify bacterial groups (Case *et al.*, 2007). FISH involves hybridisation of a specific fluorescent probe which is designed to bind to a precise region of a specific bacterial group to 16S rRNA while maintaining structure of the cell (Amann *et al.*, 1990). The number of bacterial cells can be obtained by using flow cytometry (Amann *et al.*, 1990).

In this trial, it was confirmed that ITF modulates gut microbiota composition, especially enhancing the growth of bifidobacteria. This prebiotic effect has been consistently observed in both *in vitro* and *in vivo* human intervention studies of various target groups (Wang and Gibson, 1993, Williams *et al.*, 1994, Gibson *et al.*, 1995, Bouhnik *et al.*, 1999, Kruse *et al.*, 1999, Guigoz *et al.*, 2002, Vandeputte *et al.*, 2017). Modulation of other bacterial groups are not consistent in reported studies: Kleessen *et al.* (1997) found reduction of clostridia and lactobacilli together with an increase of enterococci after inulin consumption; supplementation of both Jerusalem artichoke inulin and chicory inulin suppressed growth of *Bacteroides*/*Prevotella* and *Clostridium histolyticum* after three weeks intervention. A recent study using 16s rDNA microbiota composition profiling found that 4 weeks of inulin consumption increased the abundance of *Bifidobacterium* and *Anaerostipes* and decreased the abundance of *Bilophila* (Vandeputte *et al.*, 2017). It has been shown in various trials together with this one that ITF has a laxative effect which decreases colonic transit time and increases faecal volume output. The improvement of stool frequency was significantly correlated with increased numbers of bifidobacteria, which suggests that the laxative effect of ITF is related to the microbiota community and metabolic activity.

In previous inulin research, 10 volunteers who consumed 9 g inulin per day for two weeks had lower numbers of *Clostridium* cluster XIVa and XIVb (Erec482 probe by FISH analysis, (Harmsen *et al.*, 2002). In this current study, a slight decrease of *Clostridium* cluster XIVa and XIVb was also observed in the prebiotic treatment, however without statistical significance ($p=0.16$). Within the *Clostridium* cluster, there are proteolytic species such as *C. indolis* and *C. symbiosum*. A prolonged effect of long chain inulin has been observed in an *in vitro* comparison of fermentation of oligofructose, long chain inulin and a mixture of both (Synergy1), (Gomez

et al., 2010). This prolonged effect may result in a saccharolytic fermentation in the distal colon, where most bacterial proteolysis in the gut occurs.

Sulphide has been found to damage DNA and inhibit the ability of epithelial cells to utilise energy derived from butyrate oxidation. Its toxic effects have been thought to cause energy deficiency in colonocytes and mucosal inflammation which may contribute towards pathogenesis of the Inflammatory Bowel Disease (IBD), ulcerative colitis (Attene-Ramos *et al.*, 2006, Roediger *et al.*, 1993, Gibson *et al.*, 1991). *Desulfovibrio* is one of the dominant SRB in the human gut and increasing evidence has suggested that activity of this bacterial group correlates with IBD (Gibson *et al.*, 1991, Figliuolo *et al.*, 2017). This trial showed positive effects in that the prebiotic reduced SRB. This agrees with a randomised, double-blind, placebo-controlled, crossover trial showing the inhibitory effect of inulin on *Desulfovibrio* spp. by 16s rDNA profiling in 29 subjects (Holscher *et al.*, 2015). Prebiotics may therefore be applied usefully to IBD studies. Moreover, *Desulfovibrio* abundance has been found to be increased in the gut microbiota of autistic children, suggesting a further application for prebiotics (Finegold, 2011). Unlike the association of bifidobacterial change and bowel movement, the change of *Desulfovibrio* numbers was not correlated with other bacteria changes or bowel movement (see Appendix 4.4).

Metabolism of ITF in the gut generates SCFA *in vitro* (Wang and Gibson, 1993). Consumption of inulin and xylo-oligosaccharide mixtures showed elevated concentration of total SCFA and propionate compared to a parallel placebo group (Lecerf *et al.*, 2012a). A randomised controlled, cross over study revealed higher concentration of butyrate in faecal water after intervention with a synbiotic composed of ITF and *Bifidobacterum longum* (Macfarlane *et al.*, 2013). However, some human trials have not shown an effect of ITF on faecal water SCFA concentrations, even when significant increases in bifidobacterial growth were recorded, which may be expected to enhance SCFA production (Gibson *et al.*, 1995, Kleessen *et al.*, 1997, Brighenti *et al.*, 1999, Kruse *et al.*, 1999, Kleessen *et al.*, 2007). SCFA in the lumen are rapidly and extensively (>95%) absorbed and subsequently metabolised by the colonocyte and their concentrations in the distal colon can be affected by transit, moisture and digesta mass (Topping and Clifton, 2001). Faecal water SCFA concentrations show SCFA losses after absorption and utilisation by the host but not *in vivo* production and they do not necessarily reflect SCFA absorption by the gut (Verbeke *et al.*, 2015). Therefore, faecal water SCFA levels recorded in this study do not indicate that less SCFA was generated during prebiotic supplementation. In

blood plasma, a higher concentration of lactate was found in the prebiotic group, which may correlate with an increase of bifidobacteria as they produce lactate via the bifidus pathway.

Some human studies have found that ITF could modulate energy regulatory hormones such as elevation of blood GLP-1 and PYY, which both down-regulate energy intake and have the potential to reduce LDL-cholesterol (Piche *et al.*, 2003, Parnell and Reimer, 2009). A proposed mechanism is that ITF promote SCFA production resulting in activation of FFA2 and FFA3 with an effect on energy intake and lipogenesis (Xiong *et al.*, 2004, Karaki *et al.*, 2008). LDL-cholesterol and triacylglycerol lowering effects of ITF are not always consistent. Significant changes have been reported with studies using particular target groups such as diabetic subjects, volunteers with moderately raised total plasma cholesterol and triacylglycerol levels, or obese subjects (Yamashita *et al.*, 1984, Jackson *et al.*, 1999, Antal *et al.*, 2008, Dehghan *et al.*, 2013). A trend towards an improved lipid profile was seen in the current study, but non-significance may be due to the wide range of age and BMI and diverse baseline lipid profiles.

Urinary aromatic metabolites such as *p*-cresol and its conjugated form are gut bacteria derived metabolites from aromatic amino acids. Different levels of 4-cresol sulphate after ITF intervention were recorded in this study. A lack of significant difference between prebiotic intervention and placebo group may be due to baseline differences between volunteers; De Preter *et al.* (2007b) found subjects with high baseline *p*-cresol had a higher inhibitory response resulting from prebiotic intervention. In addition, dietary differences before each visit may contribute to varying aromatic metabolite levels.

Hippurate had a trend to be higher in the placebo group compared to the prebiotic intervention in this study. Hippurate is a colonic bacterial metabolite derived from plant phenolic compounds, aromatic amino acids and benzoic acid (Phipps *et al.*, 1998). There have been two studies demonstrating that hippurate may be positively associated with diabetes (Messana *et al.*, 1998, Kumari and Sharma, 2016). The causal relation of hippurate and physiological diseases is not fully understood.

Fermentation of ITF in *in vitro* studies does not result in appreciable production of gas, however *in vivo* there are a few volunteer trials that reported increased gastrointestinal flatus and bloating after supplementation (Wang and Gibson, 1993, Bouhnik *et al.*, 1999, Kruse *et al.*, 1999). Inulin consumption is usually either well tolerated, or only causes issues at a high dose

(>20g/d). In the current study, symptoms were increased on prebiotic consumption, however were still classed as less than mild, which would be recognised as well tolerated. There was one volunteer drop out in the study due to prebiotic supplement intolerance, however, some complaints of gas production were also noted during the placebo period. A possible reason is volunteers were having a placebo effect: they felt gastrointestinal symptoms while taking supplements and reporting these symptoms in the daily questionnaire.

In conclusion, daily supplementation of 15g ITF can shift the microbiota to a more favourable profile by stimulating bifidobacteria and repressing *Desulfovibrio* spp. It may also lower the number of proteolytic *Clostridium* cluster XIVa and XIVb. SCFA concentrations in faecal water were not promoted by ITF, however, ITF had a trend on boosting blood lactate concentrations. Urinary aromatic amino acids metabolites concentrations were lower, and improved lipid profiles in blood plasma was found in this study. A significant bulking and laxative effect was seen together with all bacterial and metabolic changes. This shows that gut bacterial metabolism in the large intestine can be shaped towards a more saccharolytic community by ITF, and this change in microbiota exerted beneficial effect on host health.

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Chapter 5 General discussion and future perspectives

5.1 General discussion

Protein supply worldwide is growing gradually, and it reached 81.23g/capita/day in 2013 according to the FAO (2017). In Europe and Northern America, it is rather stable, however, with protein supply in these two regions at 96g/capita/day and 109g/capita/day respectively since 1993 (FAO, 2017). Sometimes, supply does not necessarily relate with consumption. Dietary survey data indicates that, in European countries, protein intakes range from 67 to 114 g/d in adult men and 59 to 102 g/d in women (EFSA Panel on Dietetic Products and Allergies, 2012). Meanwhile, protein reference values are not as high as the actual intake. The recommended dietary allowance (RDA) is 56g/d for men and 46g/d for women in the US; EFSA concludes that the Population Reference Intake (PRI) is 0.66 g/kg body weight per day (Institute of Medicine (U.S.). Committee on Use of Dietary Reference Intakes in Nutrition Labeling., 2005, EFSA Panel on Dietetic Products and Allergies, 2012). Existing data show an association between high fat diet and cardiovascular disease risk; high sugar consumption is positively related to serum triglyceride levels and inversely related to insulin response; therefore, for these two macronutrients, fats and carbohydrates, reference values are given with both lower and upper limit (EFSA Panel on Dietetic Products and Allergies, 2010b, EFSA Panel on Dietetic Products and Allergies, 2010a). Though there are insufficient data to determine a Tolerable Upper Intake Level (UL) for protein, there are data indicating that high consumption of protein can induce negative consequences to host health, especially by bacterial fermentation. As discussed in previous chapters, bacterial proteolysis may produce potentially harmful metabolites such as ammonia, amines and *p*-cresol. In both **Chapter 2** and **Chapter 3**, protein fermentation metabolites production in *in vitro* models were monitored. Ammonia and BCFA concentrations were found to be significantly higher in high protein treatment. Phenols and indoles also had an increasing trend in both experiments with high protein diet simulation. Besides, gut microbiota may shift to a more proteolytic one with a long-term high protein habitual diet. In the continuous three stage fermentation, feeding high protein for more than two weeks not only promoted higher production of proteolysis metabolites, but also boosted the number of clostridia group. These possibly deleterious effects of a high protein diet could be significant in populations following special diets. Athletes and body-builders take protein supplements to help with their training and muscle gain. For example, endurance and strength-trained athletes are recommended to have 1.2-1.7 g/kg body weight per day of protein (Rodriguez *et al.*, 2009). There are some popular weight-loss diets that are high in protein. The

Atkins diet and other high protein diets with restriction of carbohydrate intake are followed in order to lose weight (WebMD, 2012, Naude *et al.*, 2014). With such high amounts of protein ingested, residual dietary protein, together with endogenous proteins that are secreted from the digestive tract, could enter the colon and provide substrate for bacterial metabolism.

Metabolism of ITF promotes specific bacterial changes in the colon, and its fermentation can boost the saccharolytic activity of the microflora. The main objective of this project was to investigate the effect of prebiotic inulin type fructans on potential negative consequences of gut bacterial proteolysis. In both **Chapter 2** and **3**, ammonia and BCFA were found in significantly lower amounts with ITF supplementation comparing to the high protein treatment. Phenols and indoles concentrations had a trend to be lower with ITF addition comparing to high protein diet simulations. In the human study, urinary aromatic metabolites were found in lower amounts with the prebiotic intervention. Besides, ITF modulated the bacteria composition towards a more saccharolytic community, with suppression of proteolytic bacteria groups. Bifidogenic effect was seen in all experiments, while enhancement of lactobacilli was only observed in *in vitro* batch cultures and continuous culture gut models. Reduction of the bacteroides group after ITF treatment was found in continuous cultured gut models. *Clostridium coccoides-Eubacterium rectale* group had a decreasing trend in the human trial which were not seen in *in vitro* fermentations. SCFA is the main end product of carbohydrate fermentation by gut bacteria. In batch cultures, prebiotics led to an increase in propionate, and in the continuous three staged culture, butyrate levels were elevated with ITF addition. Production of SCFA by gut bacteria in humans is difficult to track in faecal water due to absorption, circulation, and incorporation with host metabolism. After being absorbed, circulatory metabolites in blood can indicate some colonic production in healthy populations. Higher concentration of lactate in blood plasma with the prebiotic treatment was found. Both metabolic activity and microbiota composition were affected by ITF fermentation, and its supplementation shaped the microflora into more a saccharolytic one.

Another potential benefit of ITF that was seen in this thesis is its influence on SRB. Reduction of *Desulfovibrio* was seen in both *in vitro* experiments, although in batch culture the change in *Desulfovibrio* was not significant. This inhibitory effect of ITF on sulphate reducing bacteria was then confirmed in the *in vivo* human study. *Desulfovibrio* is one of the dominant SRB in the human gut and increasing evidence has suggested that activity of this bacterial group correlates with IBD (Gibson *et al.*, 1991, Figliuolo *et al.*, 2017).

Improvement of bowel movement with ITF has been mostly studied in constipated subjects, and changes of transit time and faecal voiding were more noticeable in this target group (Roman *et al.*, 2008, Waitzberg *et al.*, 2013, Vandeputte *et al.*, 2017). Decreased colonic transit time, increased stool frequency and volume has been documented in other *in vivo* trials in healthy populations, and this was confirmed again with 15g ITF per day in healthy population in this thesis.

Vandeputte *et al.* (2017) discovered that the beneficial effect of ITF on bowel function was positively correlated with *Bifidobacterium* and *Anaerostipes* populations, and negatively correlated with *Bilophila* population from 16s rDNA profiling data. In this study, bifidobacterial numbers were significantly higher with the ITF treatment and its change positively correlated with improvement of stool frequency, which was also significantly higher after the ITF intervention. This correlation of bifidobacterial change in number and bowel movement was the first time being observed in a human study.

According to a recent national dietary survey, British adults consume 14g dietary fibre daily, which is less than half of the UK Dietary Reference Value (DRV) of 30g per day (Food Standards Agency, 2016, The Scientific Advisory Committee on Nutrition, 2015). EFSA recommends consumption of 25g of dietary fibre to maintain regular defaecation (EFSA Panel on Dietetic Products and Allergies, 2010a). Normal healthy people should be able to benefit from ITF supplementation due to low dietary fibre intake in the general population. Besides, EFSA has a favourable opinion on dietary fibre effects on reducing blood lipids (EFSA Panel on Dietetic Products and Allergies, 2010a). ITF could modulate energy regulatory hormones such as elevating blood GLP-1 and PYY, which both down-regulate energy intake and have the potential to reduce LDL-cholesterol (Piche *et al.*, 2003, Parnell and Reimer, 2009). Blood lipids decreased after the prebiotic supplementation in **Chapter 4**, though not significantly.

In conclusion, all three experiments found favourable bacterial and metabolic changes with ITF supplementation. Furthermore, with the *in vivo* human trial, bowel function was significantly improved with ITF intervention. ITF had inhibitory effects on colonic microbial proteolysis, and could improve health and wellbeing, especially for Northern American and Western European populations who have high protein supply. Athletes, bodybuilders, and people following high protein diets for weight management could also benefit from ITF supplementation. Due to inadequate dietary fibre intake in the general population, in order to maintain regular defaecation and improve blood lipid profile, ITF can be supplemented.

5.2 Limitation and future perspectives

In **Chapters 2** and **3**, *in vitro* models were applied to study mechanisms and efficacy of ITF on reducing proteolysis in the colon before further investigation on human subjects. Batch culture fermentation is a way to test multiple substrates over a short period of time, which allows initial screening for more complicated models. Proteolysis of four dietary proteins were compared with batch culture fermentation in **Chapter 2**: different metabolism patterns were seen not only among various protein sources, but also between vegetarian and omnivore groups; this led to further testing of vegetarian and omnivore responses by use of a three stage continuous model. Casein was chosen to be the substrate for two reasons: dairy products are widely consumed by both vegetarians and omnivores; casein resulted in the highest production of aromatic metabolites in batch culture and it would be easier to reveal the inhibitory effect of ITF on aromatic metabolite production. In a human study, *p*-cresol production in subjects with high baseline of *p*-cresol were found to be suppressed better by the prebiotic intervention (De Preter *et al.*, 2007b). The three stage continuous fermentation culture system simulating all distinct regions of the human colon was done in **Chapter 3**. It was shown that protein fermentation happened mostly in the distal end of the colon, which would be difficult to research via non-invasive human studies. The *in vitro* models used here do not include absorptive function: in batch culture systems, metabolites accumulate towards the end of the fermentation; while in continuous culture gut model systems, metabolites accumulate from the proximal to the distal end. Generally, *in vitro* models can simulate the gut to aid early research screening, however, they lack host interactions such as absorption, secretion, and immune responses (Macfarlane and Macfarlane, 2007).

The FISH technique was used in this project to enumerate cell numbers with target bacteria groups that are dominant in faecal microbiota and functionally relevant (Franks *et al.*, 1998). Initially in **Chapters 2** and **3**, EC1531 was included to analyse *Escherichia coli*. No noticeable change of *E. coli* was detected in either batch culture or gut model experiments; *E. coli* counts were relatively low. Its insignificance led to the removal from the groups targeted and the introduction of more relevant bacteria groups in subsequent work. Hoyles and McCartney (2009) discovered that bacteroides were underestimated while using the Bac303 probe and the CFB286 probe can compensate for this. Furthermore, CFB286 can detect the *Prevotella* group and *Prevotella* was found with higher abundance in populations with high levels of plasma trimethylamine *N*-oxide (TMAO) which is a bacterial derived cardiovascular risk factor (Koeth

et al., 2013). Therefore, in **Chapter 4** CFB286 was used to monitor this bacteria group instead of *E.coli* in **Chapter 2** and **3**.

In **Chapter 2**, bacterial and metabolic responses varied in fermentations with three different donors. The donors were selected with restriction of antibiotics and drug use, disease and injuries, however, there are many other factors that impact on the gut microbiota and its metabolic activity: stress, lifestyle, and dietary habit (Nicholson *et al.*, 2012). In this context, dietary habit may play a very important role in microbiota function. A simple questionnaire with what are the protein sources in their diet was given to these donors: one omnivore donor consumes far less dairy products and more soy bean; one vegetarian donor does not consume dairy products at all. Two fermentation that were inoculated with these two donors' faecal samples had different responses to those relevant protein sources comparing to other batches. Further advice for conducting *in vitro* fermentation is not only restricting age and lifestyle, but also indications of dietary habit.

In this thesis, BCFA, ammonia, phenolic compounds, and indolic compounds were determined as protein fermentation metabolites. Gamma-aminobutyric acid (GABA) can be produced by gut bacteria from glutamate by glutamate decarboxylase, and is a neuro inhibitor which has shown a potential in improving host health from dietary intake (Mazzoli and Pessione, 2016). Neurotransmitter serotonin is generated from tryptophan mainly by enterochromaffin cells and some bacteria (O'Mahony *et al.*, 2015). Increasing evidence suggests that gut microbiota may regulate serotonin production in many ways (O'Mahony *et al.*, 2015). The effect, and regulatory mechanism of microbiota composition and metabolic activity on these neurotransmitters are yet to be discovered.

The cholesterol lowering effect of dietary fibre is not only associated with SCFA production, but also bile acid regulation in the gut. Some colonic commensal bacteria such as bifidobacteria, that are promoted by ITF, can assimilate bile salts (Pereira and Gibson, 2002a). Soluble dietary fibre such as β-glucan in oat is found to prevent bile acid reabsorption, and enhance faecal secretion (Gunness and Gidley, 2010). Bile acid profiles of faeces, urine, and blood samples can possibly provide useful information of cholesterol lowering effects seen here.

In the human trial, metabolite changes were not always significant which may be due to metabolism variation of gender, age, BMI, and baseline; therefore, further stratification of samples by grouping different gender, BMI range, or age range may help understand those metabolic changes better. Current data correlated health outcome and microbial changes, therefore, further analyses of metabolic and microbial changes associations may reveal relevant biological pathways, and how ITF affect colonic proteolysis.

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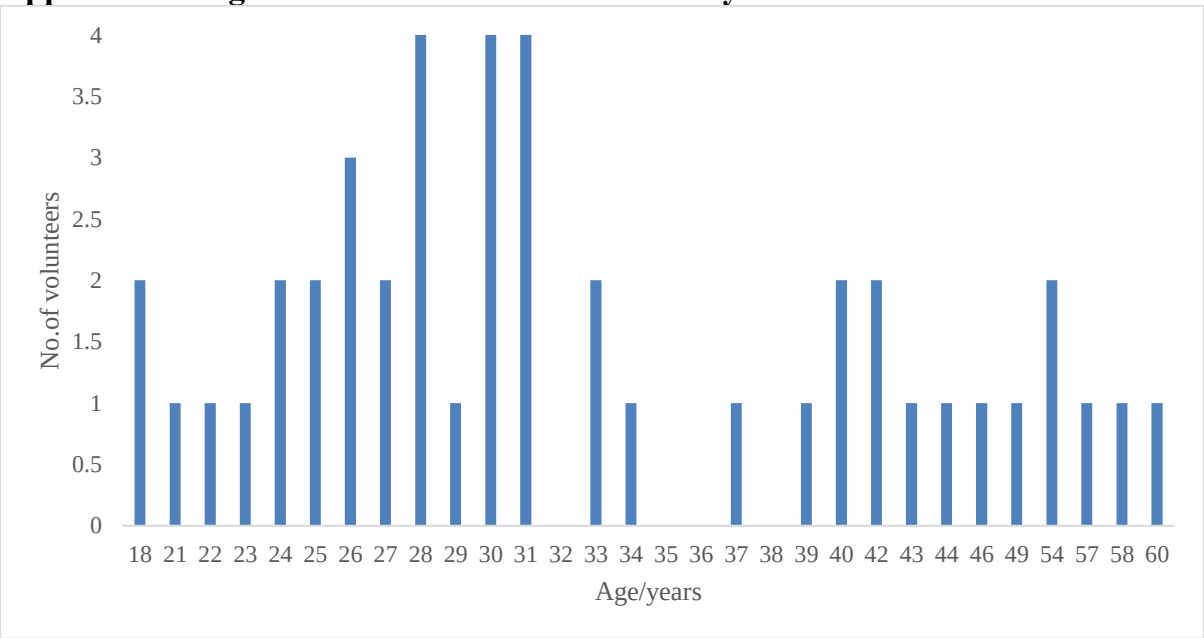
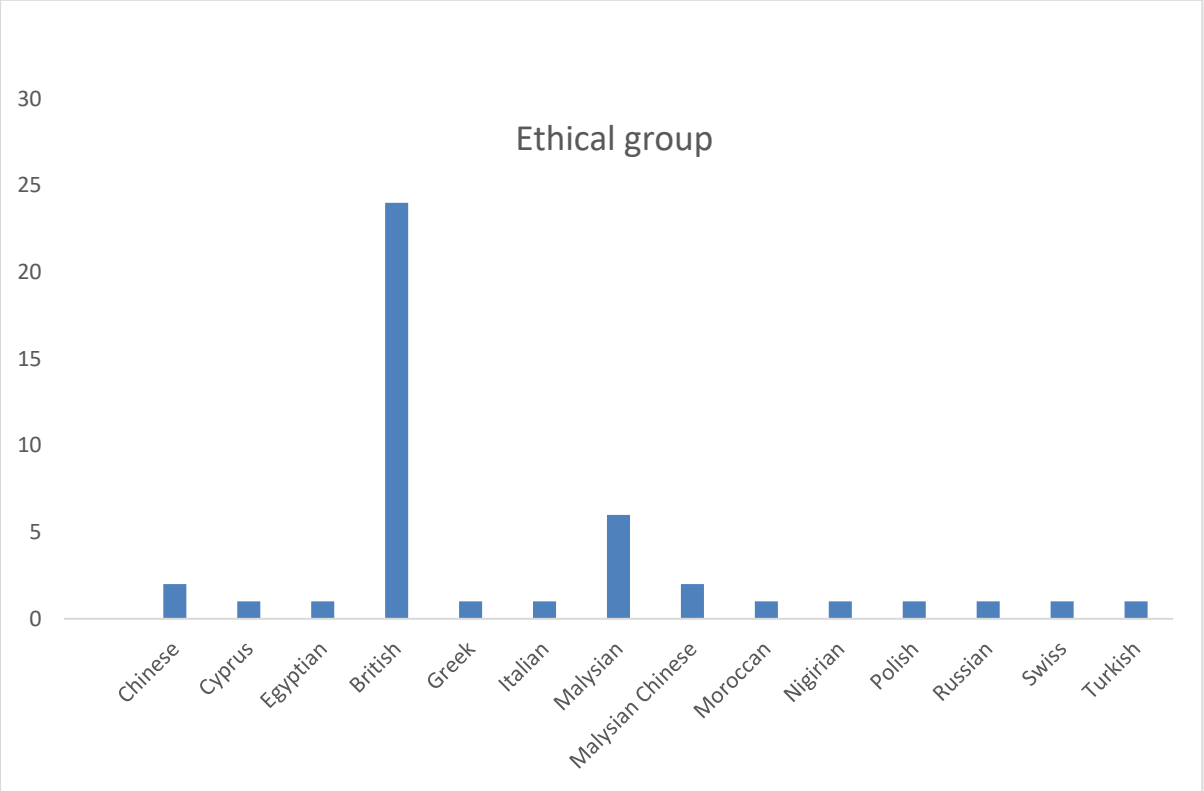
Appendices

Appendix 4.1 Age distribution of the human study



Age distribution of the human study.

Appendix 4.2 Ethnic groups of the human study



Ethnic groups of the human study.

Appendix 4.3 Weight control of the human study. There was no significant change of weight during the whole trial

	Before XZ	After XZ	Before XX	After XX
Volunteer's weight in kg	70.93±15.48	70.88±15.52	70.76±15.23	70.67±14.91

Weight control of the human study. There was no significant change of weight during the whole trial.

Appendix 4.4 Full correlations results

Correlations of bacteria numbers and stool frequency													
		EUB	Bif	Lab	Bac	Erec	Rrec	Ato	Prop	Fprau	DSV	Chis	CFB	Stool Frequency
EUB	1	.638**	.616**	.504**	.539**	.611**	.579**	.642**	.597**	.263**	.325**	.650**	0.071	0.097
		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.408	0.240
	158	158	154	148	158	158	155	157	158	124	146	150	137	148
Bif	.638**	1	.493**	.322**	.238**	.212**	.591**	.456**	.313**	0.171	.198*	.405**	-0.056	-0.051
	0.000		0.000	0.000	0.003	0.007	0.000	0.000	0.000	0.058	0.016	0.000	0.513	0.539
	158	158	154	148	158	158	155	157	158	124	146	150	137	148
Lab	.616**	.493**	1	.599**	.329**	.401**	.531**	.564**	.511**	.439**	.430**	.657**	0.048	0.053
	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.584	0.528
	154	154	154	146	154	154	151	153	154	122	143	146	133	142
Bac	.504**	.322**	.599**	1	.437**	.381**	.365**	.386**	.594**	.592**	.609**	.711**	0.017	0.081
	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.845	0.352
	148	148	146	148	148	148	146	147	148	120	138	140	127	135
Erec	.539**	.238**	.329**	.437**	1	.629**	.202*	.255**	.486**	.317**	.323**	.238**	.262**	.297**
	0.000	0.003	0.000	0.000		0.000	0.012	0.001	0.000	0.000	0.000	0.003	0.002	0.000
	158	158	154	148	158	158	155	157	158	124	146	150	137	148

Rrec	.611**	.212**	.401**	.381**	.629**	1	.310**	.417**	.592**	.214*	.300**	.377**	.278**	.330**
	0.000	0.007	0.000	0.000	0.000		0.000	0.000	0.000	0.017	0.000	0.000	0.001	0.000
	158	158	154	148	158	158	155	157	158	124	146	150	137	148
Ato	.579**	.591**	.531**	.365**	.202*	.310**	1	.622**	.299**	.271**	.236**	.564**	-0.118	-0.086
	0.000	0.000	0.000	0.000	0.012	0.000		0.000	0.000	0.003	0.004	0.000	0.173	0.302
	155	155	151	146	155	155	155	154	155	121	145	148	135	146
Prop	.642**	.456**	.564**	.386**	.255**	.417**	.622**	1	.464**	.251**	.290**	.600**	0.067	0.070
	0.000	0.000	0.000	0.000	0.001	0.000	0.000		0.000	0.005	0.000	0.000	0.437	0.402
	157	157	153	147	157	157	154	157	157	124	145	149	136	146
Fprau	.597**	.313**	.511**	.594**	.486**	.592**	.299**	.464**	1	.457**	.342**	.513**	.197*	.310**
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.021	0.000
	158	158	154	148	158	158	155	157	158	124	146	150	137	148
DSV	.263**	0.171	.439**	.592**	.317**	.214*	.271**	.251**	.457**	1	.597**	.418**	0.116	.212*
	0.003	0.058	0.000	0.000	0.000	0.017	0.003	0.005	0.000		0.000	0.000	0.235	0.024
	124	124	122	120	124	124	121	124	124	124	117	116	107	114
Chis	.325**	.198*	.430**	.609**	.323**	.300**	.236**	.290**	.342**	.597**	1	.484**	0.018	0.104
	0.000	0.016	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000		0.000	0.842	0.226
	146	146	143	138	146	146	145	145	146	117	146	138	128	138

CFB	.650**	.405**	.657**	.711**	.238**	.377**	.564**	.600**	.513**	.418**	.484**	1	0.000	0.043
	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000		1.000	0.618
	150	150	146	140	150	150	148	149	150	116	138	150	129	139
Stool Frequency	0.071	-0.056	0.048	0.017	.262**	.278**	-0.118	0.067	.197*	0.116	0.018	0.000	1	1
	0.408	0.513	0.584	0.845	0.002	0.001	0.173	0.437	0.021	0.235	0.842	1.000		
	137	137	133	127	137	137	135	136	137	107	128	129	137	148
**. Correlation is significant at the 0.01 level (2-tailed).														
*. Correlation is significant at the 0.05 level (2-tailed).														

Pearson correlation coefficient and correlation significance of bacteria population and stool frequency of all volunteers of visit 2, 3, 4, and 5.

Correlations of bacterial changes and stool frequency													
		EUB	Bif	Lab	Bac	Erec	Rrec	Ato	Prop	Fprau	DSV	Chis	CFB	Stool Frequency
EUB	Pearson Correlation	1	.817**	.584**	.368**	.718**	.675**	.561**	.593**	.738**	0.206	.253*	.484**	.234*
	Sig. (2-tailed)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.057	0.019	0.000	0.050
	N	86	86	86	86	86	86	86	86	86	86	86	86	71
Bif	Pearson Correlation	.817**	1	.499**	.483**	.549**	.508**	.475**	.544**	.562**	0.120	.224*	.485**	.256*
	Sig. (2-tailed)	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.270	0.039	0.000	0.031
	N	86	86	86	86	86	86	86	86	86	86	86	86	71
Lab	Pearson Correlation	.584**	.499**	1	.533**	.449**	.460**	.295**	.527**	.540**	.379**	.384**	.345**	0.113
	Sig. (2-tailed)	0.000	0.000		0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.001	0.349
	N	86	86	86	86	86	86	86	86	86	86	86	86	71
Bac	Pearson Correlation	.368**	.483**	.533**	1	.314**	0.209	0.141	.456**	.341**	.394**	.320**	0.178	-0.084
	Sig. (2-tailed)	0.000	0.000	0.000		0.003	0.053	0.196	0.000	0.001	0.000	0.003	0.100	0.486

	N	86	86	86	86	86	86	86	86	86	86	86	86	71
Erec	Pearson Correlation	.718**	.549**	.449**	.314**	1	.597**	.396**	.476**	.615**	.319**	.291**	.285**	0.225
	Sig. (2-tailed)	0.000	0.000	0.000	0.003		0.000	0.000	0.000	0.000	0.003	0.007	0.008	0.059
	N	86	86	86	86	86	86	86	86	86	86	86	86	71
Rrec	Pearson Correlation	.675**	.508**	.460**	0.209	.597**	1	.483**	.568**	.723**	.281**	.343**	.290**	0.209
	Sig. (2-tailed)	0.000	0.000	0.000	0.053	0.000		0.000	0.000	0.000	0.009	0.001	0.007	0.080
	N	86	86	86	86	86	86	86	86	86	86	86	86	71
Ato	Pearson Correlation	.561**	.475**	.295**	0.141	.396**	.483**	1	.535**	.587**	0.072	.240*	.589**	0.225
	Sig. (2-tailed)	0.000	0.000	0.006	0.196	0.000	0.000		0.000	0.000	0.508	0.026	0.000	0.059
	N	86	86	86	86	86	86	86	86	86	86	86	86	71
Prop	Pearson Correlation	.593**	.544**	.527**	.456**	.476**	.568**	.535**	1	.745**	.465**	.368**	.418**	0.190
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.113
	N	86	86	86	86	86	86	86	86	86	86	86	86	71
Fprau	Pearson Correlation	.738**	.562**	.540**	.341**	.615**	.723**	.587**	.745**	1	.338**	.372**	.398**	0.228
	Sig. (2-tailed)	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000		0.001	0.000	0.000	0.055
	N	86	86	86	86	86	86	86	86	86	86	86	86	71
DSV	Pearson Correlation	0.206	0.120	.379**	.394**	.319**	.281**	0.072	.465**	.338**	1	.351**	-0.016	0.025
	Sig. (2-tailed)	0.057	0.270	0.000	0.000	0.003	0.009	0.508	0.000	0.001		0.001	0.881	0.839
	N	86	86	86	86	86	86	86	86	86	86	86	86	71
Chis	Pearson Correlation	.253*	.224*	.384**	.320**	.291**	.343**	.240*	.368**	.372**	.351**	1	-0.001	0.163
	Sig. (2-tailed)	0.019	0.039	0.000	0.003	0.007	0.001	0.026	0.000	0.000	0.001		0.991	0.175
	N	86	86	86	86	86	86	86	86	86	86	86	86	71

CFB	Pearson Correlation	.484**	.485**	.345**	0.178	.285**	.290**	.589**	.418**	.398**	-0.016	-0.001	1	.277*
	Sig. (2-tailed)	0.000	0.000	0.001	0.100	0.008	0.007	0.000	0.000	0.000	0.881	0.991		0.020
	N	86	86	86	86	86	86	86	86	86	86	86	86	71
Stool frequency	Pearson Correlation	.234*	.256*	0.113	-0.084	0.225	0.209	0.225	0.190	0.228	0.025	0.163	.277*	1
	Sig. (2-tailed)	0.050	0.031	0.349	0.486	0.059	0.080	0.059	0.113	0.055	0.839	0.175	0.020	
	N	71	71	71	71	71	71	71	71	71	71	71	71	71
**. Correlation is significant at the 0.01 level (2-tailed).														
*. Correlation is significant at the 0.05 level (2-tailed).														

Pearson correlation coefficient and correlation significance of bacteria changes and stool frequency of all volunteers in both intervention

Glenn Gibson
Tel: 0118 378 8715
Sciences Email: g.r.gibson@reading.ac.uk

Hugh Sinclair Unit of Human Nutrition
Department of Food and Nutritional Sciences
University of Reading
PO Box 226
Reading RG6 6AP
Phone +44 (0)118 378 7771

Appendix 4.5 Consent Form for IFT study

Please initial boxes

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

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

3. I authorise the Investigator to inform my General Practitioner of my participation in the study. ☐

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

5. I consent to an initial blood sample being taken for screening purposes, followed by a series of blood samples throughout the study at the times indicated on the accompanying Participant Information Sheet. ☐

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

- I consent to my contact details being stored on the Nutrition Unit Volunteer Database. ☐ Yes ☐ No
- I consent to my screening information (including date of birth, height, weight, smoking status, long-term use of medication, and blood test results, such as level of cholesterol, triacylglycerol, and glucose) being stored on the Nutrition Unit Volunteer Database. ☐ Yes ☐ No
- I wish to receive a summary of the overall results once the study is complete and analysed statistically. ☐ Yes ☐ No

Participant details

Name of Participant: _____ Date of Birth: _____

Signature: _____ Date: _____

Address of Participant:

(*Please add if you wish to receive the overall results of the study, and/or you consent to be part of the Hugh Sinclair Unit of Human Nutrition Volunteer Database*)

_____

_____

Telephone number: _____

General Practitioner (GP) details

Name: _____

Address: _____

_____

Telephone: _____

Witnessed by

Name of researcher taking consent: _____

Signature: Date:

Appendix 4.6 Poster

University of Reading

VOLUNTEERS NEEDED

The happier your bacteria are, the healthier you are?

We are recruiting volunteers for a study investigating the effects of a beneficial prebiotic on bacteria composition and metabolism.

We are looking for:

Men and women (aged 18-50) who haven't received antibiotics in the past six months

You will be reimbursed for your time and expenses

This study has been reviewed according to procedures specified by the University Research Ethics Committee and has been given a favourable opinion for conduct

For further information on the IFT Study, please contact

nutritionvolunteers@reading.ac.uk
or 0118 378 7771 for IFT study

nutritionvolunteers@reading.ac.uk or 0118 378 7771 ukX.Wang6@pgr.reading.ac.uk
IFTstudy

Appendix 4.7 Research ethics application

Department of Food and Nutritional Sciences

Note All sections of this form should be completed.
Please continue on separate sheets if necessary.

University of Reading

Research Ethics Committee

Principal Investigator: GLENN GIBSON

School: School of Chemistry Food & Pharmacy

Email: g.r.gibson@reading.ac.uk

Title of Project: The effect of prebiotic Synergy1 supplementation on microbiota, protein metabolism and gastrointestinal (GI) symptoms in people consuming high protein diet (ITF study)

Proposed starting date: March 2016 ……

I confirm that to the best of my knowledge I have made known all information relevant to the Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I confirm that I have given due consideration to equality and diversity in the management, design and conduct of the research project.

I confirm that if this project is an interventional study, a list of names and contact details of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed:

…….. Date: ……

(Investigator)

…….. Date: ……

(Head of School or authorised Head of Department)

…….. Date: ……

(Student -where applicable)

Checklist

1. This form is signed by my Head of School (or authorised Head of Department) ☐

2. The Consent form includes a statement to the effect that the project has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct ☐

3. I have made, and explained within this application, arrangements for any confidential material generated by the research to be stored securely within the University and, where appropriate, subsequently disposed of securely. ☐

4. I have made arrangements for expenses to be paid to participants in the research, if any, OR, if not, I have explained why not. ☐

5. EITHER

   (a) The proposed research does not involve the taking of blood samples; ☐

   OR

   (b) For anyone whose proximity to the blood samples brings a risk of Hepatitis B, documentary evidence of immunity prior to the risk of exposure will be retained by the Head of School or authorized Head of Department. ☐

   Signed:

   …….. Date……

   (Head of School or

   authorised Head of Department)

6. EITHER

   (a) The proposed research does not involve the storage of human tissue, as defined by the Human Tissue Act 2004; ☐

   OR

   (b) I have explained within the application how the requirements of the Human Tissue Act 2004 will be met. ☐

7. EITHER

   (a) The proposed research will not generate any information about the health of participants; ☐

   OR

   (b) If the research could reveal adverse information regarding the health of participants, their consent to pass information on to their GP will be included in the consent form and in this circumstance I will inform the participant and their GP, ☐

providing a copy of the relevant details to each and identifying by date of birth

OR

(c) I have explained within the application why (b) above is not appropriate. ☐

8. EITHER

(a) the proposed research does not involve children under the age of 5; ☐

OR

(b) My Head of School (or authorised Head of Department) has given details of the proposed research to the University's insurance officer, and the research will not proceed until I have confirmation that insurance cover is in place. ☐

Signed:

…….. Date……..

(Head of School or authorised Head of Department)

This form and further relevant information (see Sections 5 (b)-(e) of the Notes for Guidance) should be returned, both electronically and in hard copy, to:

Dr Mike Proven
Coordinator for Quality Assurance in Research
Whiteknights House
Email: m.j.proven@reading.ac.uk

You will be notified of the Committee's decision as quickly as possible, and you should not proceed with the project until a favourable ethical opinion has been passed.

Application Form

SECTION 1: APPLICATION DETAILS
1.1 Project Title: The effect of prebiotic Synergy1 supplementation on microbiota, protein metabolism and gastrointestinal (GI) symptoms in people consuming high protein diets (ITF study) Date of Submission: February 2016 Proposed start date: March 2016 Proposed End Date: March 2017
1.2 Principal Investigator [supervisor name, if student project]: Glenn Gibson Department :Department of Food and Nutritional Sciences Email: g.r.gibson@reading.ac.uk Office room number: 2.45 Internal telephone: 0118 378 8715 Other applicants (role): Name: Xuedan Wang Department :Department of Food and Nutritional Sciences Email: X.Wang6@pgr.reading.ac.uk Name: Bob Rastall (Staff) Department :Department of Food and Nutritional Sciences Email: r.a.rastall@reading.ac.uk Internal telephone: 0118 378 6726
1.3 Project Submission Declaration I confirm that to the best of my knowledge I have made known all information relevant to the Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun. I understand that it is a legal requirement that both staff and students undergo Criminal Records Checks when in a position of trust (i.e. when working with children or vulnerable adults). I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed. Signed…… (Principal Investigator) Date:…… Signed…… (Student) Date:……
1.4 University Research Ethics Committee Applications

Projects expected to require review by the University Research Ethics Committee must be reviewed by the Chair of the School Ethics Committee or the Head of School before submission.

Signed…… (Chair of School Committee) Date:……

Signed…… (Head of School) Date:……

1.5

External research ethics committees

Please provide details below of other external research ethics committees to which this project has been submitted, or from whom approval has already been granted [e.g. NHS Committee]

Name of committee	Date of submission/approval	Reference	Status

SECTION 2: PROJECT DETAILS

2.1

Lay summary

[Please provide a lay summary of the project; what is being investigated and why?]

The composition and metabolism of human gut microbiota play crucial roles in health. Microbial colonisation of the gastrointestinal tract varies widely, with the large intestine having not only the highest density of microbes in terms of bacterial cells per gram but also the most metabolically active microbial community (1). Genetics, mode of birth, infant feeding patterns, antibiotic usage, sanitary living conditions and long term dietary habits contribute towards shaping the composition of the gut microbiome. Diet clearly has a major impact on variation in the gut microbiota composition, and this can be detected in faecal samples after only a few days (2, 3). The bacterial metabolism of dietary components produces much chemical diversity in the large intestine with protective or detrimental effects on disease development (3).

Dietary protein levels are relatively high in western European populations, up to 103g/d, as reported by Food and Agriculture Organization (4). This may result in high levels, entering the large gut where it can become a substrate for proteolytic bacteria. Protein specifically can provide nutrition for microorganisms but metabolites from bacterial protein breakdown can be detrimental. Protein intake from the diet might not be the only source of microbial proteolysis; the human body also secretes considerable amounts of protein into the digestive lumen which can potentially be used by the microflora. On the contrary, end products of carbohydrate metabolism can be positive for health. In this context, prebiotics are carbohydrates that are resistant to digestion and can become available for bacteria in the colon to produce SCFAs and inhibit the production of harmful metabolites. A switch towards more carbohydrate metabolism in the colon, at the expense of proteolysis therefore has positive capacity through the production of more benign metabolites.

Rationale for design

Prebiotics are dietary ingredients that target carbohydrate digesting bacteria only. Given the high intake levels of protein in Western populations, they may be useful to modulate the composition/activity of the microbial gut ecology for improved health (5).
Among prebiotic nutrients, inulin-type fructans (ITF) are well characterized and their administration promotes growth of beneficial microorganisms like *Bifidobacterium* spp. (5).These microorganisms are involved in the reduction of intestinal endotoxin concentration, improve glucose tolerance, exert benefits upon immune function and inhibit pathogens (6,7). In healthy individuals, ITF intake promotes satiety and modulates gut peptides regulating food intake (7, 8).
The aim of the present study is to investigate the effect of inulin-type fructans (ITF) on the negative consequences of colonic fermentation in individuals consuming high protein diets. The hypothesis to be tested is that their action promotes carbohydrate degrading bacteria at the expense of protein utilisers.

Primary

- To investigate the effect of ITF on the faecal microbiota of individuals consuming high protein diets, using a culture independent procedure known as Fluorescence *in situ* Hybridisation (FLOW-FISH). The main groups to be enumerated by this method will be: bifidobacteria, lactobacilli, clostridia, eubacteria, bacteroides, atopobium, proteobacteria, sulphate-reducing bacteria and *E. coli*. In addition, bacterial DNA will be extracted and used for a fuller microbial diversity (metagenomic) analysis.

- To ascertain the extent of colonic prebiotic fermentation and to investigate the effect on bacterial metabolites in faeces, urine and blood.

Secondary

- To conduct an assessment of stool frequency, stool consistency, bloating, flatulence, abdominal pain and mood changes in a human study.

References

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3. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature 505:559–563

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7. Everard A, Lazarevic V, Derrien M, Girard M, Muccioli GG, Neyrinck AM, et al 2011. Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. Diabetes 60: 2775-2786.

8. Cani PD, Lecourt E, Dewulf EM, Sohet FM, Pachikian BD, Naslain D, et al 2009. Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. American Journal of Clinical Nutrition 90:1236-1243

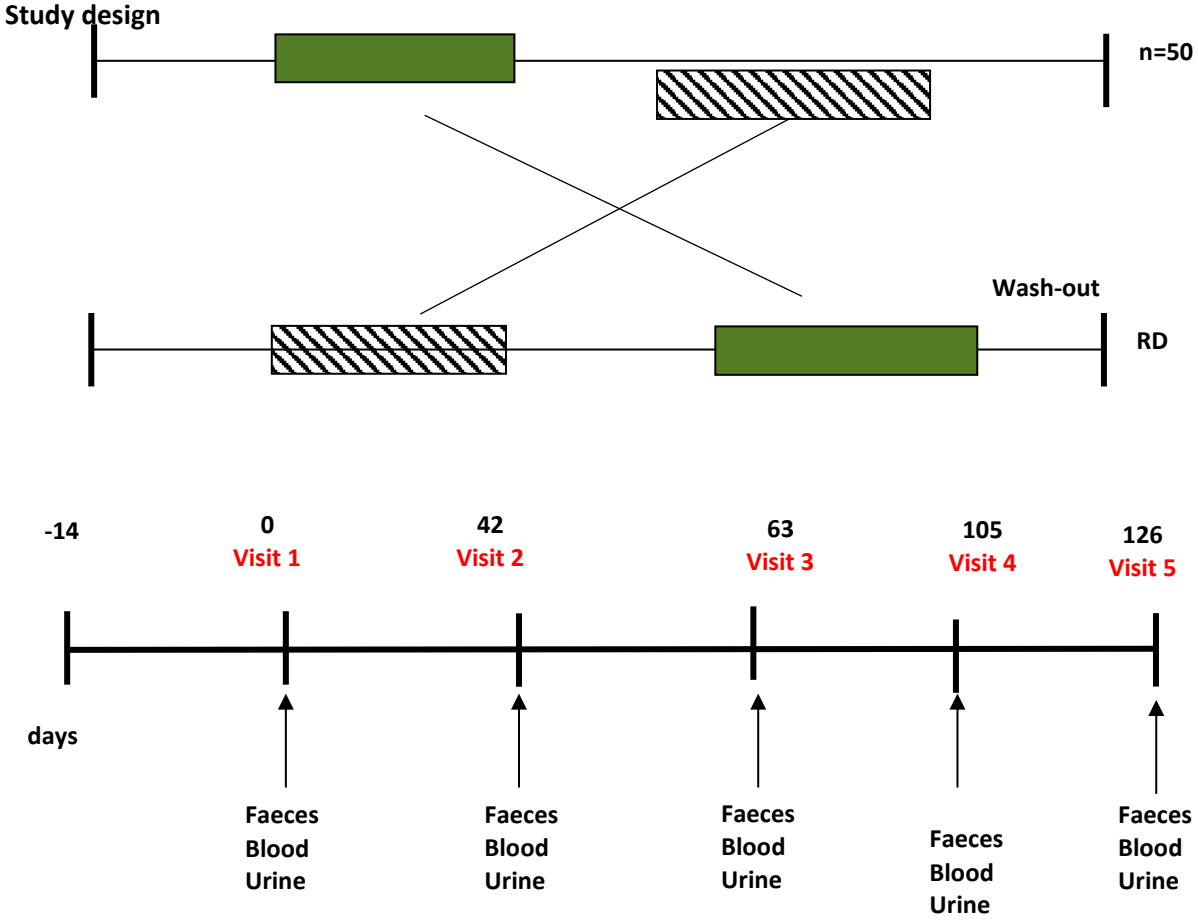
2.2

Procedure

[Outline the project's research protocol]

This will be a crossover, placebo controlled, and randomised study in 50 (18-50 years old) individuals. The study consists of 6 week randomised intervention treatment periods with the prebiotic and placebo, following by 3 weeks wash-out period and another 6 week randomised intervention treatment.

Study design



This trial will investigate the effects of two groups following an intervention diet with a prebiotic food supplement or placebo food supplement. The IFT food supplement (supplied by the Beneo company) is a chicory inulin powder produced in Belgium. Participants will be required to take 2 sachets of 7.5g prebiotic food supplement daily. This will be reconstituted with milk and participants will be encouraged to take the sachet at the same time each day. The placebo control food supplement will be maltodextrin DE 19. Maltodextrin is a standard placebo product for use in human studies involving prebiotics and has been used in similar trials by ourselves for the last 20 years. The products will be provided as a powder (served in sachets) and identical in texture, appearance and taste. The Beneo company will produce and provide barcoded sachets, blinded to the investigators and volunteers. After all volunteers have completed their study visits and all samples has been analysed by researchers, Beneo company will provide information about barcoded sachets. Compliance to food supplement (prebiotic or placebo) will be assessed by recording intake in GI diaries and participants will also be asked to return any unused sachets to the researchers at the end of the each 6-week intervention period. For participants to be considered compliant and therefore included in the study, they will be required to take the food supplement or placebo at least 6 out of every 7 days of the trial for the 6 week period and also return completed questionnaires. Primary outcome measures for the trial are microbial analysis of stools and metabolite analyses of faeces, blood and urine. It will also be essential for participants to provide these samples as described in the Trial Procedure section of this protocol. In order to be eligible for inclusion in the trial, the following drugs or therapies will not be permitted during the four weeks prior to commencing the trial and for its duration:

- ✓ Antibiotics
- ✓ Prebiotics or probiotics (in food products or as supplements)

- ✓ Other dietary supplements or drugs that may affect the luminal microenvironment of the intestine (e.g. Orlistat, Lactulose)

Primary efficacy parameters

The faecal microbiota of volunteers will be characterised, using FLOW-FISH, to determine the effectiveness of treatment in increasing the numbers of beneficial groups (e.g. bifidobacteria) at the expense of less beneficial groups (e.g. clostridia, sulphate-reducing bacteria, *E. coli*). In addition, a detailed study of the microbiota will be performed on total faecal DNA extracts, using16SrRNA sequencing.

The concentration of SCFA and lactate in faecal samples will be determined to ascertain the extent of colonic prebiotic fermentation. Other bacterial metabolites will be measured in faeces, urine and blood by metabolomic (NMR based) analysis in order to generate a detailed picture of microbial fermentation profiles.

Secondary efficacy parameters

Gastrointestinal symptoms (e.g. abdominal pain, flatulence or bloating), number of bowel movements and consistency of stools, will be assessed daily in a volunteer diary. (Appendix E).

Once consent has been obtained, participants will be randomised and included in the trial. We will over recruit by 10% to cover for drop-outs.

Randomisation will be stratified by sex and allocation will occur to one of the two treatment groups using a 1:1 ratio. The method of minimisation will be used to ensure treatment arms are balanced with respect to the number of patients in each group (https://www-users.york.ac.uk/~mb55/guide/minim.htm). Participants are free to withdraw from the study at any time, without providing a reason, if they wish to cease participation. Should this occur, all efforts will be made to report the reason for withdrawal as thoroughly as possible. Allowance has been made for withdrawal of 10% subjects in the sample size calculation. Data from withdrawn participants will be included in the intention to treat analysis.

Screening:

Participants identified as potentially suitable from the University of Reading database or by the local groups and societies will be contacted, and screened against inclusion and exclusion criteria if interested in being involved.

Volunteers will provide a baseline blood sample at a pre-screening visit to check for anaemia, and a stool sample to check microbiota composition. Faecal and blood samples will be collected at site visits at the Department of Food and Nutritional Sciences. Once consent has been obtained, all eligible participants will be asked to fill in a 4-day food diary, a food frequency questionnaire, and complete a brief medical history questionnaire. Based on the food diary, protein consumption will be calculated to evaluate if the volunteer meet the criterion. The volunteers will then be randomised and requested to participate in the run-in period.

Trial Day	Stage of Study	Treatment
-2 weeks	Pre-trial meeting	• Briefing about the study • Distribution of trial information sheets and preliminary discussions with volunteers • Provide blood and stool samples for screening • Provide food diary for screening
0	Baseline **(Visit 1)**	• Provide baseline faecal sample, urine sample and a blood sample (50ml; 5 dessert spoons) for baseline measurements • Distribution of test sachets for the 6w treatment Volunteer diaries (diaries for bowel habit and

		mood questionnaire) will be distributed for the 42 day treatment
6 weeks	**Treatment period** **(Visit 2)**	• Consume two sachets for every day of 6w treatment period • During the treatment (prebiotic or placebo), complete dietary, bowel habit and mood questionnaires and record any concomitant medication or adverse events during the trial • Provide faecal sample, urine sample and blood sample after 6w (Visit 2)
3 weeks	**Washout period** **(Visit 3)**	• Refrain from consuming probiotic and prebiotic supplements. • Do not take any product sachets • Provide faecal sample, urine sample and blood sample after 3w (Visit 3)
15 weeks	**Treatment period** **(Visit 4)**	• Consume two sachets for every day of 6w treatment period • During the treatment (prebiotic or placebo), complete dietary, bowel habit and mood questionnaires and record any concomitant medication or adverse events during the trial • Provide faecal sample, urine sample and blood sample after 6w (Visit 4)
18 weeks	**Washout period** **(Visit 5)**	• Continue to refrain from consuming probiotic and prebiotic supplements. • Do not take any product sachets • Provide faecal sample urine sample and blood sample after 3w (Visit 5)

END OF THE STUDY

Those researchers working with blood, urine and faeces will be up to date with Hepatitis A and B vaccinations.

- *Bacterial enumeration*
  Freshly voided faecal samples will be diluted 1 in 10 (w/w) with anaerobic phosphate buffer and mixed in a stomacher for 2 min. Changes in faecal bacterial populations will be assessed through the use of a culture independent procedure that assesses molecular changes in the microbiota (FLOW-FISH and metagenomics)

- *Short chain fatty acids (SCFA)*
  Samples of the faecal slurry [1 in 10 (w/w) dilution of faeces] will be taken for determination of SCFA (end products of bacterial metabolism) by gas chromatography.

All investigators coming into contact with faecal matter will have completed a Hepatitis A vaccination course. Any individuals coming into contact with faecal samples will abide by good general laboratory procedures.

- *Metabolites*

A blood sample to check for anaemia and baseline measurements will be taken during screening (50ml – 5 dessert spoons)). 5 subsequent blood samples will be taken during the trial. Volunteers will be asked to provide a venous, fasting (fasted for 10 hours), blood sample. Blood will be collected through venepuncture by a trained phlebotomist.

Blood will be extracted into sodium heparin tubes (kept on ice), diluted and centrifuged to gain plasma (for metabolites analysis).

Plasma and serum samples will be labelled as a biohazard and stored at -80^{o}C.

Samples of the faecal slurry, blood plasma and urine will be kept for metabolites determination by ^{1}H-NMR based metabolomics

The Department of Food and Nutritional Sciences is fully licensed under the Human Tissue Act 2004 and as such will adhere to the guidelines necessary for the storage of all biological material. A detailed log will be kept to record when the sample was taken, its place of storage, when analysis was conducted on the sample and how and when the sample was disposed of.

Early withdrawal

- Withdrawal Criteria:

Volunteers will be informed in the consent form and also in the volunteer information sheet that they have the right to withdraw from the study at any time without giving a reason and without prejudice. In addition they may be withdrawn at the Investigator's discretion at any time.

- Volunteers may be withdrawn by the Investigator due to:

An adverse event for which the Investigator does not consider continuing study participation safe.

Recurrent illness.
Poor tolerance
Poor adherence.

Description of the test product

The product used in this study will be *Orafti®Synergy1*. The placebo will be Maltodextrin DE 19 (a powdered carbohydrate that looks and tastes identical to the test) served in sachets. The products will be provided as a powder in sachets, therefore it can be kept at room temperature.

Serving: 2 serving per day (1 sachet: 7.5g)
Instruction of use: Sprinkle all powder of the sachet over a bowl of cereal, or take with milk, just before consumption. Be sure to ingest all powder content.

Visit schedule and assessments

Screening/Pre-Trial meeting (Baseline visit)

Informed consent will be obtained prior to the performance of any study related activities/assessments. Inclusion/exclusion criteria will be reviewed for volunteer eligibility. Here, volunteers will undergo the following evaluations:

- Demography
- Past/current medical conditions
- Weight/ Height
- Concomitant medication
- Smoking history
- Blood sample collection
- Food diary and protein consumption

Note: Blood sample collections can be arranged for another day should volunteer prefer.

In addition, volunteers deemed eligible and who want to participate will be given food and drink diary and instructed on how to complete the diary.

The volunteer will either be excluded or, if fully meeting the inclusion criteria and willing to take part in the study, will be randomised and enrolled. On Day 1, the study volunteer will be given the correct number of sachets for the first treatment period. Food and drink diaries will be collected and diary cards will be issued and the volunteer will be instructed on how to complete these diaries. Volunteers will keep a daily diary noting the number of bowel movements and the average consistency of the stools using the Bristol stool chart (hard, solid, loose or watery), as well as the occurrence of abdominal discomfort, flatulence or bloating and mood changes. Furthermore the diary will include an area for noting any illness experienced including colds and respiratory infections.

Visit 1: Initiation of the study

Volunteers meeting the inclusion/exclusion criteria will be given the correct number of sachets for the first 6 weeks of the treatment. Food and drink diaries will be collected and daily diary cards will be delivered and the volunteer will be instructed on how to complete the diary cards. Volunteers will provide blood (50ml; 5 dessertspoons) urine and faecal samples. Concomitant medications will be checked and the volunteer's weight recorded.

Blood sample collection

Volunteers will be asked to provide a venous, fasting (having fasted for 10 hours, drinking only water – but no water 1hr before arrival) blood sample (50ml; 5 dessertspoons) at each visit. Blood samples will be collected by an experienced and trained phlebotomist in the Hugh Sinclair Unit of Human Nutrition (Department of Food and Nutritional Sciences).

Faecal sample collections

Volunteers will be asked to provide a fresh faecal sample at each visit. The volunteers will be provided with an appropriate vessel for sample collection at the University. If unable to do so, they will be given the option to come in on following days until production is possible. No new treatment will be issued until stool has been provided. Samples will be processed immediately for microbial and metabolites analyses.

Urine sample collections

Volunteers will be asked to provide a 24h urine sample at each visit. Volunteers will be provided with an appropriate container for the 24h urine sample collection at the University. Samples will be processed immediately for metabolite analysis.

Visits 2: 6-weeks treatment period

At Visit 2, volunteers will provide blood, urine and faecal samples. Adverse events will be addressed, concomitant medications will be checked and the diary card will be reviewed with the volunteer. New diary cards and the correct number of sachets for the 6 weeks of the treatment will be given. In the event of withdrawal, all evaluations will be performed as per the end of the study, provided the subject is willing.

Visit 3: wash-out period

At Visit 3, volunteers will provide blood (50ml; 5 dessertspoons), urine and a faecal sample. Adverse events will be addressed, concomitant medications will be checked, weight will be recorded and the diary card will be reviewed with the volunteer. New diary cards will be distributed and volunteers will not be supplied with feeding sachets at this visit as they will enter 3 weeks of washout period. All unused products will be collected at this visit. In the event of withdrawal, all evaluations will be performed as at the end of the study, provided the subject is willing.

Visits 4: 6-weeks treatment period

At Visit 6, volunteers will provide blood (50ml; 5 dessertspoons), urine and faecal samples. Adverse events will be addressed, concomitant medications will be checked, weight will be recorded and the diary card will be reviewed with the volunteer. New diary cards will be distributed and volunteers will be supplied with sachets for the 6 weeks of the treatment – different from the product they received for the first 6 weeks.

Visit 5: wash-out period

At Visit 5, volunteers will provide blood (50ml; 5 dessertspoons), urine and a faecal sample. Adverse events will be addressed, concomitant medication will be checked, weight will be recorded and the diary card will be reviewed with the volunteer. All unused products will be collected at this visit. In the event of withdrawal, all evaluations will be performed as at the end of the study, provided the subject is willing.
Visit 5 finalises the study.

Statistical analysis:

Faeces:

Bacterial numbers will be compared after transformation to log counts. This will be done using a repeat measure ANOVA test analysing repeat measures over time using the factors: Treatment group 0 (placebo) or 1 (treatment).

Volunteer diaries:

We will use a χ^2 test which compares two factors to see if they are independent from each other (in this case the placebo and treatment values): of stool frequency, consistency, abdominal pain and intestinal bloating.

(Note: All questionnaires or interviews should be appended to this application)

2.3

Location

Where will the project take place?

The project will take place within the Department of Food and Nutritional Sciences building, within the Hugh Sinclair Unit.

If the project is to take place in Hugh Sinclair Unit of Human Nutrition, please confirm that you have informed Ms Sarah Elizabeth Hargreaves (s.e.hargreaves@reading.ac.uk). Yes, Ms Sarah Elizabeth Hargreaves has been informed.

2.4

Funding

Is the research supported by funding from a research council or other *external* sources (e.g., charities, business)? [If yes, please check for any particular requirements of the funder regarding ethical review]Yes

B E N E O

BENEO GmbH

Maximilianstrasse 10

68165 Mannheim

Germany

General Management: Hildegard Bauer, Andreas Herber, Dominique Speleers

Register Court: County Mannheim - No. HRB 701800 - VAT DE 253691060

2.5

Ethical Issues

[Please outline any potential ethical issues in the project, and how these will be addressed in the research protocol]

It is not anticipated that there will be any risk to participant safety. Synergy 1 has been used in a number of different feeding trials with various groups of volunteers and was well tolerated by all. Participation in the study does not pose any significant risk. However, same people who have taken similar prebiotics have reported an increase in gas production, with slight bloating feelings, some flatulence and mild diarrhoea. Cases of this are very rare. Volunteers will be questioned about adverse events on a weekly basis on the telephone. Please refer to Appendix H for further information

2.6

Deception

Will the research involve any element of intentional deception at any stage (i.e. providing false or misleading information about the study, or omitting information)? No

[If so, this should be justified. You should also consider including debriefing materials for participants, which outline the nature and the justification of the deception used]

2.7

Payment

Will you be paying your participants for their involvement in the study?

[Will there be pro rata payments if participants withdraw early?]

Time	Task	Payment	Total Payment
2 hours	Assessment and instruction	£ 8.50 / h	£ 17
6 hours	Sample collection	£ 7.00 / h	£ 42
13 hours	Record keeping	£ 7.00 / h	£ 91
Total 21 hours			**£ 150**

Volunteers will be paid £150 for completion of the trial. Those who withdraw will have their payment pro-rated according to time spent. Reserve volunteers will be paid for assessment and instruction if they are not required to participate in the treatment phases of the trial.

Note: excessive payment may be considered coercive and therefore unethical. Travel expenses will not be provided.

2.8

Data storage, data protection and confidentiality

[Please outline plans for the handling of data to ensure data protection and confidentiality, i.e. how and where will the data (results data, consent forms) be stored? Do any samples fall under the jurisdiction of the Human Tissue Agency act?]

Confidentiality will be maintained by allocating volunteers an identification code, which will be used to identify all samples and data obtained. Volunteer's names will not be used in any reports or publications. All data generated from the study will be held securely within a password protected file, only the study investigators will have access to this. A record of the names of the volunteers will not be held on the same file. Information matching volunteer names with identification codes will be kept in a locked filing cabinet, the investigators will only use identification codes. The only time data will be matched with volunteer names is for those volunteers that request to have their personal results discussed with them. A request for individual results to be discussed will include a review of all sample results for the individual volunteer. A list of the names and addresses of the subjects in this project will be compiled, this, together with a copy of the Consent Form, will be retained within the Department for five years after the date that the project is completed.

2.9

Consent

[Please outline procedures for obtaining consent from participants]

Potential volunteers may contact the study investigator if they are interested in participating, or if they would like any further information or to discuss any concerns. A meeting will be arranged to talk to the participants before commencement of the trial. Participants are free to withdraw from the trial at any time without giving reasons. Those who wish to participate in the study will attend a pre-trial assessment, in which informed consent will be obtained. Consent will be obtained after volunteers are asked questions about inclusion and exclusion criteria. Volunteers are free to ask the investigator questions at any point during / following this process.

SECTION 3: PARTICIPANT DETAILS

3.1

Sample Size

[What is your target sample size? Provide statistical (or otherwise) justification for this number]

By the use of a statistical power calculation it can be observed that at significance level of 5% (two-sided) will be that, a log change of 0.5 can be detected at a power of 80% with 43 volunteers. (This calculation is based on the assumption that the within volunteers standard deviation is 0.8, which was as observed by Kruse et al., 1999). Therefore 43 volunteers (18-50 years old) will be recruited for participation in this study. We will over recruit by 10% to cover for drop-outs.

18-50 year old individuals will be screened according the inclusion and exclusion criteria. From this screening we will select individuals (equal numbers of men and women) to enrol in the trial.

3.2

Will the research involve vulnerable adults (e.g., adults with mental health problems or neurological conditions)? No

[If so, you will need to consider carefully issues around working with this population, e.g. consent procedures, Criminal Records Bureau disclosures. It is highly likely that you will also need approval from an NHS Research Ethics Committee for your project]

3.3

Will your research involve young people under the age of 18 years? No

Will your research involve children under the age of 5 years? No

[If so, you will need to consider carefully issues around working with these constituencies, e.g. consent procedures, Criminal Records Bureau disclosures. You may need to consider age appropriate information sheets]

3.4

Will your research involve NHS patients or Clients of Social Services? No

[If so, you will need approval from an NHS Research Ethics Committee for your project. In the case of NHS staff, this is only required if their recruitment is by virtue of their professional role. Ideally, approval will have been received from the NHS committee prior to being submitted to the University committee. Please attach original NRES application and, where relevant, approval documents]

3.5

Recruitment

[Please describe recruitment procedures (e.g. advertising, databases) and attach any recruitment materials, such as posters, emails, letters, etc.]

Posters with study information will be distributed around the Reading area. Potential volunteers may then contact the study investigator if they are interested in participating, or if they would like any further information or to discuss any concerns. Potential participants will have a minimum of two weeks to decide whether they want to be involved in the study. A meeting will be arranged to talk to participants before commencement of the trial prior to informed consent. Participants are free to withdraw from the trial at any time. Advertisements may also be emailed, put in newspapers and on walls in and around the University.

Appendix A: CONSENT FORM

Department of Food and Nutritional Sciences
Xuedan Wang: X.Wang6 @pgr.reading.ac.uk
Whiteknights
PO Box 266, Reading RG6 6AP, UK
Telephone 07840031309

Consent Form

1. I have read and had explained to me by ……..…

   the accompanying Information Sheet relating to the project on:

   ……...

2. I have had explained to me the purposes of the project and what will be required of me, and any questions I have had have been answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation.

3. I understand that participation is entirely voluntary and that I have the right to withdraw from the project any time, and that this will be without detriment.

4. ***Researcher to delete (a) and (b) if GP will not be contacted, or (b) if no response from GP is required***

   a) I authorise the Investigator to consult my General Practitioner, and provide their name and address details overleaf.

   b) I authorise my General Practitioner to disclose any information which may be relevant to my proposed participation in the project.

5. I agree to the interview/session being video/audio taped. ***(delete if not applicable)***

6. This application has been reviewed by the University Research Ethics Committee and has been approved by UREC. It has been given a favourable ethical opinion for conduct.

7. I have received a copy of this Consent Form and of the accompanying Information Sheet.

Name: ……

Date of birth [if GP details given]: ……

Signed: ……...……

Date: ……...……

GP details

Name: ……

Address: ……

……...……

Appendix B

Department of Food and Nutritional Sciences
Xuedan Wang: X.Wang6 @pgr.reading.ac.uk
Whiteknights
PO Box 266, Reading RG6 6AP, UK
Telephone 07840031309

GP letter

Dear *Name of GP*,

Name of Patient (and date of birth) has expressed an interest in participating in a dietary intervention trial at The University of Reading. The trial will investigate the effect of a prebiotic Synergy1 on on protein metabolism by gut bacteria in healthy adults.

This will be a double-blind, crossover, placebo controlled, randomised study in individuals aged 18-50 years old. The study will consist of a treatment period (prebiotic or placebo for 6 weeks), a wash-out period (3 weeks) and another treatment period (prebiotic or placebo for 6 weeks). The subject information sheet has been enclosed for your information.

The product used in this study will be Synergy1 served in sachets, twice daily. The placebo will be maltodextrin served in sachets, twice daily. Synergy1 and maltodextrin are completely safe. As such the trial is not considered of risk to the volunteers.

This human study has been subject to ethical review, according to the procedures specified by the University Research Ethics Committee, and has been given a favourable ethical opinion for conduct.

The trial will commence on (*Trial start date)*

If you have any questions about the trial please do not hesitate to contact me.

We appreciate your help.

Yours sincerely,

Xuedan Wang

Appendix C

Department of Food and Nutritional Sciences
Xuedan Wang: X.Wang6 @pgr.reading.ac.uk
Whiteknights
PO Box 266, Reading RG6 6AP, UK
Telephone 07840031309

PARTICIPANT INFORMATION SHEET

The effect of prebiotic Synergy1 supplementation on microbiota, protein metabolism and gastrointestinal (GI) symptoms in people consuming high protein diet (ITF study)

You are being invited to take part in a research study. Before you decide if you want to take part it is important that you understand what is involved. Please read the following information and discuss with others if you wish. Please ask us if there is anything you do not understand or if you would like any additional information. Take time to decide whether or not you wish to take part.

Aim

The aim of this study is to evaluate the effects of prebiotic Synegy1 on intestinal bacteria and protein metabolism in individuals aged 18-50 years. About 50 volunteers will take part in this study. In addition, a further 5 volunteers will be recruited as reserves. Equal numbers of men and women will be included in the study cohort.

Before you decide whether to take part in the study, please read the following information carefully. If you want to know anything about the study, which is not written here, please ask the investigator.

What are prebiotics?

- Prebiotics are non-digestible carbohydrates that exert bacteria changes in the intestine and bring about health benefits
- They are used in foods to increase gut bacterial numbers
- They have been found to improve the intestinal bacterial composition of the general population
- They are safe for human consumption
- They have been consumed by humans for hundreds of years
- The prebiotic used in this study Orafti®Synergy1 is produced by Beneo company which is also sponsoring this study.

Proposed benefits of prebiotics

- Reduce the number / activities of disease causing bacteria
- Influence satiety
- Improve immune response
- Reduce risk of gastrointestinal illness such as travellers' diarrhoea, irritable bowel syndrome, infections
- Increase absorption of minerals

Why is this study being carried out?

There is an increasing shift towards use of diet to control protein metabolism and other health parameters. Prebiotics are safe interventions that could help.

Inclusion criteria/exclusion criteria

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

- Aged between 18 and 50 years of age.
- Willing to participate in the entire study (signed informed consent required).
- Subjects will be eligible for the study if male or female (non pregnant), aged 18– 50 years, with high consumption of protein (105g/day).

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

- History or evidence of intestinal disease; such as tumour, Irritable Bowel Syndrome, *etc*., within the previous 5 years.
- Received antibiotics in the previous six months
- Not willing to cease the consumption of probiotic or prebiotic preparations for the duration of the study
- Former participation in another study involving prebiotic or probiotic preparations within the previous 2-weeks, or intention to use such products during the course of the study (please note sensory evaluations may be still permitted after discussion with the Investigator)
- History of malignancy within the previous 5 years (with exception of well-treated basal cell carcinoma or *in situ* cervical carcinoma).
- Diabetes
- Smoker
- Lactose intolerant
- Allergic to gluten
- Currently prescribed immunosuppressive drugs. Participants will be required to withdraw should they begin taking any of the ineligible medication.
- *Intention* to use *regularly* other medication which affects gastrointestinal motility.
- History of alcohol or drug misuse.
- Using statins
- Suffer from any major conditions involving the following:

✓ Head, Ears, Eyes, Nose and Throat
✓ Dermatological/Connective tissue
✓ Neurological
✓ Lymphatic
✓ Urogenital/Rectal
✓ Abdominal
✓ Respiratory
✓ A previous cardiovascular event within the last 6 months, presence of secondary dyslipemia related to thyroid dysfunction, used any drug affecting lipid metabolism in previous 3 months, a history of alcohol abuse.

What will I be asked to do?

- All participants will be asked to fill out a health screening questionnaire and inclusion/exclusion criteria will be reviewed for volunteer eligibility.
- Informed consent from yourself will be required
- On giving consent and passing initial screening, participants' height and weight will be measured and they will be required to give a blood sample to screen for anaemia and baseline measurements (50ml; 5 dessertspoons)
- Once the study begins, participants will be randomly allocated to consume the prebiotic supplement or a placebo (maltodextrin) for 6 weeks and after the first wash out period participants will switch to placebo or prebiotic supplement. Neither you nor investigator will know which of the supplements you are taking. After the each 6 weeks (placebo or treatment) there will be a 3 week washout period where no product will be consumed.
- Participants will be required to consume the product as two doses before breakfast and after dinner. The products will be provided as a powder in sachets, therefore can be kept at room temperature, and also can be taken abroad.
- Participants will provide one stool sample on weeks 0, 6, 9, 15 and 18 of the study to look for changes in gut bacteria. The same times will be used for blood sampling to assess blood metabolites.
- Volunteers will be asked to provide a 24h urine sample at each visit.
- Volunteers will be given a container for faecal collection and shown to private facilities within the department to give a stool sample. If unable to do so, they will be given the option to come in the days following until production is possible. No new treatment will be issued until stool has been provided.
- All blood samples will be taken in the Hugh Sinclair unit of the Department of Food and Nutritional Sciences
- **Maintenance of normal dietary patterns throughout the study is essential** and participants will be required to complete food and drink logs throughout the study
- Any adverse medical events which occur during the trial (e.g. headache, gut symptoms) should be recorded in a diary along with medication taken
- All incidence of respiratory infections and colds should also be reported
- Daily stool habit should be recorded in a diary
- Please note that participants will be removed from the study if they develop acute gastrointestinal illness (e.g. food poisoning) or intolerance to the supplement or if they do not comply to above stated restrictions

Are there any risks?

The product used in this study will be the prebiotic Synergy1, the placebo will be maltodextrin served in sachets, twice daily. Synergy1 and maltodextrin do not pose any risk to participants.

Blood samples will be collected by experienced staff trained for this purpose at the University (Hugh Sinclair Unit). There is a chance of a little discomfort and bruising when having blood taken but every care will be taken to minimise this.

Confidentiality

Confidentiality will be maintained by allocating volunteers an identification code, which will be used to identify all samples and data obtained. Volunteer's names will not be used in any reports or publications. All data generated from the study will be held securely within a password protected file, only the study investigators will have access to this a record of the names of the volunteers will not be held on the same file.

Information matching volunteer names with identification codes will be kept in a locked filing cabinet, the investigators will only use identification codes. The only time data will be matched with volunteer names is for those volunteers that request to have their personal results discussed with them. A request for individual results to be discussed will include a review of all sample results for each volunteer. A list of the names and addresses of the subjects in this

project will be compiled, this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Restrictions during testing

- Participants must not consume pre- or probiotic supplements or food products containing them during the study.

Examples of these food products are:

- Danone Actimel yoghurt drink
- Yakult milk drink
- Danone Activia yoghurt
- Kellog's Rice Crispies multigrain
- Weetabix
- Muller Vitality Yoghurt/Drinks
- Warburton's healthy inside bread

General Information

- You will receive £150 for completing the trial and £20 if you are a reserve volunteer who does not receive a treatment. Volunteers that drop out will have their payment pro-rated to cover the part of the study completed.
- Stool sample pots will be provided and advice on how to take stool samples will be given
- Analysis of faecal, urine, and blood samples will occur at the University of Reading
- You will be provided with breakfast on visit days
- If at any time you wish to withdraw from the study you are completely free to do so without giving a reason
  The information collected will be used for research purposes only. All information will be confidential and individuals' names will not be used in any reports resulting from this work.
- Once the study has been completed you can request your results
- All unused samples will be destroyed after the completion of the study and sample analysis.

The University has appropriate insurance and is well used to carrying out these types of trials. If there is a complain then this should be addressed to Professor Glenn R Gibson, Head of FMSU, University of Reading g.r.gibson@reading.ac.uk
The investigators thank you for taking time to read this. If you have any queries please feel free to contact:

Xuedan Wang X.Wang6@pgr.reading.ac.uk

Study Timetable

Proposed dates	Stage of Study	Treatment
	Baseline Pre-trial meeting/ Screening	• **Briefing about the study** • **Provide screening blood and stool samples** • **Dispensing food and drink diary cards**
Weeks 0	Visit 1 Start of study	• Provide base-line faecal, urine and blood samples • Collecting food and drink diary cards • Dispensing diary cards • Dispensing product • During the 6 week period – product will be taken twice daily • Diaries to be completed daily
Weeks 1-6 (interim period))	**Visits 2** **6 Week** Treatment period	• Diaries to be submitted • Provide faecal, urine and blood samples and given new diary cards. No product provided for washout period. • Dispensing diary cards • Diaries to be completed daily
Weeks 6-9	**Visit 3** **3 week** Post Washout period	• Provide faecal, urine and blood samples (visit 3) • Diaries to be submitted • Dispensing diary cards • Dispensing product • During next 6 week period – product will be taken twice daily • Diaries to be completed daily
Weeks 9-15 **(**interim period**)**	**Visits 4** 6 Week Treatment period	• Diaries to be submitted • Provide faecal, urine and blood samples and given new diary cards. No product provided for washout period. • Given new diary cards • Diaries to be completed daily
Weeks 15-18 (end of treatment period)	**Visit 5** 3 week Post Washout period	• Provide faecal, urine and blood samples (visit 3) • Diaries to be submitted

Note: Volunteers will take all treatments during the course of the study but not at the same time. Volunteers will be provided with breakfast on visit days.

FOODS TO AVOID

Whilst participating in this trial there are some foods that we would ask you to please refrain from consuming

Probiotics

- (found in many yogurts)

Additional Prebiotics

Added to

-Supplemented bread

-Supplemented Cereals

-Supplemented fruit juices

-Additional supplements

Appendix D: Pre-study Questionnaire

Pre-study General Health Questionnaire and Volunteer details

Name: _____

DOB: _____

Gender: _____

Address: _____

_____

_____

_____

_____

Telephone: _____

Height: _____ (cm)

Weight: _____ (kg)

Waist circumference: _____ (cm)

Questions to assess general health

Does the following apply to you?

	Yes	No	Don't know	If Yes – Please describe
History of drug or alcohol abuse				
Excessive Alcohol consumption ***** *******Should not drink regularly (most days or everyday) more than :* ***Men: 3-4 Units a day*** *(not more than a pint of strong lager,beer or cider;)* ***Women: 2-3 Units a day*** *(not more than a standard 175 ml glass of wine)*				
Smoker				
Lactose intolerance				
Pregnancy, lactation or planning pregnancy				
Involvement in drug/ medication study in last month				
Intake in pre or probiotics within last month				
Gluten allergy				
Use of antibiotics in previous 6 months				
Colonic irrigation (within last 3 months)?				
Medication active on GI tract (within last 3 months)				
Family history of colorectal cancer in under 50's				

Please state any prescribed medication you are currently taking below:

_____

_____

_____

_____

Do you suffer from any conditions involving the following?

	Yes	No	Don't know	If Yes – Please describe
Dermatological/Connective tissue				
Head, Ears, Eyes, Nose and Throat				
Cardiovascular				
Respiratory				
Abdominal				
Urogenital/Rectal				
Gastroenterological				
Lymphatic				
Neurological				
Coeliac disease				

All details will be kept strictly confidential

Appendix E: Volunteer diaries

Volunteer Diary

Page 1 of 10

Volunteer No. _____

Period No. _____ Day No. to Day No.

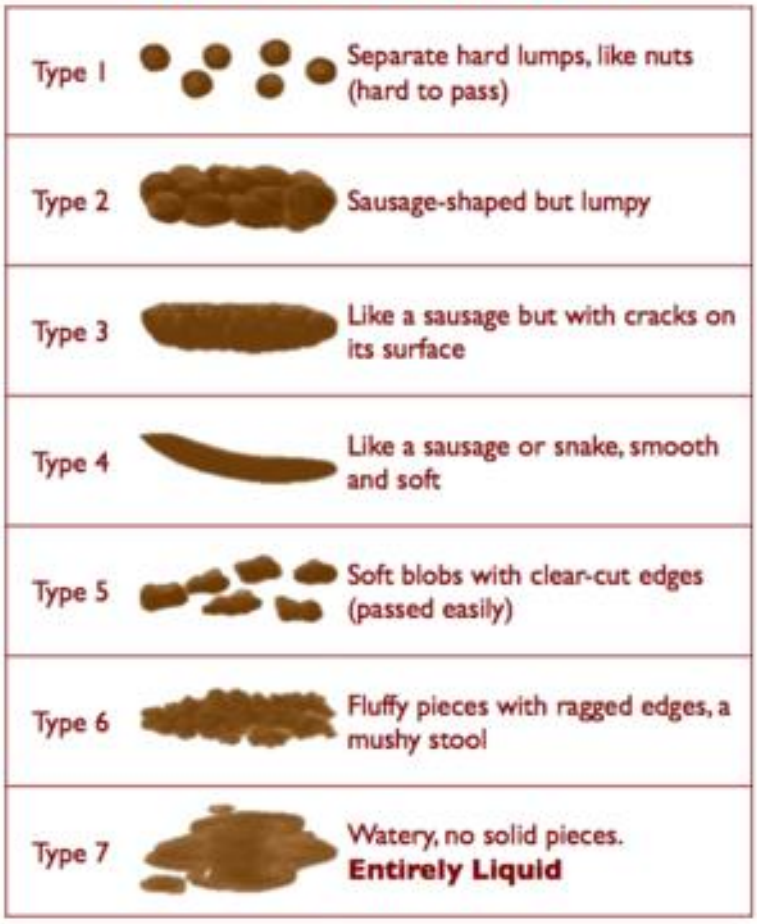
Please fill in the diary carefully and completely for each day. If you are unsure how to answer, please give the best information you can. Please return completed diary to Xuedan Wang on your next visit.

To be filled in by investigator only!

Date started at:

Next visit at:

Bristol Stool Chart



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

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

Volunteer No.

Study day	**Date d/m**	**Number of stools**	**Stool consistency as per Bristol chart(page 2)**			**Abdominal pain**			
		If 0 please include	Stool 1	Stool 2	Stool 3	**none**	**mild**	**moderate**	**severe**
e.g.	15/01	1	4	x	x	✓			
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									

 Volunteer No.

Study day	**Date d/m**	**Stomach or intestinal bloating**				**Flatulence**			
		none	**mild**	**moderate**	**severe**	**none**	**mild**	**moderate**	**severe**
e.g.	**15/01**	✓				✓			
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									

Volunteer No. _____

Study day	**Date** **d/m**	**Happy**			**Alert**		
		Less than normal	**Normal**	**More than normal**	**Less than normal**	**Normal**	**More than normal**
e.g.	**15/1**		✓			✓	
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							
21							

Volunteer No. _____

Study day	**Date d/m**	**Energetic**			**Stressed**		
		Less than normal	**Normal**	**More than normal**	**Less than normal**	**Normal**	**More than normal**
e.g	**15/01**		✓			✓	
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							
21							

 Volunteer No.

Medication

Please enter the intake of any medication taken during the study with name, dosage per day, date started and date stopped.

Medication	**Dosage**	**Date started - stopped**
e.g1. paracetamol	**500mg twice**	**15/1 – 15/01**
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		

 Volunteer No.

Adverse Events

Please enter the occurrence of all adverse events and any respiratory infections, colds or flu-like symptoms with start and end date and time.

Adverse Event	**Date – started**	**Date – stopped**
e.g1. headache	**15/01**	**15/01**
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		

Volunteer No. _____

Date:

Time	**Food/drink**	**Details**	**Amount**
E.g. 6.30			
7.30	Cereal	Weetabix	3
	Semi-skimmed milk		1 mug full
	Toast	Wholemeal	2 slices
	Tea	Tea, Milk	1 mug full

 Volunteer No. _____

Final Questions

Did you experience any of the following after taking your product:

Did you experience an after taste?

Yes _____ No _____

Did you experience a feeling of fullness?

Yes _____ No _____

Did you experience difficulty in taking the product?

Yes _____ No _____

Department of Food and Nutritional Sciences
Xuedan Wang: X.Wang6 @pgr.reading.ac.uk
Whiteknights
PO Box 266, Reading RG6 6AP, UK
Telephone 07840031309

Appendix F: Advertisement

VOLUNTEERS NEEDED

We are recruiting volunteers for a study investigating the effects of a beneficial prebiotic on bacteria composition and metabolism.

We are looking for:

Men and women (aged 18-50) consuming high protein

If so, would you be willing to participate in our prebiotic feeding study?

For further information please contact
Xuedan Wang (Holly)
on X.Wang6@pgr.reading.ac.uk or 07840031309

Appendix G

Recruitment Letter

Dear

We are looking to recruit men and women aged 18-50 years old, who haven't received any antibiotics for the past 6 months , on to a dietary intervention trial at The University of Reading. The product we will be using is a prebiotic called Synergy1. Prebiotics are non-digestible fibre and safe for human consumption. This trial will investigate the effect of the prebiotic on gut bacteria and protein metabolism.

The study will consist of a 4 month randomised crossover treatment period with prebiotic or placebo.

Please review the enclosed information sheet and contact myself (study investigator) if you are interested in participating, or would like any further information or to discuss any concerns. There will be a minimum of two weeks to decide whether you would like to be involved in the study and a meeting will be arranged before the commencement of the trial prior to giving informed consent. Participants are free to withdraw from the trial at any time.

The trial will commence on (*Trial start date)*

If you have any questions about the trial please do not hesitate to contact me.

We appreciate your help.

Yours sincerely,

Xuedan Wang

Appendix H

Information relating to safety aspects of the prebiotic

Please find attached the pdf file from Beneo confirming both prebiotic and placebo in this study is safe to take. Any concerns/questions, please feel free to call at any time.

Product Sheet

Orafti® Synergy1

DOC.A3-40*01/13

Specification

Description

Orafti® Synergy1

- is an **enriched chicory inulin** powder* containing a carefully selected DP distribution (DP = Degree of Polymerisation = chain length of the molecules);
- is a combination of chicory inulin molecules with selected chain lengths, enriched by a specific fraction of oligofructose produced by partial enzymatic hydrolysis of chicory inulin;
- is a food ingredient consisting of inulin, oligofructose, fructose, glucose and sucrose;
- is exactly the same product as the one used in the clinical research that shows that the daily intake of 8 g significantly increases calcium uptake in humans (patent granted).

Chicory inulin is consisting of polysaccharides which are based on fructose units linked together by ß(2-1)linkages. Almost every molecule is terminated by a glucose unit. The total number of fructose or glucose units of chicory inulin ranges mainly between 2 and 60.

Chicory Oligofructose is consisting of oligosaccharides which are based on fructose units linked together by ß(2-1)linkages. Part of these molecules are terminated by a glucose unit. The total number of fructose or glucose units (= Degree of Polymerisation or DP) of chicory oligofructose ranges mainly between 2 and 8.

Compositional Specifications

Analytical Methods : see our Technical Brochures.

Inulin*	(92 ± 2) g/100g
Glucose + fructose + sucrose*	(8 ± 2) g/100g
Dry Matter (grav.)	(97 ± 2) g/100g
Carbohydrate content*	> 99.5 g/100g
Ash (sulphated)*	< 0.2 g/100g
Conductivity (w = 15 g/100g)	< 250 µS/cm
Heavy Metals*	Pb ≤ 0.02 mg/kg As ≤ 0.03 mg/kg Cd, Hg each ≤ 0.01mg/kg
pH (w = 10 g/100g DM)	5.0 to 7.0

* expressed on Dry Matter (grav.).

Microbiological Specifications

All values expressed on Dry Matter.
Analytical methods : see our Technical Brochures.

Mesophilic bacteria - total count	max. 1000 cfu per g
Yeasts	max. 20 cfu per g
Moulds	max. 20 cfu per g
Thermophilic aerobic spores	max. 1000 cfu per g
Anaerobic H_2S producing thermophilic spores	max. 25 cfu per g
Enterobacteriaceae	negative in 1 g
Bacillus cereus	max. 100 cfu per g
Coagulase positive staphylococci	negative in 0.1 g
Escherichia coli	negative in 1 g
Clostridia	negative in 1 g
Salmonella	negative in 250 g
Listeria	negative in 25 g

BENEO-Orafti - Aandorenstraat 1, B - 3300 Tienen Belgium - Tel +32 (0)16 801 301 - Fax +32 (0)16 801 308 - info@BENEO-Orafti.com - www.BENEO-Orafti.com

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Information

Nutritional information: European Union

All values are average values expressed per 100 g commercial product.

Carbohydrates	9	Protein	negligible
Sugars[1)]	9	Fat	negligible
Dietary Fibre[2)]	88		
Caloric value[3)]	212 kcal / 857 kJ	Vitamins and Minerals	negligible

1) Sugars = glucose + fructose + sucrose + inulobiose (difructose)

2) As measured by AOAC method 997.08 excluding inulobiose (difructose).The EU Directive 2008/100/EC (amending 90/496/EEC) introduced a new definition of dietary fibre starting with three monomeric units. AOAC Method 997.08 allows the identification and quantification of undigestible dimers. This therefore creates a difference in calculated dietary fibre based on EU and US definitions with subsequent effect on sugars. For inulin, no change from the current situation is needed.

3) based on a caloric value of 2 kcal/g for all dietary fibres, as defined in EU Directive 2008/100/EC (amending 90/496/EEC). The Directive 90/496/EEC will be replaced by Regulation (EU) 1169/2011 from 13 December 2014 on.

Nutritional information: US

All values are average values expressed per 100 g commercial product.

Carbohydrates	97[1)]	Protein	negligible
Sugars	8	Fat	negligible
Dietary Fibre[2)]	89		
Caloric value[3)]	166 kcal / 693 kJ	Vitamins and Minerals	negligible

1) including dietary fibre
2) measured by AOAC method 997.08
3) based on a caloric value of 1.5 kcal/g for inulin and oligofructose.

Nutritional information: other countries

For caloric values national legislation is applicable. In general, the caloric value for inulin is within a range between 1 and 1.5 kcal/g and for oligofructose 1.5 kcal/g as confirmed by a number of national authorities.

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Information

Other Information (see also our Technical Brochures)

DP ratio	DP3-9: (50±10)% (HPAEC) DP≥10: (50±10)% (HPAEC)
Aspect*	Fine, white to slightly yellow powder
Behaviour*	Hygroscopic
Taste*	Slightly sweet (~25% comp. to sucrose), without aftertaste
Solubility in water*	About 5% at room temperature
Dispersibility in water*	See document "Solubility and Dispersibility Characteristics of Orafti Powdered Products"
Properties and Applications	See our Technical Brochures.
Particle Sizes*	See document « particle sizes »
Density*	Approx. (600 +/- 50) g/l
Proposed labeling in ingredient list	«Oligofructose enriched Inulin» or «inulin, oligofructose»
Safety	Safe. Not toxic. Not dangerous. Is, like other fine powders, when mixed with air and ignited, capable of causing an explosion.
Further information	Excessive consumption may cause laxative effects.
Packaging	Paper bags 25 kg on pallets 1000 kg.
Optimal storage conditions	Cool (<25°C) and dry (<60%RH), in its original airtight packaging.
Shelf life	3 years from date of production (see also best before date on packaging label and CoA) Guaranteed: minimum 18 months upon delivery from production plant
Transport conditions	According to document 'Transport Conditions'

Kosher	Certified, Orthodox Union
Halal	Certified, Halal Feed and Food Inspection Authority (Belgium)
Plant origin	Suitable for vegetarians & vegans
Produced by	BENEO-Orafti - address below

Represented by :

To the best of our knowledge, the information in this product sheet is reliable.
(*) Indicative values listed under "Other information" on this page cannot be the basis for complaints, unless a specific requirement from the customer has been agreed by BENEO-Orafti on one of these values.

BENEO-Orafti - Aandorenstraat 1, B - 3300 Tienen Belgium - Tel +32 (0)16 801 301 - Fax +32 (0)16 801 308 - info@BENEO-Orafti.com - www.BENEO-Orafti.com

PDB 20222 en
release 16, 15.06.15

AGENAMALT 20.222 PRODUCT DATA SHEET
Maltodextrin DE 19

GENERAL DESCRIPTION

- AGENAMALT 20.222 is a spray-dried saccharification product.
- AGENAMALT 20.222 is a free-flowing, slightly hygroscopic powder that solves readily in water and consists of a special carbohydrate composition.

• Appearance:	fine, white powder
• Odour:	neutral, pure
• Taste:	slightly sweet

NUTRITIONAL VALUES (in g/100 g product)

• Engery:	1620 kJ
• Fat:	0
thereof saturated fatty acids:	0
• Carbohydrates:	95
thereof sugars:	9
• Dietary fibres:	0
• Protein:	0
• Salt:	< 0,10
• Bread units:	approx. 7,9

ANALYSIS DATA

• Dry substance:	min. 95,0 %
• pH-value:	4,0 – 5,5
• DE-value (Dextrose Equivalent):	18,0 – 20,0 % in d.s. (in dry substance)
• Bulk density loose:	450 – 550 g/l
• Sulphate ash:	approx. 0,2 % in d.s.
• Carbohydrate composition (HPLC):	
Glucose:	approx. 3,5 % in d.s.
Maltose:	approx. 5,5 % in d.s.
Maltotriose:	approx. 6,5 % in d.s.
Oligosaccharides:	rest
• Microbiology:	
Total plate count:	max. 1.000/g
Coliforms:	negative/g
E.coli:	negative/g
Yeasts:	max. 10/g
Moulds:	max. 10/g
Salmonella:	negative/250 g

STORAGE AND MINIMUM SHELF-LIFE
- If properly stored under dry conditions (max. 70 % relative humidity): min. 60 months

PACKING
- In multiply paper-bags with PE-lining of 25 kg (1 pallet = 30 bags = 750 kg)
- Big-Bags on request (400 kg, 550 kg, 600 kg, 800 kg)

CUSTOMS TARIFF NUMBER
- 1702 9050

FOOD LEGISLATION (AUSTRIA)
- AGENAMALT 20.222 is a foodstuff and corresponds to the current regulations in the European Union as well as in Austria.
- Designation within a list of ingredients according to VO 1169/2011 idgF: "Maltodextrin".

PROPERTIES AND APPLICATIONS
- Particularly because of its excellent solubility in water, its neutral taste and its good digestibility AGENAMALT 20.222 is used in a great variety of food products.

- Typical applications:

• Flavours:	carrier for spray-dried and blended fruit flavours
Baby-food:	easily digestible carbohydrate
Spices:	neutral carrier
Clear dry soups:	improvement of free flowing properties and dosage rate
Sweet products:	reduces the sweetness, reduces the tendency of saccharose solutions to crystallize