An investigation into the properties of non-digestible carbohydrates that selectively promote colonic propionate production.

Hannah Charlotte Harris
BSc (Hons), MSc

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

School of Medicine, Dentistry and Nursing
College of Medical Veterinary and Life Sciences (MVLS),
University of Glasgow

June 2016

Abstract

Short chain fatty acids (SCFA), including propionate, are produced by the bacterial fermentation of carbohydrates in the colon. Propionate has many potential roles in health, including inhibiting cholesterol synthesis, *de novo* lipogenesis and increasing satiety. The profile of SCFA produced is determined by both the substrate available and the bacteria present and may be influenced by environmental conditions within the lumen of the colon.

Whilst it may be beneficial to increase colonic propionate production, dietary strategies to achieve this are unproven. Adding propionate to food leads to poorer organoleptic properties, and oral propionate is absorbed in the small intestine. The optimum way to selectively increase colonic propionate would be to select fermentable carbohydrates that selectively promote propionate production. To date, few studies have undertaken a systematic assessment of the factors leading to increased colonic propionate production making the selection of propiogenic carbohydrates challenging.

The aim of this thesis was to identify the best carbohydrates for selectively increasing propionate production, and to explore the factors which control propionate production. This work started with a systematic review of the literature for evidence of candidate carbohydrates, which led to a screen of 'propiogenic' substrates using *in vitro* batch fermentations and mechanistic analysis of the impact of pH, bond linkage and orientation using a range of sugars, polysaccharides and fibre sources.

A new unit for SCFA production was developed to allow comparison of results from *in vitro* studies encompassing a range different methodologies found in the literature. The systematic review found that rhamnose yielded the highest rate and proportion of propionate production whereas, for polysaccharides, β-glucan ranked highest for rate and guar gum ranked highest for molar production, but this was not replicated across all studies. Thus, no single NDC was established as highly propiogenic. Some substrates appeared more propiogenic than others and when these were screened *in vitro.* Laminarin, and other β-glucans ranked highest for propionate production. Legume fibre and mycoprotein fibre were also propiogenic. A full complement of glucose disaccharides was tested to examine the role glycosidic bond orientation and position on propionate production. Of the glucose disaccharides tested, β(1-4) bonding was associated with

increased proportion of propionate and α(1-1) and β(1-4) increased the rate and proportion of butyrate production.

In conclusion, it appears that for fibre to affect satiety, high intakes of fibre are needed, and which a major mechanism is thought to occur via propionate. Within this thesis, it was identified that rather than selecting specific fibres, increasing overall intakes of highly fermentable carbohydrates is as effective at increasing propionate production. Selecting carbohydrates with beta-bonding, particularly laminarin and other β(1-4) fermentable carbohydrates leads to marginal increases in propionate production. Compared with targeted delivery of propionate to the colon, fermentable carbohydrates examined in this thesis have lesser and variable effects on propionate production. A more complete understanding of the impact of bond configurations in polysaccharides, rather than disaccharides, may help selection or design of dietary carbohydrates which selectively promote colonic propionate production substrates for inclusion in functional foods. Overall, this study has concluded that few substrates are selectively propiogenic and the evidence suggests that similar changes in propionate production may be achieved by modest changes in dietary fibre intake.

Table of Contents

List of Tables

List of Figures

List of Accompanying material

Appendix 1- Participant information sheet

Appendix 2- Participant consent form

Acknowledgement

Thank you to all the stool donors, which have allowed me to have a PhD.

I would like to acknowledge BBSRC DRINC for funding me to do this project.

I would like to thank Prof Christine Edwards and Dr Douglas Morrison for offering me the opportunity to do this PhD, and the time, patience, support, and encouragement you have given me along the way.

Thank you to Douglas for all of the guidance as well as technical help with the GC: FID throughout this project.

I would like to thank Christine for all of the help and support, not only with the academic side of things but also personal aspects during this PhD. Without this, I would not have been able to complete this process; I will always appreciate the help and support you have given me over the last couple of years.

Thank you to all the students and staff in the nutrition department who have helped make this an enjoyable time. To 'the Greeks' who have tried in vain to teach me Greek, Majid for feeding me copious amounts of food, Hani, Michael, and in particular, Mhairi all of whom have listened to me moan and helped me to keep sane!

Thank you to my friends and family for being there no matter what- even though you all think I spend my days playing with poo!

Thank you to Chris, for being there during the good, the bad, and the boring!

Publications

Molecular determinants of short chain fatty acid production: influence of glycosidic bond configuration. H Harris, C.A Edwards, D.J. Morrison. Proceedings of the nutrition society (2015)

Standardisation of Units for Short Chain Fatty Acid Production by *in vitro* fermentation. H Harris, C.A Edwards, D.J. Morrison. Proceedings of the nutrition society (2014)

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature _____

Printed name _____

Definitions/Abbreviations

AUC	Area under the curve
BCFA	Branched chain fatty acids
BMI	Body mass index
C2	Acetate
C3	Propionate
C4	Butyrate
CAZyme	Carbohydrate active enzyme
CB1	Cyclin B1
CBMs	Carbohydrate-binding molecules
CCK	Cholecystokinin
CE	Carbohydrate esterases
CHD	Coronary heart disease
Cmax	Maximal concentration
CRP	Chicory root pulp
CVD	Cardiovascular disease
CV%	Coefficience of variance
DF	Dietary fibre
dH_2O	Distilled water
DP	Degree of polymerisation
DW	Dry weight
EMP	Embden-Meyerhof-Parnas
FFA	Free fatty acid
FFAR	Free fatty acid receptor
FISH	Fluorescent *in situ* hybridisation
FOS	Fructooligosaccharide
GC: FID	Gas Chromatography: Flame Ionising Detector
GH	Glycoside hydrolases
GIP	Glucose-dependent insulinotropic polypeptide
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide-1
GOS	Galactooligosaccharide
GPCR	G protein coupled receptor

GT	Glycosyl Transferases
HDACi	Histone deacetylate inhibitor
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
IBD	Inflammatory bowel disease
IPE 0.8	Inulin propionate ester degree of polymerisation 0.8
IGN	Intestinal gluconeogenesis
IQR	Interquartile range
kDa	Kilodaltons
LC-AX	Long-chain arabinoxylan
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
mmol/g CHO/ day	mmol/g carbohydrate / day
mmol/g CHO/ hour	mmol/g carbohydrate / hour
MVLS	Medical, veterinary and life sciences
MOS	Mannooligosaccharide(s)
MW	Molecular weight
NA	Not applicable
Na- Propionate	Sodium propionate
NDC	Non-digestible Carbohydrate
NE	No effect
NI	Not included
OF	Oligofructose
OPA	Orthophosphoric acid
OXM	Oxyntomodulin
PA	Propionic acidaemia
PBMC	Peripheral blood mononuclear cells
PCC	Propionyl-CoA carboxylase
PDX	Polydextrose
PMN	Polymorphonuclear
PolyFermS	Polyfermenter intestinal model system
PP	Pancreatic polypeptide
PUL	Polysaccharide utilisation loci
PYY	Peptide YY

qPCR	Quantitative real time PCR
RS	Resistant starch
SACN	Scientific advisory committee on nutrition
SA: V	Surface area: volume ratio
SCFA	Short Chain Fatty Acid
SD	Standard deviation
SEM	Standard error of the mean
SHIME	Simulator of the human intestinal microbial system
SI	Small intestine
SUS	Starch utilisation system
T2D	Type two diabetes
TAG	Triglyceride
THF	Tetrahydrofolate (FH_4)
TIM	TNO intestinal model
UK	United Kingdom
USA	United States of America
VIP	Vasoactive Intestinal Peptide
WW	Wet weight

Chapter 1 Literature Review

1.1 Background

The considerable variety of foods in the human diet gives rise to a number of bioactive molecules which impact upon health. One example of a food component that varies considerably between diets is dietary fibre which encompasses a range of different non digestible carbohydrates (NDC). Dietary fibres have been associated with reduction in the risk of type 2 diabetes (T2D), total cholesterol and body mass index (BMI) (de Munter et al., 2007, Consortium, 2015). The mechanisms of these effects may differ between types of NDC and have not been fully elucidated. The inability of the enzymes in the small intestine (SI) to digest NDC, and their fermentation by the colonic bacteria to produce short chain fatty acids (SCFA) indicate that bacterial products may play a crucial role.

Comprehension of the complex colonic bacterial ecosystem, has accelerated in recent years, particularly with the shift away from culture dependent techniques to those that are more DNA/RNA based. For example, Walker's group considered the effects of pH on bacterial populations *in vitro* using FISH which is dependent on matching DNA or RNA with bacterial probes (Walker et al., 2005). Qin and colleagues used metagenomics to investigate the variety of the bacteria in different people (Qin et al., 2010) and David et al considered the impact of short term dietary changes on the bacterial populations also using metagenomics (David et al., 2014).

This transition in methodology has provided greatly improved knowledge of the types of bacteria and enzymes that are present in the colon, but more limited information on their active functionality. Metagenomic analysis provides information on the capacity, but not the actual activity of the enzymes present, whereas transcriptomics provides information on mRNA expression. However, as transcriptomics is often performed on stool samples, it may be misleading and not representative of events in the proximal colon, where most of the metabolism occurs (Kinross et al., 2011, David et al., 2014). Metabolomics, which can be performed with range of possible techniques including NMR and GC-MS, scans the metabolites in samples of blood (Wikoff et al., 2009), urine or faeces (Yap et al., 2008) and can help unravel the overall impact of different diets on bacterial metabolism. These methods generate large amounts of data which require complex bioinformatics and rely on metabolite pattern comparison rather than quantification of fermentation products (Kinross et al., 2011).

Another approach is targeted metabolomics where specific types of bacterial products are studied, a key example being short chain fatty acids (SCFA). Acetate, propionate, and butyrate are the main SCFA produced in the colon. These are key bacterial metabolites which have several possible down-stream roles in human health, including effects on colonic enterocytes, liver metabolism, inflammation and energy metabolism (den Besten et al., 2013b, Wong et al., 2006). The main focus of this thesis is on the production of propionate. Propionate has been reported to influence cholesterol production (Wolever et al., 1991, den Besten et al., 2013a), gluconeogenesis (De Vadder et al., 2015) and activates colonic free fatty acid receptors (FFAR) associated with increased expression of the satiety hormones; peptide YY (PYY) and glucagon like peptide-1 (GLP-1) (Le Poul et al., 2003, Lin et al., 2012). Recently, direct administration of high levels of propionate into the colon using a synthetic ester vehicle produced many of these effects, and inhibited body weight gain, and reduced plasma cholesterol levels (Chambers et al., 2014). These studies will be discussed in detail later in this introduction.

SCFA production can be differentially manipulated by feeding non-digestible carbohydrates (NDC) which differ in their physicochemical properties. For example, glucans containing beta glycosidic bonds had higher propionate production compared with those with predominantly alpha bonds (Hughes et al., 2008, Laurentin and Edwards, 2004). Due to a lack of mechanistic analysis, the specific drivers leading to the production of propionate are not known, making selection of substrates for selectively increasing propionate production difficult. Understanding the determinants of propionate production would enable the development of strategies to increase the amount of propionate produced in the colon or its release from synthetic vehicles such as that used by Chambers and colleagues. This thesis describes a series of studies to elucidate the factors that increase propionate production and strategies for enhancing colonic propionate levels in humans.

1.2 The colonic ecosystem

The colon was previously considered to be an organ solely used for the absorption of water, salts, and nutrients and the formation of stool to remove waste. The colon is however much more than this, it is a diverse ecosystem which is linked to many aspects of and disturbances in physiology ranging from obesity, (Ley et al., 2006) and immunological disorders (Maslowski et al., 2009b) to autism (Mulle et al., 2013). The colonic bacterial

ecosystem is also capable of a wide range of metabolic processes many of which remain to be fully explored.

The activity and possible influences of the colonic bacterial ecosystem have been studied for several decades but research has been hampered by the difficulties in growing many fastidiously anaerobic bacteria with complex growth requirements. However, the field has been revolutionised by the development of culture independent techniques with the rapid, and now affordable sequencing of bacteria using next generation sequencing (NGS) and complex bioinformatics. This highly detailed analysis, which does not require growth and isolation of individual bacteria, has shed intense light onto the complexities of the human microbiome. For example, approximately 3 million bacterial genes being identified, (Qin et al., 2010) however the active functionality of these bacteria and how their metabolism can be modified remain relatively unclear.

In the MetaHIT study of stool samples collected from individuals across Europe, it was reported that an individual colon contains approximately 160 out of over 1000 possible species of colonic microbiota, of which Bacteroidetes and Firmicutes were the most prevalent. The functions of the genes identified were also assessed and after the housekeeping genes , the second key group encoded for enzymes involved in the utilisation of NDC (e.g. pectin and sorbitol and glycans) (Qin et al., 2010). Bacteria differ greatly in the enzymes they express to ferment dietary components and their capabilities to use different substrates (discussed below) (Martens et al., 2011). This is beneficial as humans are unable to digest NDC using their own enzymes. Thus there is a symbiotic relationship between humans and their commensal bacteria. The humans gain a range of bioactive molecules including SCFA, and extra energy from substrates they cannot salvage themselves; the bacteria gain a suitable living environment with a regular source of energy and nutrients. As there are many possible variations in the composition of the microbiome of an individual, there are large differences in the composition of the colonic bacterial ecosystem between individuals. Factors such as age, body mass index, and diet have all been associated with differences in the bacterial composition. However, approximately 90% of the ecosystem is made up of a core set of 57 different bacterial species, suggesting that the foundation of the gut microbiome is relatively conserved (Qin et al., 2010) with five dominant phyla of bacteria. Of these, the vast majority belong to the phyla Bacteroidetes or Firmicutes and the remaining are Actinobacteria, Proteobacteria and Verrucobacteria. At genus level; *Bacteroides, Prevotella, Ruminococcus, Lactobacillus,*

and *Bifidobacterium* are also highly abundant within the colon and are associated with the increased production of SCFA (Eckburg et al., 2005, Arumugam et al., 2011, Qin et al., 2010, Le Chatelier et al., 2013).

1.3 Fermentation and the production of SCFA

The colonic microbiota obtains energy from substrates such as NDC and proteins by fermenting them to SCFA, branched chain fatty acids (BCFA, isovalerate, isocaproate, isoleucine), carbon dioxide, methane, and hydrogen, as well as secondary products such as ethanol, succinate, and lactate which can be further utilised by other bacteria for SCFA production (Reichardt et al., 2014, Wong et al., 2006). BCFA are isomers of SCFA formed by the fermentation of amino acids with valine fermentation producing isobutyrate, leucine forming isovalerate as well as isocaproate, and isoleucine forming 2-methylbutyrate (Windey et al., 2012).

There are two main types of colonic fermentation; saccharolytic and proteolytic. Saccharolytic fermentation is the most common type occurring in the colon (particularly in the proximal region), and occurs when there is an abundance of sugars, mostly contained within NDC and produces mostly SCFA as reduced products. Proteolytic fermentation occurs mainly in the distal colon when there is a scarcity of sugars for saccharolytic fermentation as they have been utilised in the proximal colon. Proteolytic fermentation of proteins, peptides, and glycoproteins leads to the production of BCFA, phenols, and amines. Proteolytic fermentation produces some SCFA, but the majority of acetate, propionate, and butyrate is produced by saccharolytic bacteria.

Smith and Macfarlane., (1997) conduced *in vitro* fermentations of amino acids and observed that the fermentation of 10 mmol/l valine fermentation generated 2.9 mmol/l of isobutyrate whereas fermentation of 10 mmol/l leucine yielded 1.2 mmol/l of isovalerate (Smith and Macfarlane, 1997). Mortensen et al., (1990) carried out similar analysis and found that the fermentation of valine alone (100 mmol/l) generated 23.2 mmol/l of isobutyrate, and leucine produced 15.6 mmol/l of isovalerate. In contrast, when 100 mmol/l of lactulose was fermented low concentrations of BCFA were produced with 4.0 mmol/l isobutyrate and 1.9 mmol/l of isovalerate which was lower than that of acetate, propionate and butyrate (679.2 mmol/l, 93.2 mmol/l, and 27.7 mmol/l). This demonstrates

that BCFA production by NDC fermentation is low or that the BCFA are rapidly used by the bacteria fermenting the carbohydrate.

1.4 Extent of SCFA production

SCFA are carboxylic acids consisting of 1 to 6 carbons with acetate (C2), propionate (C3), and butyrate (C4) being produced in the highest concentrations by colonic fermentation (Figure 1-1). Less prominent SCFA include; formate (C1), valerate (C5), caproate (C6) and the BCFA; isobutyrate (iC4), isovalerate (iC5), and isocaproate (iC6).

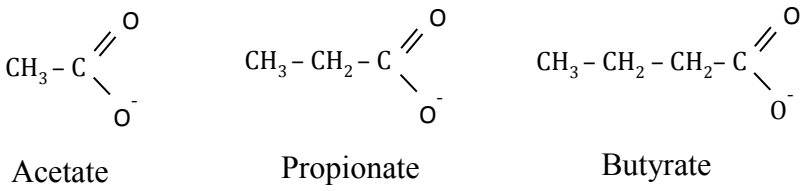

Figure 1-1: The structure of the carboxylic acids; acetate, propionate, and butyrate

The majority of SCFA production occurs in the proximal colon and decreases distally. As most of the SCFA are rapidly absorbed (95% of production) with absorption rate increasing as SCFA concentrations increase, quantification of colonic SCFA production is challenging (Wong et al., 2006, Fleming et al., 1991, Hadjiagapiou et al., 2000, Rechkemmer and von Engelhardt, 1988). The majority of SCFA in the colon (pH range of 6.7 - 5.4) are in the deprotonated anionic form as SCFA have a pKa of 4.8 (Sellin, 1999, Fallingborg et al., 1989). Therefore they are absorbed either by the Na^+/ H^+ exchanger and SCFA-bicarbonate antiporters which can be Na^+ dependent or independent (Tyagi et al., 2002), or transporters, such as the monocarboxylate transporter 1 and SCL5A8 transporters (Hadjiagapiou et al., 2000). In contrast, SCFA in the protonated form (acetic acid) enter the colonic epithelial cells by diffusion (Sellin, 1999).

SCFA within the colon can also act as ligands for free fatty acid receptors (FFAR) located on the luminal epithelium of the colon, as well activating FFAR located in various sites around the body (see Section 1.6.3).

Direct estimation of the colonic production of SCFA is difficult due to the inaccessibility of the proximal colon and rapid absorption of the SCFA. Rectal infusion studies have shown that the rate of absorption increases as the chain length decreases so faecal concentrations of SCFA are unlikely to represent the actual SCFA produced (Vogt and Wolever, 2003).

This disparity in faecal and caecal SCFA has been shown in studies in rats, where faecal SCFA concentrations (umoles/g dry weight) were approximately 80% less than observed in the caecum (Edwards and Eastwood, 1992). Investigations of colonic SCFA are often conducted in animals, and two studies have directly measured colonic SCFA in sudden death patients (Cummings et al., 1987, Macfarlane et al., 1992). The molar proportions of SCFA varied little between different colonic regions, however, SCFA concentration reduced along the colon with 26.7 mmol/kg in the proximal colon to 14.2 mmol/kg at the distal colon (Cummings et al., 1987). In the later study from the same group (Macfarlane et al., 1992) the colonic SCFA were compared between a methanogenic sudden death victim and another who did not produce methane. The SCFA in the caecum were 8 fold higher than in the distal colon. In the methanogenic colon, the methanogenic bacteria increased towards the distal colon, and in the non-methanogenic colon, they were replaced with sulphate reducing bacteria. Thus the faecal samples may have much less SCFA and a different bacterial profile to proximal colon even in humans, due to differences in absorption and substrate availability.

Cummings et al., (1987) estimated that the colonic concentration of SCFA is approximately 80-150mmol/kg with acetate, propionate, and butyrate being produced in the approximate ratio; 60:20:20 although this can be altered with dietary modifications (Cummings et al., 1987). Modifications in the ratio of SCFA have been observed *in vitro* by Laurentin and Edwards., (2004) where pyrodextrinised starch increased the proportion of propionate at the expense of acetate and the feeding trial by Vogt et al., (2004) where consumption of 25g rhamnose increased serum proportions of propionate.

Recent measurements of faecal SCFA concentrations in adults confirm a similar mean ratio, but indicate significant variation between individuals and studies (Verbeke et al., 2015). After absorption in the colon, the SCFA are transported to the liver along the portal vein where portal blood concentrations of 262.8 μM acetate and 30 μM of propionate and

butyrate have been observed (Bloemen et al., 2009). Acetate is not preferentially used in the gut or liver and is found at the highest concentrations in the peripheral blood of 70-150μM (compared to propionate and butyrate). In contrast, propionate is removed by the liver, where it is involved in functions such as gluconeogenesis (see below), resulting in approximately 4-6 μM in the peripheral blood. Butyrate in comparison is largely utilised as a fuel at the colonic epithelium and also undergoes uptake in the liver, resulting in peripheral concentrations of approximately 2-4 μM (Cummings et al., 1987, Bloemen et al., 2009).

1.5 SCFA functionality

1.5.1 Luminal functions

SCFA have a plethora of roles within the colonic lumen before undergoing rapid absorption. SCFA are weak acids with a pKa of ~4.8, and increased concentrations reduce the pH of the lumen. In a study where healthy volunteers ingested a pH measuring capsule the distal small intestine had a pH of ~7, the caecum, ascending and transverse colon had a pH of ~5.6, and the pH rose to 6.5 in the descending and sigmoid colon (Fallingborg et al., 1989). However, these subjects were fasted and so this may not take into account SCFA production, which would promote more acidic colonic pH (Fallingborg et al., 1989). A reduced luminal pH has been shown to be protective against pathogenic bacteria such as *Clostridium perfringens* which are unable to survive at a pH below pH 5.0 (Wang and Gibson, 1993).

SCFA have also been shown to have potential beneficial roles in the inhibition of secondary bile acid formation. Fadden et al., (1997), conducted batch fermentations with lactulose and wheat bran and assessed impact on concentrations of deoxycholic acid and lithocholic acids, both of which are formed by the 7α-dehydroxylation of primary bile acids. Fermentation of lactulose reduced the pH to less than 5.5 preventing the formation of the secondary bile acids, whereas this did not occur with wheat bran which was not well fermented (Fadden et al., 1997). Christl et al., (1995) assessed the effect of starch fermentation on the same secondary products, but controlled the pH at 6 or 7 (Christl et al., 1995). Here, fermentation of starch at pH 6 led to reduced concentrations of deoxycholic (35%) and lithocholic acid (31%) compared with fermentation at pH 7 for 24 hours. During fermentation of starch, SCFA concentrations increased compared to the control

(Christl et al., 1995). This suggests that the reduced pH occurring by starch fermentation prevented the production of secondary bile acids via the inhibition of the bacterial enzyme, 7α-dehydroxylase, which is present in bacteria such as Clostridial cluster XVIa (Hofmann, 1999). Populations of Clostridial cluster XVIa have been shown to be affected by diet. For example, in a human feeding trial where African Americans consumed an 'African' diet (low fat- high fibre), and Africans consumed an 'American' diet (high fat- low fibre) for 2 weeks. The switch from an African to an 'American' diet, was associated with increased prevalence of Clostridial cluster XVIa bacteria, a 400% increase in secondary bile acid formation and increases in bile acid conjugators, deoxycholic and lithocholic acid. This high fibre diet, like in the study of Christl et al., (1995), was also associated with increased SCFA production. This is further evidence that reductions in secondary bile acid synthesis are associated with increased SCFA production, thus indicating the impact of diet on bile acid production (O'Keefe et al., 2015).

SCFA also alter colonic motility with acetate, propionate and butyrate having slightly different effects. Squires et al., (1992) used colonic explants from the rat and infused with them with 100 mM of a 'SCFA cocktail' of acetate, propionate and butyrate, or varying concentrations of each SCFA alone (10 mM and 100 mM). Contractile motility was reduced throughout the colon with the SCFA cocktail. Motility of the proximal and middle colon were also reduced when 100mM of the individual SCFA were infused, for example motility in the proximal and distal colon was 17.3 and 5.0 contractions per 20 minutes whereas for the control this was 37.2 and 25.8 contractions per 20 minutes. These effects were dose dependent and butyrate was more effective in the caecum whereas acetate and propionate had greater effects than butyrate in the more distal colon (Squires et al., 1992).

A more recent investigation of guinea pig colon perfused with 0.1 ml / min buffer containing 10 to 30 mM of acetate, propionate or butyrate reported similar results. The SCFA had different effects of motility in the colon where butyrate dose dependently increased the number of contractile propagations (indicating propulsion), but reduced the number of non-propagating contractions (indicating mixing). In contrast propionate and to a lesser extent acetate reduced contractile propagations but increased the number of non-propagating contractions. This indicates that butyrate may be associated with propulsion and propionate is involved in mixing within the colon (Hurst et al., 2014). SCFA also reduced motility in the electrically stimulated rat distal colon, and the guinea pig terminal ileum which was bathed in a solution of acetate, propionate or butyrate. SCFA dose

dependently reduced the contractions of the rat distal colon (0.3 mM to 100 mM) and concentrations of 100 mM reduced peristalsis of the guinea pig ileum. To test if these effects were associated with the colonic receptors FFAR2, the distal colon of FFAR2 knock out mice was electrically stimulated in the presence of SCFA. Colonic motility was not altered in knock out mice, indicating that the role of SCFA on gut motility is independent of the receptor FFAR2 and reduces motility by other mechanisms within the colonic lumen (Dass et al., 2007).

This is contradictory to the study by Jouët et al., (2013) in humans where the effect of SCFA infusion on motility in the colon was measured with 7 manometric channels connected to pressure transducers and a barostatic bag which was swallowed by healthy individuals. Individuals were given an infusion containing 75 mM of acetate, propionate, and butyrate at pH 7 and pH 4.5 directly into the colon, as well as saline at both pHs and observed no effect on colonic motility. However, there was no reported preparation of the bowel, or prior fasting, therefore it is possible that there may have been existing colonic SCFA prior to infusion, masking any effects (Jouet et al., 2013). This indicates that it may be difficult to extrapolate from the controlled *in vitro* animal gut assessment to the *in vivo* response in humans due to the high concentrations used *in vitro* and the reduced control of human studies.

Individually, SCFA have an assortment of functions occurring in the lumen, within the colonic epithelial cell layer, as well as post absorption. The individual roles of the SCFA are discussed below.

1.5.2 Acetate

Approximately 60% of SCFA produced is acetate, which has little uptake in the liver. The remainder of the acetate produced enters the periphery, and acetate is the only SCFA that reaches the systemic blood in easily measurable concentrations (Bloemen et al., 2009).

When in the cell cytoplasm acetate, via acetyl- CoA, can be used for fatty acid (FA) and cholesterol synthesis. Acetate is the preferential substrate for lipogenesis in the colonocytes (compared to butyrate) (Zambell et al., 2003). Rectal infusion studies in healthy adults have also observed inhibition of acetate incorporation into FA in the presence of propionate, where 6mM Na-propionate led to a 30% reduction in plasma FA

concentrations when compared to acetate without propionate infusion (Wolever et al., 1995). Wolever and colleagues used rectal infusion studies in healthy individuals and demonstrated that propionate mediated suppression of cholesterol synthesis occurs as a result of the inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) (Wolever et al., 1991, Wolever et al., 1995). The ability of propionate to oppose the effect of acetate was also demonstrated *in vitro* using isolated rat hepatocytes, in which the effect of propionate dose (0.1-25 mM) on ^{14}C acetate, $^{3}H_2O$ and 1-^{14}C Mevalonate (marker of the HMG-CoA pathway) mediated production of FA and cholesterol. Cholesterol synthesis was dose dependently reduced from 2.5mM for all precursors tested. In contrast, FA synthesis via acetate was only dose dependently decreased by propionate (2.5 mM) FA synthesis by $^{3}H_2O$ and 1-^{14}C Mevalonate were not altered (Wright et al., 1990). These studies therefore indicate the potential importance of the acetate to propionate ratio on the regulation of FA and cholesterol synthesis.

Acetate also has a selection of potential different tissue specific effects including being able to cross the blood-brain barrier to reach the hippocampus. This was seen in the investigation by Frost et al., (2014) where an intraperitoneal injection of ^{11}C- acetate stimulated the arcuate nucleus and labelled acetate accumulated in the hypothalamus, but not in other areas of the brain. Within this study it was also observed that pro-opiomelanocortin expression (anorectic hormone) increased and agouti-related peptide expression (orexigenic) decreased which would indicate that acetate could potentially have FFAR2 independent effects on satiety (see below)(Frost et al., 2014).

Acetate is also involved in the inhibition of lipolysis in mouse adipocytes, and is possibly due to the activation of FFAR2 as this was attenuated in an FFAR3 knock out mouse model (Ge et al., 2008). This is interesting as FFAR3, which is closely related to FFAR2 (see below), is also activated by acetate but has been shown to increase expression of leptin (Xiong et al., 2004) which is associated with increased lipolysis (Zeng et al., 2015). Acetate is also implicated in beta-cell function where in the recent study by Tang et al., (2015) acetate dose dependently (0.1-1 mM) inhibited insulin secretion occurring after GLP-1 exposure. These effects were not present with beta-cells from FFAR2$^{-/-}$FFAR3$^{-/-}$ knockout mice, suggesting a role for these receptors in these acetate mediated roles in beta-cell function (Tang et al., 2015).

1.5.3 Propionate

Approximately 90% or more of propionate reaching the liver via the portal vein is utilised in the liver, with the remainder reaching the peripheral tissues (Peters et al., 1992, Bloemen et al., 2009). As discussed previously, propionate inhibits the uptake into the liver of colonic acetate for the production of cholesterol, reducing the rate of cholesterol synthesis. Propionate and butyrate are also involved in the regulation of insulin-stimulated and basal lipogenesis. Heimann et al., (2015) demonstrated that propionate and butyrate inhibited basal, and insulin mediated lipogenesis, as well as increasing insulin sensitivity and basal glucose uptake in rat adipocytes. The insulin mediated effects were detected with 1 mM of propionate whereas for basal effects 10 mM of propionate was required, suggesting increased sensitivity to propionate in the presence of insulin (Heimann et al., 2015).

Propionate, as well as regulating cholesterol and lipid production, can contribute carbon to the *de novo* synthesis of glucose - gluconeogenesis. Gluconeogenesis occurs once propionate enters the citric acid cycle as succinyl-CoA (Wiltrout and Satter, 1972, den Besten et al., 2013a) and is particularly important in ruminants that obtain a high proportion of glucose by hepatic gluconeogenesis. For example, in lactating cows approximately 45% of all glucose was produced directly from propionate (Wiltrout and Satter, 1972). Hepatic and intestinal gluconeogenesis (IGN) are also important in man for generating glucose in the fasted state, or during diets that are low in carbohydrate, and occurs when propionate is converted to succinate (Nuttall et al., 2008, Veldhorst et al., 2009). Unlike hepatic gluconeogenesis, IGN is linked to the activation of FFAR3. FFAR3 activation generates the release of vasoactive intestinal peptide (VIP), a neurotransmitter involved in signalling from gut epithelial cells to the brain. This then leads to the activation of glucose-6-phosphatase, resulting in the *de novo* production of glucose (De Vadder et al., 2015). Colonic butyrate is also involved in gluconeogenesis by releasing cAMP, activating the expression of IGN genes, (propionate does this via FFAR3) which then leads to IGN, with propionate being utilised as a substrate (De Vadder et al., 2015).

IGN and the downstream metabolic effects have also been investigated in IGN knockout mice (*I-G6pc*$^{-/-}$ mice). De Vadder et al., (2015) fed these mice a variety of diets and observed a reduced tolerance of glucose and insulin compared to wild type controls, particularly after a propionate enriched diet. It was also observed that these knock-out mice

had increased weight gain compared to the wild type, although there was no change in food consumption (De Vadder et al., 2015). IGN was also observed to have an effect on weight gain in an earlier investigation by De Vadder et al., (2014) where *I-G6pc*$^{-/-}$ and wild type mice were fed a high fat/high sugar diet with or without fructooligosaccharide (FOS, a fermentable carbohydrate). It was observed that the *I-G6pc*$^{-/-}$ mice had greater body weight gain and reduced glucose clearance compared to the wild type mice after feeding of both diets (De Vadder et al., 2014). Similar effects were also observed by Penhoat et al., (2011) who fed the same knock-out mouse model (with IGN inhibition) a protein enriched diet, and observed increased food intake, and decreased body weight compared to wild type mice (Penhoat et al., 2011). This indicates that IGN may play a role in the reduction of weight gain and increased glucose tolerance that has been identified after consumption of NDC and the down-stream fermentation metabolites that are produced (De Vadder et al., 2014, De Vadder et al., 2015).

Propionate has been shown to prevent tumorigenesis *in vitro* with BaF3 cancerous cells which are present in the liver. Propionate dose dependently reduced the proliferation of these cells ($\log^{-6}$ to $\log^{-2}$ M propionate). This also occurred with butyrate and to a lesser extent acetate (Bindels et al., 2012). Propionate also has roles in the regulation of the cell cycle acting as a histone deacetylate inhibitor (HDACi), but this occurs more with butyrate (Hinnebusch et al., 2002). This is discussed below in section 1.5.4.

1.5.3.1 *Propionic acidaemia*

Although propionate is generally considered as advantageous, for some humans this is not the case as they are unable to metabolise propionate. This inability to metabolise propionate is caused by the disorder known as propionic acidaemia (PA). PA is an autosomal recessive disorder where the enzyme propionyl-CoA carboxylase (PCC) is absent, or does not function. PCC converts propionyl-CoA to methylmalonyl-CoA in the pathway leading to the citric acid cycle. PCC deficiency prevents individuals with PA from utilising BCFA, cholesterol side chains, some amino acids, and odd chain fatty acids, such as propionate leading to accumulation of propionic acid in all organs (Baumgartner et al., 2014, Grunert et al., 2013). Globally, PA effects 1:50000 to 1:100000 individuals, although this is higher in areas with a higher prevalence of incest (Zayed, 2015). PA is often diagnosed within the first week of life and is associated with neurological deficits, such as low IQ and developmental delay, gastrointestinal issues, such as vomiting and

ketoacidosis, cardiomyopathy, anaemia, and failure to thrive. Treatment for this disorder is the consumption of a low protein diet, L-carnitine, and antibiotics to reduce the gut bacteria activity (Zayed, 2015, Baumgartner et al., 2014, Grunert et al., 2013).

1.5.4 Butyrate

Butyrate is the primary energy source in the colon, where *in vitro* assessment has demonstrated that butyrate provides up to 70-80% of the energy consumed by the colonocytes (Roediger, 1980). Butyrate has several actions in the turnover of colonic cells, many of which are paradoxical. The butyrate paradox was partially explained within the study by Belcheva et al., (2014) where mice predisposed to colonic polyps and cancer were fed a high or a low carbohydrate diet. Here, low concentrations of butyrate increased cellular proliferation, and at high concentrations, butyrate did not increase cell proliferation (Belcheva et al., 2014). The regulatory effects of butyrate are likely due to the ability to act as an HDACi of which it has the highest propensity of all the SCFA (Waldecker et al., 2008a). Hinnebusch et al., (2002) observed anti-proliferative effects of propionate and butyrate by inducing cell cycle arrest via the activation of p21 in cells from the HT-29 cancerous cell line, although this translated to increased apoptosis only after butyrate exposure, not propionate exposure (Hinnebusch et al., 2002).

Butyrate has immunoregulatory functions analogous with its role as an HDACi, for example Aoyama et al., (2010) observed that 4 mM of butyrate caused neutrophil apoptosis (Aoyama et al., 2010). Butyrate, as well as propionate is connected with regulation of the immune response where it induces the production of regulatory T-cells by upregulating FOXP3 from dendritic cells (Furusawa et al., 2013, Arpaia et al., 2013). This role of butyrate has also been observed where administration reduces inflammatory markers in mouse models of colitis and in the release of regulatory T-cells after the exposure to lipopolysaccharides (LPS) (Arpaia et al., 2013).

Propionate and butyrate influence immunity and cellular proliferation independently of their role as an HDACi. When an inulin type fructan was fed to leukemic mice for 13 days, reduced expression of proinflammatory cytokines and chemokines such as IL-4, IL-8, and MCP-1 compared to the leukemic mouse on a standard diet was observed. Leukemic cells from these mice were also exposed to varying concentrations of acetate, propionate, and butyrate. This led to a dose dependent decrease in hepatic cell proliferation occurred via

free fatty acid receptor 2 (FFAR2) activation by propionate and butyrate (Bindels et al., 2012). These studies highlight the complexities regarding the regulatory roles of propionate and butyrate that occur based on their ability to function as a HDACi as well as their ability to activate FFAR2/3 (see below).

1.6 SCFA receptors in the colon

SCFA have recently been shown to act as agonists of a selection of de-orphanized G Protein coupled receptors (GPCR). There are a variety of GPCR in the colon; these include GPR109A, GPR120, GPR40, GPR41, GPR43, and olfactory receptor 78 (Olfr78). This functionality of these receptors is discussed below.

1.6.1 Medium and long chain receptors

Medium and long chain fatty acids are the natural ligands of free fatty acid receptor 1 (FFAR1; also known as GPR40) which potentially plays a role in satiety and glucose homeostasis via the expression of GLP-1 (Habib et al., 2013).

FFAR4 (GPR120) is activated by long chain fatty acids (13-21 carbons) and is expressed on a variety of cell types including: adipose, lung, and the colonic epithelium (Hirasawa et al., 2005). FFAR4 is observed to be up-regulated in obesity, high fat diets and may have an effect on appetite regulation and glucose homeostasis (Cornall et al., 2011, Habib et al., 2013), as well as increasing GLP-1 expression in mice (Habib et al., 2013, Hirasawa et al., 2005).

1.6.2 GPR109A

GPR109A is located on the colonic epithelium, particularly in the distal regions of the colon, adipose tissue, macrophages, and dendritic cells. This receptor is activated by niacin (Vitamin B3) and butyrate, and has effects within the colon (Thangaraju et al., 2009, Singh et al., 2014). In colon cancer GPR109A is downregulated, although transfection of GPR109A to the human colon cancer cell line KM12L4 has shown that the activation of the receptor leads to apoptosis of these cancerous cells. This occurred with activation by both niacin, and butyrate, although this was more apparent with butyrate. Although butyrate is an HDACi, the observed apoptosis was postulated to be as a result of blockage

of the NFκB pathway of inflammation and not due to changes in acetylation (Thangaraju et al., 2009). GPR109A also has butyrate-mediated effects on inflammation where it has been identified as being protective during colonic inflammation (Singh et al., 2014).

1.6.3 GPR43 (FFAR2) and GPR41 (FFAR3)

The SCFA receptors: GPR43 and GPR41 (FFAR2 and FFAR3) are of interest as they have roles in the mediation of satiety hormones, inflammation, and glucose homeostasis, all of which are associated with obesity. SCFA are the natural ligands for these receptors, with propionate able to activated FFAR2 with an EC_{50} of 290 μM and FFAR3 with an EC_{50} of 127 μM (Le Poul et al., 2003, Brown et al., 2003). FFAR2 and FFAR3 are only 39% similar but differ in their modes of action by having different pathways of activation (Le Poul et al., 2003, Brown et al., 2003).

All of the SCFA have the ability to activate these receptors with high ligand affinity however; the extent differs for each SCFA. Propionate has the highest affinity for both, GPR43 (FFAR2) and GPR41 (FFAR3). Ligand binding abilities of acetate, propionate, and butyrate for GPR43 (FFAR2) are in the order; propionate > acetate = butyrate, whereas for GPR41 (FFAR3) ligand binding affinities are in the order; propionate = butyrate > acetate (Le Poul et al., 2003, Nilsson et al., 2003, Brown et al., 2003).

FFAR2 and FFAR3 are located on a large selection of cells and tissues but their tissue distribution around the body is not equal (Le Poul et al., 2003). Both receptors are widely expressed throughout the body. They have been identified in the spleen, bone marrow, colon (Kaji et al., 2011), adipose, breast tissues (Le Poul et al., 2003) and pancreatic cells (Tang et al., 2015). Interestingly, these receptors are also expressed on immune cells such as peripheral blood mononuclear cells ([PBMC], monocytes, macrophages, B-lymphocytes) and polymorphonuclear cells ([PMN], mast cells, eosinophils, leukocytes and neutrophils) (Al-Lahham et al., 2012, Le Poul et al., 2003). Both receptors are expressed on these cells, however FFAR2 is associated with greater expression on immune cells compared with FFAR3 (Brown et al., 2003, Nilsson et al., 2003, Kaji et al., 2011). As these receptors are expressed on the enteroendocrine L-cells of the colon, this allows the SCFA to have receptor mediated effects prior to being absorbed from the lumen (Fig 1-2) (Karaki et al., 2008).

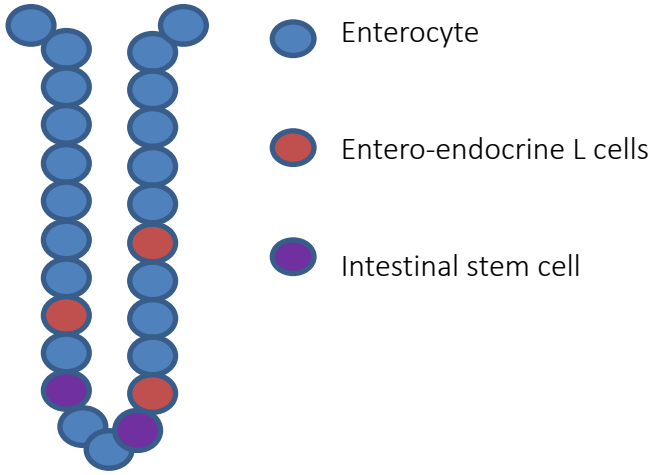

Figure 1-2: Simplistic diagram of a colonic crypt

The majority of the cells in the colonic crypt are enterocytes, and butyrate is their source of energy. Entero-endocrine L cells are less common (~1% of the cells in the colon) and more sporadically located within the colonic crypt. Within these L –cells, the receptors FFAR2 and FFAR3 are located and are involved in the expression of satiety hormones such as; GLP-1 and PYY (Karaki et al., 2008, Tolhurst et al., 2012)

As well as activation by different ligands, in different locations within the body, the expression of these receptors can be manipulated by the diet and body mass. This occurs as dietary fibre consumption increases SCFA production, and different NDCs generate different proportions of SCFA. For example, FOS consumption is associated with increased SCFA production, leading to increased FFAR2 expression. This was demonstrated in the *ex vivo* experiment by Kaji et al., (2011) where a 5% FOS diet was fed to mice for 28 days. The consumption of FOS increased the density of FFAR2 by 300% in the proximal colon and 50% in the distal colon of the mice compared to the control. This indicated that that increased consumption of NDC increases the number of cells not only in the proximal, but also distal colon leading to the release of GLP-1. Assessment of the human colon also identified that the sigmoid colon had the highest densities of cells expressing FFAR2 and GLP-1 (Kaji et al., 2011). This suggests that selecting slowly fermentable substrates may be advantageous for activation of the more distally located receptors. It has also been observed that obese individuals have an increased expression of FFAR3 compared to lean controls. This is likely to be due to reduced methylation of the *FFAR3* gene thus increasing gene expression, and as a result receptor expression (Remely et al., 2014). These receptors are of particular interest due to their down-stream effects. The signalling and transduction pathways of FFAR2 are better understood than those of

FFAR3 (Sleeth et al., 2010), and both receptors have a variety of actions. In mouse models with FFAR2, FFAR3 or both receptor knockouts (Tang et al., 2015), different effects were seen. For example FFAR2$^{-/-}$ had no effect on insulin secretion with 1M acetate and FFAR3$^{-/-}$ showed slight attenuation in insulin secretion compared to the wild type, whereas the double knock out mice had no insulin secretion compared to wild type and similar effects were also seen with blood glucose (Tang et al., 2015). This suggests that these receptors may have synergistic roles, at either a genetic or a protein level and that in single knock out models the other receptor may 'compensate' for the other (Lin et al., 2012, Tang et al., 2015). Some of the functions of FFAR 2/3 activation are discussed below (Figure 1-3).

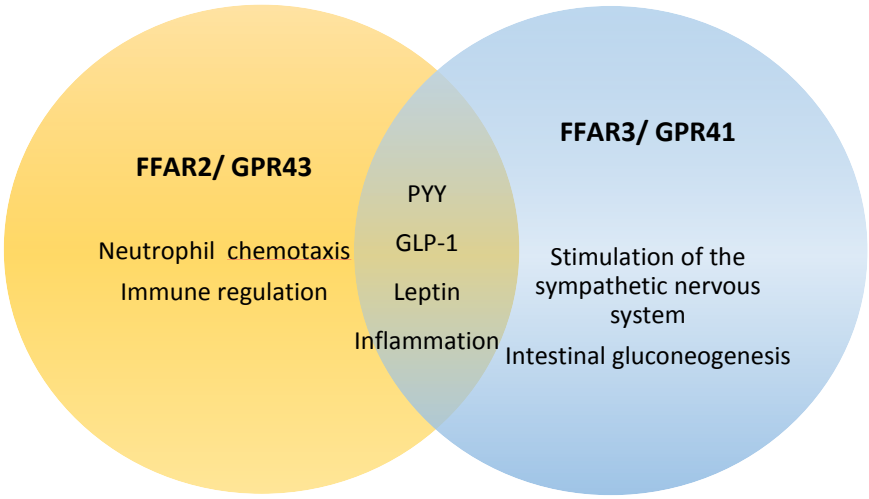

Figure 1-3: Key roles of FFAR2 and FFAR3.

Common and differential roles of stimulation of the FFAR. (Kaji et al., 2011, Lin et al., 2012, Maslowski et al., 2009b, De Vadder et al., 2014, Byrne et al., 2015, Xiong et al., 2004).

1.6.3.1 *Inflammation*

FFAR2 and FFAR3 are involved in the regulation of inflammation and the immune response where much of the mechanistic insight has been derived from knockout mouse models. Maslowski et al., (2009) determined in FFAR2$^{-/-}$ mice that after consumption of acetate, these mice had increased inflammatory markers with onset of allergic airway disease and inflammatory arthritis compared to the wild type group. These effects were attributed to FFAR2 located on neutrophils as there was a reduced effect of acetate on

neutrophil count, cytokine, and chemokine response in response to an inflammatory challenge on FFAR2$^{-/-}$ neutrophils but not FFAR2$^{+/+}$ neutrophils (Maslowski et al., 2009a). This role of these receptors in inflammation is also supported by the investigation by Kim et al., (2013) where feeding acetate to wild type mice increased neutrophil counts after inducing inflammatory stress compared to those without acetate. An appropriate immune response (neutrophil infiltration, inflammatory cytokines) after exposure to the pathogenic bacteria *Citrobacter rodentium* and with an ethanol challenge occurred with wild type mice; however, this did not occur in FFAR2$^{-/-}$ and FFAR3$^{-/-}$ mice. These effects indicate that the presence of SCFA and colonic receptors are required to deal with the initial onset of inflammation as reduced neutrophil frequency and cytokine presence was observed in the knock out mice post infection. This suggests that the immune roles of these receptors are not restricted to immune cells but may be relevant during the onset of inflammation within the colon (Kim et al., 2013). The activation of FFAR2 and FFAR3 by acetate and propionate may also have led to the onset of downstream inflammatory signalling pathways MEK/ERK, and p38 MAPK after infection by *C. rodentium.* This is as these SCFA, and propionate to a greater extent, led to approximately 30% of colonic epithelial cells expressing p-ERK in wild type but not in knock out mice, indicating that the SCFAs and receptors may have immunoregulatory roles in the colon (Kim et al., 2013). Exposure to propionate, butyrate, and to a lesser extent, acetate *ex vivo* in the colonic cells of mice with induced colonic inflammation has also been shown to reduce inflammation via inhibition of NFκB and reduced production of proinflammatory cytokines such as IL-6 and TNF-α by neutrophils. Within the same study the human colonic cell line COLO320DM was exposed to LPS where propionate and butyrate, and to a lesser extent acetate reduced TNF-α production and increased Il-8 production (Tedelind et al., 2007). The observation that topical administration of butyrate in individuals with ulcerative colitis reduced inflammation (albeit mildly), suggests that SCFA via FFAR2 and FFAR3 may have immunoregulatory roles within the colon and have the ability to reduce generalised inflammation such as that caused by IBD (Vernia et al., 1995).

1.6.3.2 *Anorexigenic gut hormones*

FFAR2 and FFAR3 may be involved in the satiety response, and have been shown to be central to the release of PYY (Karaki et al., 2008), and GLP-1 in mouse models (Lin et al., 2012). Investigations using FFAR2$^{-/-}$ and FFAR3$^{-/-}$ mice (single knock out) showed that GLP-1 expression was reduced with stimulation of acetate (100%) and propionate (50%)

in FFAR3$^{-/-}$ mice compared with wild type mice (Tolhurst et al., 2012). This effect was more prevalent in FFAR2$^{-/-}$ mice than FFAR3$^{-/-}$ mice indicating that FFAR2 is the main receptor mediating GLP-1 release in the colon (Tolhurst et al., 2012). These results are similar to those of Psichas et al., (2014) who observed that propionate injections dose dependently increased GLP-1 and PYY in wild type mice, but this was attenuated in FFAR2$^{-/-}$ mice (Psichas et al., 2014). Samuel et al., (2008) also identified reduced PYY expression in FFAR3$^{-/-}$ mice compared to wild type mice, although this required bacterial colonisation to occur. Therefore, this indicates that these effects were likely as a result of SCFA produced by bacterial fermentation of their polysaccharide rich diet. Paradoxically, FFAR3$^{-/-}$ mice had reduced expression of leptin and reduced weight gain compared to wild type mice (Samuel et al., 2008). This also suggests that the production of SCFA by bacterial fermentation increases satiety via the receptor mediated release of PYY and GLP-1. This is also supported by evidence from a study where propionate was directly administered to the colon in a human feeding trial using an inulin -propionate ester which also led to increased concentrations of PYY and GLP-1 (Chambers et al., 2014).

1.6.4 Olfr78

Olfr78 is an olfactory receptor activated by acetate and propionate (Pluznick et al., 2013). Olfr78 is expressed in a number of different systems such as the brain, blood vessels, kidney and colon. Within the colon, Olfr78 expression increases distally and is expressed on colonic enteroendocrine L-cells also expressing PYY, GLP-1, FFAR2, and FFAR3. For activation of these receptors high concentrations of propionate are required, for example the EC_{50} of FFAR3 is 11 μM and 900 μM for Olfr78. This along with their expression on L-cells led to the hypothesis of Fleischer et al., (2015) that they may play a role in the regulation of satiety hormones, PYY and GLP-1, this however has not been tested (Fleischer et al., 2015).

Olfr78 and FFAR3 have opposing effects. Using different knockout mice Pliznick et al., (2013) observed antagonistic effects of Olfr78 on FFAR3 mediated blood pressure reduction. In wild type mice, administration of 0-50 mM propionate dose dependently decreased blood pressure by 30 mmHg, and this effect remained in Olfr78$^{-/-}$ mice. However, when FFAR3$^{-/-}$ mice were exposed to 10 mM propionate, blood pressure increased significantly compared to the wild type (Pluznick et al., 2013). Although little is known about Olfr78, this suggests that they may possibly have regulatory effects on the

actions of SCFA. It would however be interesting to assess how the release of PYY and GLP-1 is effected as a result of Olfr78 of which to my knowledge has not been tested.

1.7 **Satiety**

Satiation is the feeling of fullness that results in the termination of a meal and satiety is the feeling of fullness experienced after a meal (postprandial feelings) which delay the consumption of the next meal (Duca and Covasa, 2012). Feelings of satiety are stimulated throughout the gut through receptor mediated monitoring of luminal and circulating nutrients, leading to production of anorexigenic (enhances feelings of satiety) or orexigenic (increase food intake) hormones. Figure 1-4 outlines the effects and process of food consumption throughout the gut. Table 1-1 outlines some of the effects of hormones produced during feeding or fasting on satiety and energy intake. Some of these effects on feeding are short term, affecting gastric emptying and the initiation or termination of a meal, whereas others are long term, altering the intake of subsequent meals (Wren et al., 2001, Batterham et al., 2003b, Edholm et al., 2010).

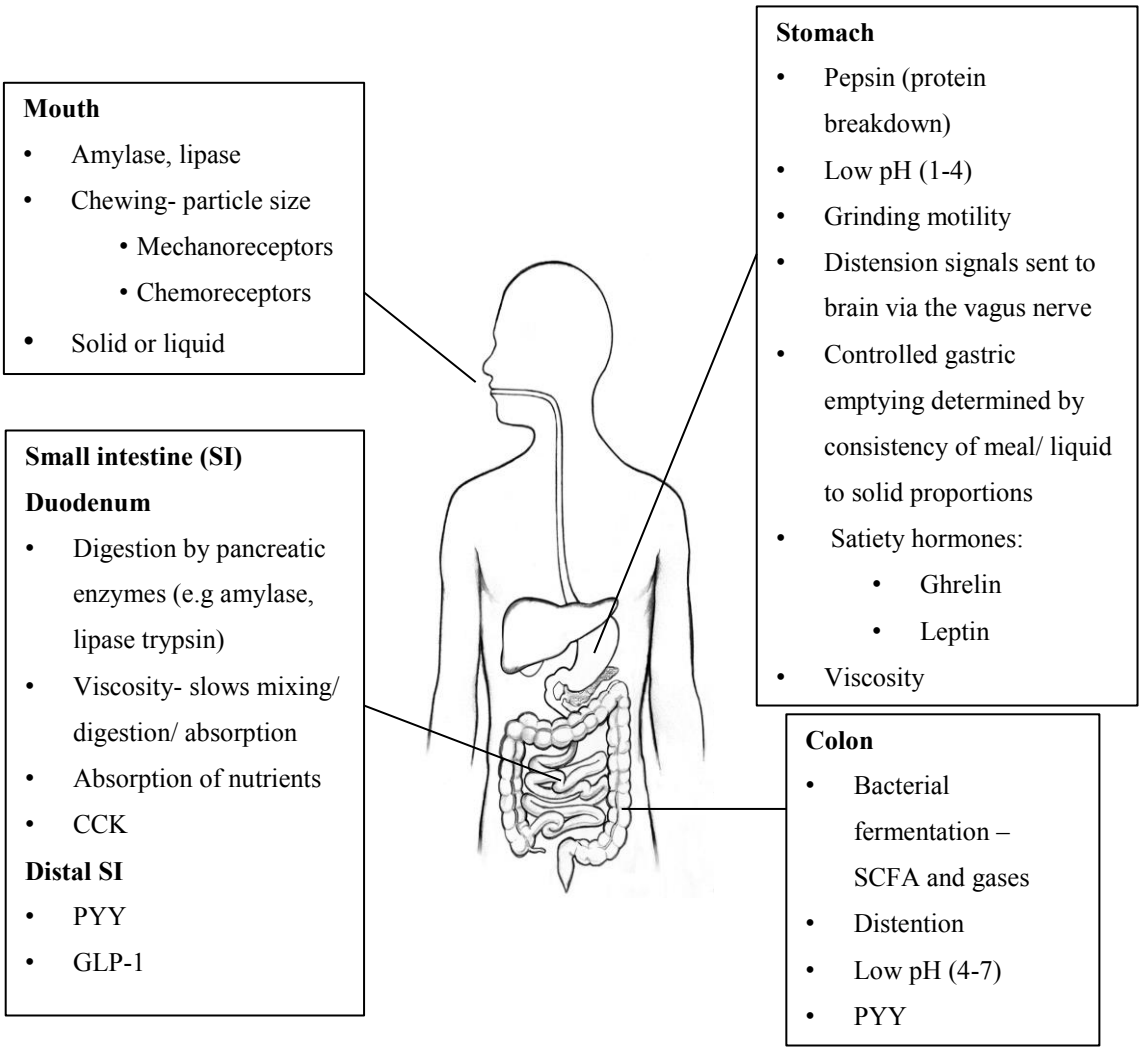

Figure 1-4: Events in the gastrointestinal tract which may influence digestion and satiety.

CCK – Cholecystokinin, PYY – Peptide YY, GLP-1- Glucagon- like peptide-1, OXM – Oxyntomodulin

Table 1-1- Gastrointestinal hormones associated with the regulation of energy intake

Hormone	Effects	Location	Reference
Ghrelin	• Increased under fasting conditions • Enhances food intake • Concentrations increase after calorie restricted weight loss but not after Roux-En-Y gastric bypass • Concentrations reciprocal to those of leptin • Short term effects on satiety • Decreased by OXM	Stomach	(Wren et al., 2001), (Cummings et al., 2002)
Leptin	• Increased by high body fat (obesity) • Weight loss by calorie restriction decreases concentrations • Increased with meal and insulin production • Concentrations are reciprocal to those of ghrelin • Long term effects on satiety	Adipose tissue, Stomach,	(Xiong et al., 2004, Lin et al., 2012) (Weigle et al., 2003)
CCK	• Increased 15 minutes after feeding, stimulates pancreatic secretion • Reduces energy intake and duration of feeding, stimulates gall bladder contraction • Slows on gastric emptying and also acts centrally in hypothalamus • Short term effects on satiety	Small intestine	(Mathus-Vliegen and de Groot, 2013, Kissileff et al., 1981).
PP	• Increased after feeding • Reduces energy intake and increases satiety short and longer term (12 hours)	Pancreas	(Batterham et al., 2003b)
PYY	• Reduces energy intake and enhances satiety • Short term, and long term effects • Reduces ghrelin concentrations • Basal concentrations are reduced in obesity	Distal SI and colonic L-cells, Co-located with GLP-1	(Habib et al., 2013, Batterham et al., 2003a)
GLP-1	• Reduces energy intake • Enhances satiety • Reduced gastric emptying	Distal SI and colonic L-cells, Co-located with PYY	(Habib et al., 2013, Verdich et al., 2001, Edholm et al., 2010)
OXM	• Reduces energy intake • Short term effects • Decreases concentrations of ghrelin	Intestinal cells	(Cohen et al., 2003)

CCK – Cholecystokinin, PP – Pancreatic polypeptide, PYY – Peptide YY, GLP-1- Glucagon- like peptide-1, OXM – Oxyntomodulin

1.8 Dietary fibre

One of the main influences on SCFA production is the colonic fermentation of NDC which are nearly all encompassed in the most recent definition of dietary fibre, agreed in the Codex Alimentarius in 2009 (Moller, 2011) is:

> *"Dietary fibre denotes carbohydrate polymers[1] with 10 or more monomeric Units[2], which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories:*
> *- Edible carbohydrate polymers naturally occurring in the food consumed.*
> *- Carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological benefit to health, as demonstrated by generally accepted scientific evidence to competent authorities.*
> *- Synthetic carbohydrate polymers that have been shown to have a physiological benefit to health, as demonstrated by generally accepted scientific evidence to competent authorities.*
> ***NOTES:***
> *[1] Includes also lignin and other compounds if quantified by AOAC 991.43.*
> *[2] Decision on whether to include carbohydrates with a degree of polymerization from DP 3 to 9 should be left to national authorities".*

Epidemiological evidence associates dietary fibre with improved health Table 1-2. The Scientific Advisory Committee on Nutrition (SACN) recommend that adults should consume 30 g of dietary fibre per day (SACN., 2015). Consumption of fibre in the British diet is much lower than this, with the National Diet and Nutrition Survey identifying that approximately 13.7 g/day NSP (~17.8 g/ day of dietary fibre) is consumed within the British diet (Bates et al., 2014). Table 1-2 outlines epidemiological evidence highlighting the importance of dietary fibre in different ethnicities as well as different habitual diets (Eastern and Western). Of importance is the association of increased dietary fibre and reduced weight, BMI, and waist circumference, particularly as current recommendations for fibre intake are not met, which may be related to the increased prevalence of obesity. Over the last 35 years the prevalence of obesity has doubled worldwide with 13% of all adults being obese and 39% of adults being overweight (WHO, 2015). Obesity is associated with the increased prevalence of type two diabetes (T2D) where 90% of the people affected are obese or overweight and 12.4% of all obese adults are affected (Public Health England., 2014). As described in Table 1-2, T2D prevalence also decreases with increased dietary fibre consumption. Obesity may also increase the risk of some cancers, for example, 20.3% of uterine and 11.1% of colon cancers were associated with obesity in a cohort study of the UK population (Bhaskaran et al., 2014).

Table 1-2: Epidemiological studies assessing association of dietary fibre intake and health.

Reference	Population	Fibre consumption	Impact of fibre	Fibre type observations
(Du et al., 2010)	89432, European individuals	10 g/day increase	↓ weight change, ↓ waist circumference NE on BMI	Cereal fibre beneficial compared to fruit and vegetable fibre
(Consortium, 2015) The InterAct Consortium	28460, European individuals	<18.9 g/day vs > 29.7 g/day	↓ Total cholesterol ↓ Risk of T2D NE on BMI	Cereal fibre beneficial compared to fruit and vegetable fibre
(Consortium, 2015) The InterAct Consortium	Meta-analysis of 20 investigations of men and women, different diabetes status and a selection of different ethnicities (e.g European, Asian, Nauru)	10 g/day increase	↓ Risk of T2D	Cereal fibre beneficial compared to fruit and vegetable fibre
(Jiang et al., 2012)	934, Chinese with T2D and 918 Chinese healthy individuals	≤9.1 g/day vs >9.1 g/day men and ≤8.4 g/day and >8.4 g/day women	↓HbA1c ↓ Total cholesterol ↓LDL cholesterol	
(Larsson and Wolk, 2014)	69677 Swedish individuals	≤18.2 g/day vs ≥28.3 g/day men and ≤20.4 g/day and ≥30.5 g/day women	↓ Stroke ↓Cerebral infarction NE on BMI	Fruit and vegetable fibre are the most beneficial
(Fujii et al., 2013)	4399 diabetic Japanese	Associations based on diabetic and non-diabetic food diaries. Average fibre intake was 12.83 g/day	↓ Waist circumference ↓HbA1c ↓Total, LDL and HDL cholesterol ↓BMI	
(de Munter et al., 2007)	161737 American (USA) women	~4.35 vs ~41.25 g/ whole grain per day	Whole grain ↓ T2D ↓BMI	Beneficial effects from the bran
(de Munter et al., 2007)	Meta-analysis of 6 investigations (USA men and women, Finnish)	Two servings a day of whole grain	Whole grain ↓ T2D	

(Kim and Je, 2014)	Meta-analysis of 7 investigations including; Europe, USA, middle east	10 g/day	↓ risk of mortality	
(Threapleton et al., 2013)	Meta-analysis of 22 investigations with men, women, smokers, and vegetarians from Europe, UK, Scandinavia, Japan, USA, and Australia	7 g/ day	↓ risk of CHD ↓risk of CVD	
(Liu et al., 2003)	74091 American (USA) women	13 g/day vs 20 g/day DF from whole grain and 18 g/day and 15 g/day. DF from refined grain	↓BMI ↓Weight gain	Changes with whole grain fibre, not refined fibre
(Thane et al., 2009)	3663 British adults	0- ≥48 g of whole grain /day	NE on BMI but ↓ BMI in men when BMI is over 30 NE on waist circumference	
(Wanders et al., 2011)	Meta-analysis of 188 investigations with long and short term consumption of different dietary fibres	Energy intake: supplement of 17 g/day Body weight: supplement of 11.1 g/day	↓ energy intake ↓ body weight	

T2D = Type two diabetes, CHD = coronary heart disease, CVD = cardiovascular disease, BMI = body mass index, LDL = low-density lipoprotein. HbA1c is a marker of plasma glucose concentration

The positive effects of dietary fibre have also been observed in acute and long term feeding trials on animals, healthy, and overweight adults (Table 1-4). However, the results between studies are often conflicting even for the same dietary fibres. This may be due to different doses or comparing different cohorts of individuals/ animals. For example beta-glucan consumption (10% w/w) in mice decreased body weight gain and food intake, after three weeks and increased production of acetate and propionate (Arora et al., 2012). However, consumption of 30 g of oat β-glucan by healthy individuals had no effect on satiety or desire to eat (Lyly et al., 2009). This was in contrast to a human feeding trial by Vitaglione et al., (2009) where 3 g of barley β-glucan for a breakfast led to reduced hunger

and energy intake in a subsequent meal. It was also observed that plasma ghrelin decreased and PYY increased up to 180 minutes after the breakfast with β-glucan (Vitaglione et al., 2009). Different effects were also observed in overweight individuals after consumption of guar gum, which contains beta, linked mannoses and galactoses (Adam and Westerterp-Plantenga, 2005). Here, a breakfast containing 2.5 g of guar gum increased plasma GLP-1, but no effect on satiety was observed (Adam and Westerterp-Plantenga, 2005) (Table 1-4).

Similar differences were also seen with assessment of inulin (10%) where consumption in mice decreased body weight gain and food intake after 6 weeks (Arora et al., 2012). Effects differed in studies of healthy humans where 24 g of inulin had no effect on GLP-1 or GIP but decreased ghrelin and increased SCFA production. Although a standard lunch was provided as part of the protocol, energy intake was not assessed and therefore it is difficult to assess whether the changes in hormone levels led to differences in energy intake (Table 1-4) (Tarini and Wolever 2010)

Mixed effects of dietary fibre on satiety, energy intake and body weight were also evident in the systematic review of 188 human feeding studies of various type of dietary fibre (Table 1-3)(Wanders et al., 2011). Both acute studies with a maximal duration of 7 hours, and long- term effects, (maximum mean duration, 20 weeks) were assessed. Forty three percent of the acute studies observed an overall energy intake reduction after eating fibre. All included studies assessing viscous fibres such as pectin (74 individuals, average dose- 14.2 g) and pectin rich fibres (33 individuals, average dose- 3.6 g) observed a reduction in overall subjective appetite (Table 1-3). In contrast, studies which fed β-glucan- rich fibres, dextrin, fructan, and resistant starch showed no overall effect on subjective appetite. The impact of physicochemical properties was also assessed and 59% of studies of the more viscous fibres showed reductions in subjective appetite compared to only 14% of studies using less viscous fibres although objective criteria for this division on viscosity were not provided. When the energy intake (assessed with an *ad libitum* meal) of the short term studies was compared, 100% of studies assessing β-glucan- rich fibres, dextrin, resistant starch and pectin observed reductions in energy intake. Energy intake was also reduced in 39% more studies using viscous fibres and 34% more studies with soluble fibre than non-viscous and non-soluble fibres (Wanders et al., 2011).

Long-term studies (with an average duration of 8.4 weeks) observed less of an effect of fibre type on recalled or *ad libitum* energy intake, in which 63% of all studies observed decreased energy intake. An advantage of the long-term studies is the ability to assess the effect of long-term fibre intake on bodyweight. Overall, 54% of studies assessed observed a reduction in body weight due to increased fibre intake (Table 1-3)(Wanders et al., 2011).

This systematic review also identified only a weak correlation (slope = -0.014χ) between increased fibre dose and decreased body weight (per 4 week period). Acute fibre doses were approximately 9 g/day and long-term doses were approximately 14 g/day (Wanders et al., 2011). The requirement of high doses of fibre has also been identified where the oligofructose consumption was dose dependently increased (Pedersen et al., 2013). Doses above 35 g/day of oligofructose were required to observe any effects on gut released satiety hormones. In comparison with the average fibre intake of adults of 13 g/day (NSP) doses equivalent to or higher than the UK daily intake of fibre to have an effect (Wanders et al., 2011, Pedersen et al., 2013, Bates et al., 2014).

Wanders et al., (2011) concluded that all types of fibre had some effect on appetite and body weight (Table 1-3), however the extent depended on the physicochemical properties of the dietary fibre. All fibre types reduced appetite and body weight; however, reduced appetite occurred more with more viscous fibres. In contrast, acute energy intake was reduced only when the fibres were more viscous, soluble and fermentable. All of the fibre types tested (except for more viscous fibre) reduced long-term energy intake (Wanders et al., 2011). Therefore, these data go towards explaining the mixed effects of dietary fibre on health in studies which use varied doses and physicochemical structures of dietary fibre (Table 1-6 – Table 1-6) (Wanders et al., 2011).

Consumption of high doses of fibre are also associated with colonic effects which may alter hunger and satiety and differ with the physicochemical properties of fibre sources. For example, consumption of highly fermentable, non-viscous, soluble fibres such as oligofructose have been associated with increased flatulence and bloating in contrast to cellulose (Daud et al., 2014). Consumption of the soluble, fermentable and viscous fibre, psyllium for two weeks in crackers (providing ~23 g/fibre a day) increased bloating, flatulence and abdominal pain, but did reduce *ad libitum* food intake (Table 1-5). In contrast, wheat bran had no effect on any of these parameters (Stevens et al., 1987). These studies indicate that factors other than the SCFA produced could alter energy intake. This

is particularly true for highly fermentable and soluble fibres, which result in increased abdominal symptoms (Daud et al., 2014). As a result replicating the doses of dietary fibre used in human feeding trials is likely to be unfeasible and difficult for the general population to maintain (Wanders et al., 2011).

Table 1-3: Physicochemical properties of fibre and overall effects on appetite, energy intake and body weight from a systematic review of 188 studies (Wanders et al., 2011).

Fibre type	Subjective appetite	Acute Energy intake	Long term energy intake	Body weight
More viscous	Strongly Reduced	Reduced	No effect	Reduced
Less viscous	Reduced	No effect	Reduced	Reduced
More soluble	Reduced	Reduced	Reduced	Reduced
Less soluble	Reduced	No effect	Reduced	Reduced
More fermentable	Reduced	Reduced	Reduced	Reduced
Less fermentable	Reduced	No effect	Reduced	Reduced
Total fibre	Reduced	Reduced	Reduced	Reduced

Table adapted from (Wanders et al., 2011). Reduced = 1-50% of studies observed an effect, strongly reduced 51-100% of studies observed an effect, No effect = 0% of studies observed an effect.

Table 1-4: Intervention studies in animal models investigating the effect of fibres on satiety associated parameters.

Study	Fibres investigated	Intervention	Satiety and food intake	Gut hormones	SCFA production
(Kaji et al., 2011)	Fructo-oligosaccharide	Rats fed ~16 g/day for 29 days Fructo-oligosaccharide in diet		↑ FFAR2, ↑ GLP-1 in proximal colon	
(Arora et al., 2012)	Inulin	Mice fed High fat diet + 100 g/kg inulin for 8 weeks	↓ Body weight gain from 3 weeks, ↓ Food intake from 6 weeks		↑ Acetate ↑ Propionate
(Van den Abbeele et al., 2011)	Inulin	10% of feed (w/w%) was inulin for 21 days in humanised rats			↑ SCFA production ↓ Acetate %
(Arora et al., 2012)	β-glucan	Mice fed High fat diet + 100 g/kg β-glucan for 8 weeks	↓ Body weight gain from 3 weeks, ↓ Food intake from 4 weeks		↑ Acetate ↑ Propionate
(Zhou et al., 2008)	Resistant starch	Rats fed 530.7g resistant starch for 10 days		↑ GLP-1, ↑ PYY, ↑ Glucose ↓ Insulin	
(Van den Abbeele et al., 2011)	Long-chain arabinoxylan (LC-AX)	10% of feed (w/w%) was LC-AX for 21 days in humanised rats			↑ SCFA production ↓ Acetate % ↑ Propionate %

LC-AX - Long-chain arabinoxylan, MOS – Mannooligosaccharide, PDX – Polydextrose, HDL- high density lipoprotein. Blank space – it was not measured

Table 1-5: Human intervention studies investigating the effect of fibres on satiety associated parameters

Study	Fibres investigated	Intervention	Satiety and food intake	Gut hormones	SCFA production
(Tarini and Wolever, 2010)	Inulin	56 g High fructose corn syrup + 24 g inulin as a drink (compared with High fructose corn syrup) Single blind cross over design		NE glucose, insulin, c-peptide, GIP, GLP-1. ↓ FFA at 4 hours ↓ Ghrelin 4 – 6 hours	↑ Acetate ↑ Propionate ↑ Butyrate
(Lyly et al., 2009)	Oat β-glucan	30 g oat β-glucan added to drink Crossover with 1day between sessions	NE on feelings of satiety or desire to eat		
(Vitaglione et al., 2009)	Barley β-glucan	Consumed bread containing 3 g barley β-glucan for breakfast. Cross over randomised design with one week wash out	↓ Hunger ↑ Fullness ↑ Satiety ↓ Energy intake	↓ Glucose ↓ Ghrelin ↑ PYY	
(Ellis et al., 1991)	Guar gum	Breakfast of different molecular weight guar gum sandwiches containing 7.6 g Randomised blinded Cross-over, 3 days apart		↓ Insulin NE glucose	
(Lyly et al., 2009)	Guar gum	10 g guar gum added to drink Crossover with 1day between sessions	↑ Feelings of satiety ↓ Desire to eat		
(Adam and Westerterp-Plantenga, 2005)	Galactose and guar	50 g galactose + 2.5 g guar at breakfast in a drink Crossover design with placebo separated by 1 week		↑ Plasma GLP-1, ↑insulin at 120 mins, ↑feelings of satiety	

(Stevens et al., 1987)	Psyllium	Consumed equivalent of 23 g/day for 2 weeks as a cracker. Double blinded cross-over study	↑ Flatulence, ↑Bloating, ↑ Abdominal pain, NE nausea, ↓Energy intake		
(Vogt et al., 2004a)	Rhamnose	Consumed 25 g rhamnose for 28 days Semi randomised crossover design, separated by 3 months			↑ Propionate (serum) NE faecal or urinary SCFA
(Vogt et al., 2004a)	Lactulose	Consumed 25 g Lactulose for 28 days Semi randomised crossover design , separated by 3 months			NE breath, faecal or urinary SCFA
(Bodinham et al., 2013)	High-amylose maize type 2 RS	24 g dietary fibre at breakfast and lunch in Randomised single blind cross over study with placebo separated by 1 week		Breakfast: ↓GLP-1, NE insulin Lunch: ↓ Insulin, NE GLP-1 NE: Glucose, C-peptide	
(Cherbut et al., 1997)	Maize fibre	15 g fibre/ day of maize fibre for 4 weeks Cross-over, single blind randomised study with one month interval		NE glucose, insulin ↓ total cholesterol	
(Cherbut et al., 1997)	Potato fibre	15 g fibre/day of maize fibre for 4 weeks Cross-over, single blind randomised study with one month interval		NE glucose, insulin, total cholesterol	
(Stevens et al., 1987)	Wheat bran	Consumed equivalent of 23 g/day for 2 weeks as a cracker. Double blinded cross-over study	NE Flatulence, bloating, abdominal pain, nausea, or energy intake		
(Lyly et al., 2009)	Wheat bran	21.8 g Wheat bran added to drink Crossover with 1day between sessions	NE on feelings of satiety or desire to eat		
(Lyly et al., 2009)	Wheat bread	50 g Wheat bread consumed Crossover with 1day between sessions	↑ Feelings of satiety ↓ Desire to eat		

(Asano et al., 2004)	Manno-oligosaccharide	1 g/day or 3 g/day MOS each for 2 weeks, Crossover with a 2 week interval period			NE
(Beards et al., 2010b)	Maltilol	50 g increasing to 100 g of chocolate over 6 weeks. Some of the chocolate replaces with maltilol. Randomised double blind and placebo controlled			↑ Acetate ↑ Propionate ↑ Butyrate
(Beards et al., 2010b)	Maltilol + polydextrin	50 g increasing to 100 g of chocolate over 6 weeks. Some of the chocolate replaces with maltilol + polydextrin Randomised double blind and placebo controlled			↑ Acetate ↑ Propionate ↑ Butyrate
(Astbury et al., 2013)	Polydextrose (PDX)	Consumed a drink containing PDX ranging 6.3-25 g Cross-over, single blind randomised study with a 7 day interval	NE fullness, hunger or desire to eat ↓ Energy intake at test meal NE on subsequent EI Total energy intake reduced with 25g PDX		
(Finley et al., 2007)	Pinto beans	Consumed 130 g pinto beans per day for 12 weeks. Randomised parallel study		↓ total cholesterol ↓ HDL ↓LDL	

LC-AX - Long-chain arabinoxylan, MOS – Mannooligosaccharide, PDX – Polydextrose, HDL- high-density lipoprotein. Grey space – it was not measured

Table 1-6: Intervention studies investigating the effect of fibres on satiety associated parameters in overweight subjects

Study	Fibres investigated	Intervention	Satiety and food intake	Gut hormones	SCFA production
(Daud et al., 2014)	Oligofructose	10 g of Oligofructose for 8 weeks Randomised, single blind parallel study		↑ PYY, NE on GLP-1, insulin, and glucose	↑ Breath hydrogen, ↑ Acetate ↑ Butyrate
(Parnell and Reimer, 2009)	Oligofructose	21 g oligofructose/day for 12 weeks Randomised, double blind	↓ Body weight ↓ Fat mass ↓ Energy intake ↓ Insulin change ↓ Glucose AUC = NE	↓Ghrelin ↑ PYY ↑ GLP-1	
(Chambers et al., 2014)	Inulin	10 g/day inulin consumed for 24 weeks Randomised, double blind, parallel design		↓ Total cholesterol, ↓HDL Improved liver function	
(Adam and Westerterp-Plantenga, 2005)	Galactose and guar	50 g galactose + 2.5 g guar at breakfast crossover design with placebo separated by 1 week	NE on feelings of satiety	↑ Plasma GLP-1, Delayed peak insulin,	
(Finley et al., 2007)	Pinto beans	Consumed 130 g pinto beans per day for 12 weeks Randomised parallel study		↓ Total cholesterol ↓ HDL ↓ LDL	

LC-AX - Long-chain arabinoxylan, MOS – Mannooligosaccharide, PDX – Polydextrose, HDL- high-density lipoprotein. Grey space – it was not measured

1.8.1 Propionate feeding trials

1.8.1.1 *Oral propionate interventions*

It is difficult to carry out oral propionate feeding trials due to its low palatability. Propionate is commonly added to bread in the form of calcium propionate (Ca-Propionate) to prolong shelf life by preventing mould and bacterial growth (Liljeberg et al., 1995). However, concentrations of Na-propionate in bread are much lower than has been suggested to have an effect on satiety (~16 mmoles) (Liljeberg et al., 1995, Darzi et al., 2012). Details of investigations of the effects of oral propionate are shown in Table 1-7. Human consumption of approximately 50 mmoles propionate in a single meal had beneficial effects on glucose and insulin tolerance, as well as increasing satiety (Liljeberg and Bjorck, 1996). Similar effects were observed by Darwiche et al., (2001) where 50 mmol propionate reduced gastric empting, blood glucose and plasma insulin concentrations.

At high concentrations, poor organoleptic properties observed with Na-propionate may confuse the outcome. Thus, breads with palatable concentrations of Na-propionate were tested. A dose of 6 mmol was deemed palatable based on a visual analogue scales after ingestion, but consumption did not affect glucose, insulin, energy intake or feelings of fullness (Darzi et al., 2012). In contrast, a propionate dose of 16 mmol within bread was palatable and increased feelings of satiety, decreased blood glucose and plasma insulin, indicating that 16 mmol is to lowest palatable dose of Na-propionate to exert beneficial effects (Liljeberg et al., 1995). This also does not take into account the 'standard loaf' of each country tested. The investigation by Liljeberg et al., (1995) was conducted in Sweden, where sourdough bread is commonly consumed. The study by Darzi et al., (2012) also used sourdough, however unlike in Sweden sourdough bread is not commonly consumed in the UK. It is possible that different cultures may have had different tolerances for propionate within the bread, altering the perceived feelings of palatably with different propionate doses within the bread. Palatability on food intake and hunger have been measured using 25 mmol acetate (in the form of vinegar). The dose of acetate was consumed in a single drink (unpalatable), or two drinks (palatable) during a breakfast meal. All treatments increased feelings of fullness, decreased appetite, but subsequent meal intake was significantly reduced in the unpalatable group compared to the palatable group, indicating that the palatability affects food intake (Darzi et al., 2011).

Many of these effects may be due to reduced gastric emptying observed after propionate consumption. Delayed gastric empting is associated with increased feelings of satiety, as well as being better insulin and glucose sensitivity (Darwiche et al., 2001, Liljeberg and Bjorck, 1996). These effects may be due to local mechanisms and differ from the effects of propionate produced from fermentation of carbohydrate in the colon (Darzi et al., 2012).

1.8.1.2 *Effect of colonic propionate on satiety*

As discussed in Table 1-4 – Table 1-6 and Section 1.9, propionate is produced in the colon by the fermentation of NDC. Much of the information gained on the ability of a SCFA to be produced is by using *in vitro* analysis (discussed in Section 1.9), or as part of feeding studies. Feeding studies do not provide much insight into colonic propionate production, as SCFA concentrations are often measured using faecal or serum samples (Vogt et al., 2004a). Faecal samples are representative only of the distal colon due to the rapid absorption of propionate in the gut, and as much of the propionate is used by the liver, little reaches the peripheral blood (Bloemen et al., 2009). However, consumption of 3g oat beta- glucan (a substrate considered to be propiogenic [Hughes et al., 2008]) added to bread, has similar effects to propionate feeding studies (Vitaglione et al., 2009). When this bread was consumed for breakfast there were increased feelings of satiety, reduced energy intake, and decreased blood glucose concentrations. However, the SCFA were not measured so the mechanism of this effect is unclear (Vitaglione et al., 2009). Rectal infusion containing propionate has been used to assess FA synthesis and plasma cholesterol, but effects of satiety were not measured (Wolever et al., 1995). Therefore, there is a need to correlate health outcomes with SCFA produced from fermentation. An estimation of the amount of propionate formed by colonic fermentation can be made with stable isotope analysis. There are relatively few studies using isotopes to assess SCFA production; however, Pouteau et al., (1998) assessed acetate production from colonic lactulose fermentation by measuring the dilution of labelled acetate infused into the blood of healthy adults. Similar methods could be used could be used to assess propionate production (Pouteau et al., 1998).

Table 1-7: Outcomes of oral propionate consumption in human feeding trials

Study	Intervention+	Propionate dose*	Palatability	Effect of propionate	Effect of propionate on energy intake
(Darzi et al., 2012)	Propionic acid bread consumed (n=20, healthy)	6.1 mmol	Palatable[1]	NE plasma glucose NE plasma insulin	NE energy intake during subsequent ad libitum meal. NE fullness[1]
(Ruijschop et al., 2008)	Milk based drink with calcium-propionate (N=43 healthy)	10.4 mmol (~53 mM, 0.6% in 150 ml)	↓ Palatability[1]	NI	↑ Fullness[1] ↓ Hunger[1] NE food intake in subsequent ad libitum meal
(Frost et al., 2003)	Consumed high fat diet with propionate (n=10, healthy)	~31 mmol (3 g propionate in 50 g available CHO)	Palatable amount used although participants complained of nausea	↑ GLP-1 ↓ Gastric emptying NE glucose ↑cMax insulin	NE energy intake
(Todesco et al., 1991)	Na-propionate bread (n=6, healthy)	~34 mmol (3.3 g Na propionate in 50 g CHO)	NI	↓ Glucose	
(Liljeberg et al., 1995)	High and low concentration Na-propionate bread consumed (n=11, healthy)	Low ~ 16 mmol (21.3 g/100 g DW) High ~49 mmol (60.8 g/100 g DW)	↓ Palatability of high concentration propionate bread. NE palatability low concentration (c.f. control)[1]	Both breads ↓ Plasma glucose, insulin more so in high concentration	↑ Satiety[1]
(Liljeberg and Bjorck, 1996)	Na- propionate bread consumed (n=12, healthy)	~49 mmol (60.8 g/ 100 g DW)	Previous investigation found palatability to be poor (Liljeberg et al., 1995)	↓ Blood glucose ↓ Serum insulin	↑ Satiety[1]
(Darwiche et al., 2001)	Na- propionate bread consumed (n=9, healthy)	~49 mmol	NI	↓ Gastric emptying ↓ Blood glucose ↓ Serum insulin	NI
(Todesco et al., 1991)	Na-propionate bread (n=6, healthy) (1week duration)	~ 103 mmol (9.9 g in 150 g CHO per day)	NI	↓ Glucose NE cholesterol	NE energy intake

1= measured by visual analogue scale * When dose was not provided food consumed, by individuals, dose was calculated from information provided within the text when possible. + one day acute study unless otherwise stated. NI = not included, NE = no effect, VAS = visual analogue scale Cmax = maximal concentration, NA= not available.

More recently, an inulin-propionate ester (IPE), delivering very high doses of propionate directly to the colon (31 mmol, 2.36 g in 10 g) has been developed. Overweight individuals consumed 10 g/day of IPE for 24 weeks, which led to a 13.8% decrease in energy intake, decreased weight gain and decreases in visceral and hepatic body fat percentage were observed. No effects on glucose, insulin, PYY or GLP-1 concentrations were observed with long-term supplementation, however acute supplementation of IPE (10g) by the same group has had mixed effects of PYY and GLP-1 release. For example, Chambers et al., (2014), observed increased hormone expression between 240 and 420 minutes and Byrne et al., (2016) at the same dose did not identify any effect in hormone expression. Both studies did observe a reduction in food intake, indicating that in the case of the IPE these effects are potentially occurring independently of the satiety hormones (Chambers et al., 2014, Byrne et al., 2016).

1.9 Properties influencing selective SCFA production- the challenge of increasing propionate production

In order to increase colonic production of propionate by manipulating the types and amounts of dietary fibres consumed, the key determinants of propionate production need to be identified. There are many different factors that can influence the production of propionate, or any other SCFA. Table 1-8 outlines the properties that alter the production of SCFA, which are not only dependent on the dietary fibre consumed, but also other host factors such as the colonic pH and the profile of the colonic microbiota. These factors are not independent of one another. As discussed earlier, increased bacterial fermentation reduces the colonic pH, resulting in differences in bacterial activity. On the other hand, the distal colon has a higher pH due to reduced fermentation of carbohydrate and increased BCFA production indicating proteolytic fermentation (Section 1.3).

Table 1-8: Potential factors that influence the production of SCFA

Substrate Factors	Host Factors
Substrate availability	Colonic pH
Saccharide composition	Small bowel transit time
Solubility	Colonic residence time
Molecular weight	
Chain length	Gut microbiota composition
Molecular bonding and branching	

1.9.1 Saccharide Structure

To-date there are very few studies systematically examining the factors leading to colonic production of propionate (or any other SCFA). Most studies on SCFA production use complex dietary fibres for analysis, and as a result little is known about the role of sugar composition and / or bonding.

1.9.1.1 *Monosaccharide sugars*

Very few monosaccharides reach the colon, but they can be produced by bacterial catabolism of disaccharides and polysaccharides. Determining the sugars which increase individual SCFA production would enable more selective substrates to be used for propionate production. *In vitro* fermentation experiments have observed that the fermentation of different sugars is associated with different concentrations and patterns of individual SCFA. Using a batch *in vitro* fermentation model Gietl et al., (2012) observed that fermentation of L-rhamnose (a pentose sugar) and D-mannose (hexose sugar) generated 60.74 and 52.20 mmol/l of total SCFA, whereas the pentose sugars; L-xylose and D-arabinose generated 24.9 mmol/l and 38.18 mmol/l total SCFA. Differences in propionate production were also identified with rhamnose which selectively increased propionate production (19.09 mmol/l) when compared with a selection of sugars, such as glucose (6.07 mmol/l), and L-xylose (2.87 mmol/l) (Gietl et al., 2012). Differences in sugar composition and total SCFA production have also been observed by Mortensen et al., (1988) using 10 different sugars. D-xylose, D-ribose, D-glucose generated concentrations above 900 mmol/l, whereas D-mannose, L-rhamnose, and, D-arabinose generated between 280 and 520 mmol/l total SCFA. Mortensen et al., (1988) used 10x more substrate than Gietl et al., (2012) but SCFA concentrations were approximately 5x higher than that of (Gietl et al., 2012). Here, rhamnose, like the investigation by Gietl et al., (2012) led to the most propionate production, and sorbitol produced the most butyrate (Mortensen et al., 1988). This shows that when considering the data, without further processing to account for volume and mass of substrate used, comparing SCFA production between studies can be challenging.

Increased propionate production after rhamnose consumption has also been observed in human feeding studies. Vogt et al., (2004a), (2004b), and (2006) carried out a number of investigations on rhamnose consumption and propionate production. When healthy

subjects were fed 25g of rhamnose over the course of one day as part of a meal, serum propionate area under the curve (AUC) was increased compared to glucose and/or lactose (1.63 vs 2.4 and 3.0 μmol.h/l) (Vogt et al., 2004b). When the same group fed the same dose of rhamnose to healthy men for 28 days, once again, serum propionate was increased, but there was no observed difference in faecal or urinary SCFA concentrations (Vogt et al., 2004a). Vogt et al., (2006) conducted further analysis on the previous investigations and observed that four weeks of rhamnose consumption led to reduced TAG production, but there was no effect on total cholesterol, glucose or insulin (Vogt et al., 2006). In a more recent study, consumption of up to 25.5 g/day of rhamnose, over 7 days by healthy individuals had no effect on serum SCFA concentrations, fasting insulin or subjective appetite ratings, but did decrease plasma insulin concentrations (Darzi et al., 2015).

The effect of isomeric configuration differs between sugars. D-glucose increased total SCFA production compared to L-glucose, but there was no effect of isomeric configuration on propionate production (Mortensen et al., 1988). In contrast, D-arabinose increased propionate production compared to L- arabinose (16.91 mmol/l vs 6.19 mmol/l). The same trend was also observed for increased propionate from fermentation of the D isomer of xylose (Gietl et al., 2012). However, rhamnose is an L isomer suggesting that isomeric configuration is not the sole determinant of whether increased propionate is produced.

1.9.1.2 *Disaccharide sugars*

Most disaccharides do not reach the colon (although there are exceptions to this, such as lactulose) and are normally digested and/or absorbed in the human small intestine. However, disaccharides in *in vitro* fermentation studies are useful for the assessment of the SCFA producing properties of both the sugars and their linkages. Lactulose was utilised by Sayer et al., (2007), and Arrigoni et al., (2005) as a highly fermentable control yielded mainly acetate (Sayar et al., 2007, Arrigoni et al., 2005). There is poor consensus between different investigations in determining whether a disaccharide sugar is propiogenic. For example, sucrose, a disaccharide consisting of glucose and fructose, has been identified as being highly acetogenic, propiogenic, and butyrogenic in different investigations, all of which used *in vitro* batch fermentation. Wang and Gibson., (1993), identified that sucrose yielded 82% acetate (of total SCFA), Gietl et al., (2012), observed 83% propionate and Ferguson and Jones., (2000) observed 50% butyrate (of total SCFA) after sucrose fermentation (Wang and Gibson, 1993, Gietl et al., 2012, Ferguson and Jones, 2000). An

issue with the analysis of the properties of disaccharides is that investigations have not been systematic, i.e. different sugars and bonding have not been individually assessed.

Few investigations have systematically fermented disaccharides to assess the impact of the glycosidic bond linkage on propionate production. Gietl et al., (2012) conducted batch fermentations on four different disaccharides consisting of 1-4, or 1-6 bonding in both the alpha and beta orientation, and observed no difference in the production of propionate (Gietl et al., 2012). Sanz et al., (2005) did a small investigation, with a stool sample from only one donor, fermenting of all the possible glucose-glucose disaccharides for 12 hours. With glycosidic linkages (1-1), (1-2), (1-3), and (1-4) the beta orientation increased propionate, whereas for (1-6) linkages the alpha orientation increased production. Different linkages of galactobiose (galactose-galactose) and mannobiose (mannose-mannose) were amongst other disaccharides tested. It was observed that 3α (galactobiose) increased butyrate production compared to other galatobioses tested, and 2α (mannibiose) increased propionate compared to 3α, 4α, and 6α- mannobiose. The bacterial compositional changes because of these disaccharides was investigated and no changes in the bacterial population were observed (Sanz et al., 2005a). This indicates that, in this one individual, changes because of glycosidic bond linkage altered SCFA production independently of changes in the microbiota; however, how this relates to the bacterial functionality was not measured. This does highlight the requirement of robust systematic investigations to assess the role of disaccharide structure and bonding as drivers for selective SCFA production.

1.9.1.3 *Poly and Oligo-saccharide structure*

Few *in vitro* fermentations have been carried out to systematically assess how the sugar composition of a NDC affects SCFA production. Karppinen et al., (2000) conducted *in vitro* fermentations and observed that for oat, rye, and wheat bran the more fermentable the substrate the more SCFA was produced. This was not the case for inulin, which was the most rapidly fermented of the substrates tested (within 4 hours) but did not produce the most SCFA at 24 hours. Of the sugars in oat bran, glucose was most highly fermented with 78% being utilised over 24 hours of fermentation, compared with only 9% of arabinose. This differed from inulin where fructose then glucose were most highly utilised during fermentation with approximately 64% of glucose and 99% of fructose being utilised. This

difference in preference of sugar for fermentation may have altered SCFA production (Karppinen et al., 2000).

The relationship between SCFA production and the constituent sugars has also been tested in the seaweed, ulvan. Differentiation of the sugars producing each SCFA showed that aldobiuronate producing acetate, propionate and butyrate in the ratio 55.5 : 21.3 : 15.7., rhamnose yielded the highest proportion of propionate (51%), and glucoronate yielded a high proportion of butyrate (23%) (Bobin-Dubigeon et al., 1997).

Although little analysis has been carried out to determine what makes a substrate particularly propiogenic, different NDCs have been linked to the production of individual SCFA. Acetate is consistently the most abundant SCFA produced. Oligofructose is associated with increased acetate production (Khan and Edwards, 2005, Ferguson and Jones, 2000, Rycroft et al., 2001a). Laminarin (a β glucan component of seaweed), and pyrodextrinised starch are associated with increased propionate production (Deville, 2007, Laurentin and Edwards, 2004). Resistant starch is associated with increased production of butyrate (Laurentin and Edwards, 2004, Zhou et al., 2013) and as well as propionate, beta-glucans are associated butyrate production (Kim and White, 2009, Kaur et al., 2011). The reason that these NDCs selectively increase production of acetate, propionate, or butyrate is unclear. Differences in SCFA production are difficult to predict because of the complexity and differences in physicochemical properties of different substrates, which likely alter the colonic bacteria required to utilise them. These differences include solubility, molecular weight, chain length, the bonds present and branching.

1.9.2 Solubility

Solubility is thought to a play a role in the determination of SCFA production. Soluble fibre is generally more fermentable than insoluble fibre. Insoluble fibres are generally considered to act as a stool bulker and do not undergo extensive fermentation, however there are exceptions (e.g resistant starch) (Wong et al., 2006, Slavin, 2013). Dietary fibre sources usually consist of a combination of insoluble and soluble fibre with approximately 65% being insoluble (Wong et al., 2006).

Characterising a carbohydrate on the basis of whether it is soluble or not is simplistic, based on chemical analysis with solvents and carbohydrates are rarely 100% soluble or

insoluble (Mortensen and Nordgaard-Andersen, 1993). The association between solubility and fermentation is likely due to the properties within the carbohydrate such as if it is viscous, interacting with water molecules like pectin, has a high molecular weight or remains solid like wheat bran. Cellulose is an insoluble fibre, which does is not viscous and is poorly fermented yielding low concentrations of SCFA (Mortensen et al., 1991, Mortensen and Nordgaard-Andersen, 1993, Yu et al., 2013). Pectin, ispaghula, and guar are all examples of carbohydrates that are characterised as being soluble fibres and produce viscous solutions. They are also highly fermentable and yield high SCFA concentrations, particularly propionate and butyrate. This may be due to a greater accessibility of the sugar bonds for fermentation. This however does not take into account the differences in the sugar and bonding structure that these carbohydrates have (Khan and Edwards, 2002, Mortensen and Nordgaard-Andersen, 1993, Rycroft et al., 2001a). However, oligofructose is very soluble but is not viscous and unlike pectin, guar, and ispaghula it is not associated with the increased production of propionate and butyrate but it is associated with the production of acetate (Rycroft et al., 2001a).

Solubility is associated with the MW of a substrate. Kim and White., et al (2010) found that a curvilinear relationship between MW and solubility, this did not translate to differences in individual SCFA production although total production was slightly higher for the low MW substrate (Kim and White, 2010). This was contradicted by an investigation by Ramasamy et al., (2014) who fermented chicory root pulp (contains inulin), and as MW increased, so did solubility. The differences in solubility observed did not lead to down-stream differences in acetate, propionate, or butyrate production, but soluble fibre increased total SCFA production by 30%. Here, insoluble fibre was only fermented after all of the soluble fibre had been utilised by the bacteria (Ramasamy et al., 2014).

Solubility is likely to affect total SCFA production by increasing fermentation; however, it is unlikely to be the key determinant of individual SCFA production. The predominant physicochemical properties to determine acetate, propionate, or butyrate production, remain to be elucidated.

1.9.3 Molecular weight (MW)

The MW of a fermented carbohydrate is thought to influence the resultant SCFA production. Kim and White have conducted several studies to assess the role of MW on SCFA production using *in vitro* batch fermentations with healthy stool donors (Kim and White, 2010, Kim and White, 2011a, Kim and White, 2011b). They identified mixed effects of MW on SCFA production when testing a selection of different MW oat β-glucans (53-898 kDa). Both the highest and lowest MW resulted in the lowest total SCFA production, and to a lesser extent, reduced acetate production. Propionate and butyrate production were unaffected by MW (Kim and White, 2011b). Total SCFA production related to MW resulted in a curvilinear association (Figure 1-5).

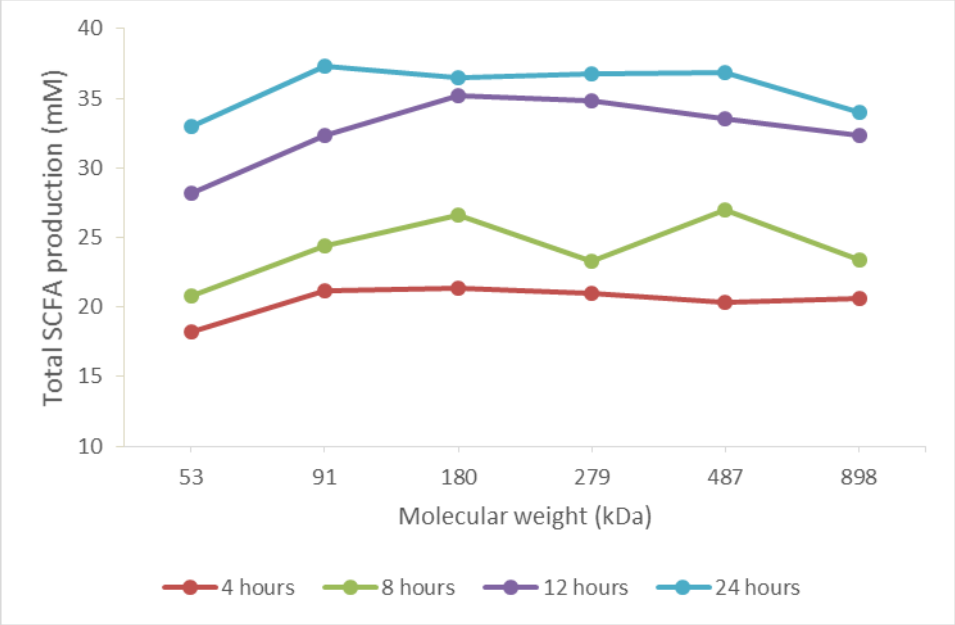

Figure 1-5: SCFA production and molecular weight of oat β-glucan.

4- 24 hour data obtained from (Kim and White, 2011b). 0 and 2 hours was omitted due to no observable effects as a result of MW seen at these time points.

This group also researched the effect of β-glucan of different MW added to wheat flour on the *in vitro* production of SCFA (Kim and White, 2011a). Total SCFA production was not altered by MW, although propionate and butyrate proportions were significantly higher for the low MW compared to the high MW (66 vs 361 kDa) (Kim and White, 2011a). This effect could be due to altered solubility, in the study by Kim and White., (2011) it was observed that solubility of the β -glucan showed a similar pattern with the extreme ends of the MW containing more soluble fibre, than the other MW β -glucans. This however was not significant, and may explain the slight curvilinear response to different MW observed (Kim and White, 2011b). The decrease in total SCFA production may also be due to steric

hindrance preventing the bacterial hydroxylases from accessing and utilising the sugars. This could occur as a result of increased number of bacterial enzymes utilising the substrate, preventing access (Valjamae et al., 1998). Steric hindrance, physical inaccessibility due to the crowded molecular structure around the bond which the enzyme targets, could also occur due to the substrate structure, such as increased viscosity, making it less accessible to the bacterial hydrolases (Pickardt et al., 2004). This would then prevent fermentation occurring, and as a result reduce the production of SCFA.

Similar effects were also seen after assessment of different MW of guar gum (15 kDa – 1100 kDa) on SCFA production in *vitro* (Stewart and Slavin, 2006). The 400 kDa guar gum generated the highest total SCFA, acetate, propionate, and butyrate production. Low MW guar gum (15 kDa) led to selectively less propionate production compared to the 20kDa and 400 kDa, but not the 110 kDa guar gum. This, similar to the studies with oat β-glucans, and suggests that there is a curvilinear response of SCFA production based on MW (Stewart and Slavin, 2006). It was hypothesised in these studies that these differences in SCFA production were as a result of differences in the solubility of the β-glucans or due to the rapid fermentation of the lower MW β-glucans (Kim and White, 2011b, Kim and White, 2011a, Stewart and Slavin, 2006) . Stewart and Slavin., (2006) also postulated that the differences in the capabilities of the bacteria to utilise the different MW substrates could possibly be altering the SCFA production (Stewart and Slavin, 2006).

The effect of MW on bacterial composition, independently of any changes in SCFA production, has also been investigated. Al-Tamimi et al., (2006) fermented varying fractions of sugar beet arabinoxylan and found no difference in SCFA production, but observed changes in the bacterial composition. A high MW increased numbers of lactobacilli and a low MW decreased *Clostridium perfringens/ histolyticum* sub group numbers (Al-Tamimi et al., 2006). Although these differences in SCFA production due to MW have been seen, this does not take into account molecular structure such as the linear chain length or the branching of fibre. Linear chain length has been shown to alter SCFA production when this was assessed with galacto-oligosaccharide (GOS). For example *in vitro* investigations by Gietl et al., (2012) and Cardelle-Cobas et al., (2009) used GOS with different degrees of polymerisation (DP) found that DP did not alter SCFA production or bacterial composition (Gietl et al., 2012) (Cardelle-Cobas et al., 2009). In contrast, Ladirat et al., (2014) determined that a low DP GOS (DP of 2-3) resulted in low total SCFA, although they did note that a DP of 4, 5 or 6 did not generate any changes in SCFA

production compared with each other. The chain length has been shown to influence bacterial composition and SCFA production (Ladirat et al., 2014). Inulin with DP of 10 and β(1-2) bonding reduced bifidobacteria numbers compared to lactulose β(1-4) as well as having a decreased acetate concentration compared to GOS (DP = 2). GOS also increased S*treptococci* compared to FOS (DP [4-5], β1-2), but decreased propionate production compared to lactulose (Rycroft et al., 2001a). DP has been shown to effect SCFA production, FOS and inulin with a DP between 10-20 yielded high total SCFA, a high proportion of propionate and low proportions of acetate and butyrate compared to inulin and FOS with lower DPs (Stewart et al., 2008). These studies demonstrate that a specific chain length or branching is unlikely to determine individual SCFA production.

Carbohydrate branching also had differing effects on SCFA production. Hopkins et al., (2003) fermented arabinoxylans crosslinked with ferulic acid and noted decreased total SCFA production with branching, but no difference on individual SCFA production (Hopkins et al., 2003). This difference in total production is attributed to different bacterial species being required to utilise the bonds that form the cross-links. Decreased butyrate production has been detected when dextrans of various MW and branching are investigated. Unlike with arabinoxylans, there was no difference in total SCFA production with differences in branching or MW dextrans when they were tested *in vitro* (Sarbini et al., 2011). The pyrodextrinisation of starch which introduces branching as well as β-linkages to starch has also been observed *in vitro* (Laurentin and Edwards., 2004). These low MW pyrodextrinised starches increased production of propionate and decreased acetate compared to the native starches of potato, lentil and cocoyam. The reason for this was unknown but it was postulated that the solubility might have had an effect.

1.9.4 Dietary intake and bacterial populations

The NDC that reach the colon may alter the colonic microbial environment. This may be due to enriching the populations of bacteria that can utilise certain substrates or by changing pH or other inhibitory metabolites which affect some but not all bacteria. The profound effect of a habitual diet on the colonic microbiota has been investigated in both children (De Filippo et al., 2010), and adults (Yatsunenko et al., 2012). Children consuming a Western diet which was high in fat and protein and low in fibre were compared with those consuming a rural African 'Neolithic' diet consisting of low fat and protein and high fibre (De Filippo et al., 2010). Children on the different diets had the

same core phyla of Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria, which covered 94.2% of the bacterial phyla present, demonstrating that the basic bacterial genome is relatively stable. Differences were apparent with the Western diet with Firmicutes and Proteobacteria prevailing and for the Neolithic diet Actinobacteria, Bacteroidetes (particularly *Prevotella* [53% of the bacteria] dominating) (De Filippo et al., 2010). This was also corroborated by Yatsunenko et al., (2012) who found microbiome and enzyme activity of two different high carbohydrate diets; those of the Amerindians in the Amazon and rural Malawians to be similar, however they were distinct from the Western diet of US Americans, particularly in adulthood. Interestingly, bacterial composition did not differ between Western children and children from more traditional and 'Neolithic' backgrounds during breast feeding, suggesting that these later differences are possibly due to diet and not environmental or genetic factors (De Filippo et al., 2010, Yatsunenko et al., 2012).

1.9.4.1 *Modulation of the colonic bacterial profile*

Human feeding trials as well as *in vitro* investigations have demonstrated that different foods and increasing NDC consumption alter the bacterial profile. Fava et al., (2013) demonstrated in overweight individuals that increasing overall carbohydrate consumption for 24 weeks increased *Bacteroides,* and *Bifidobacterium* spp populations (Fava et al., 2013). This was in contrast to a 18 week investigation of overweight and obese individuals where consuming 43 g/day of cereal fibre had no influence on bacterial profiles (Weickert et al., 2011). This discrepancy in the role of different foods on bacterial changes is also demonstrated in feeding studies, which have been assessed using different bacterial methods (Table 1-9). The different results could also be due to the large quantities of food required to have an effect on the bacterial profile. Martinez et al., (2010) investigated the effect of the consumption of type 2 and type 4 resistant starch by 13 healthy individuals. Type 4 resistant starch influenced the bacterial profile reducing Firmicutes and increasing Bacteroidetes, Actinobacteria and *Bifidobacterium*, whereas type 2 resistant starch had no effect (Table 1-9), suggesting the physicochemical properties may lead to different bacterial profiles. Within this investigation, it was also observed that these effects were short lived, and once consumption of the starches had ceased the bacterial population returned to its initial state (Martinez et al., 2010). Investigations from the same group (Sanz et al., [2005, 2006]) found different effects of FOS on bacterial production *in vitro*. For example, bifidobacteria increased in some (Sanz et al., 2005b, Sanz et al., 2006b), but

not all investigations (Sanz et al., 2006a), although all studies did not find any effect on total bacteria or lactobacilli. This along with Table 1-9 demonstrates that the modulation of the bacteria with diet is complex and in many cases, for profound effects on the bacterial profile, large quantities of individual fibres for prolonged periods are required to maintain these changes.

Table 1-9: Carbohydrate consumption and the bacterial composition

References	Food	Intervention	Bacterial changes	Bacterial analysis
(Kruse et al., 1999)	Inulin	8 healthy individuals 34 g/day 8 weeks	↑ *Bifidobacterium* NE total	FISH
(Gibson et al., 1995)	Inulin	4 healthy individuals 15 g/day for 15 days	NE Total bacteria ↑bifidobacteria NE Bacteroides NE Clostridia NE Lactobacilli	Selective media plating
(Gibson et al., 1995)	OF	8 healthy individuals 15 g/day for 15 days	NE Total bacteria ↑ bifidobacteria ↓ Bacteroides ↓ Clostridia ↓ Lactobacilli	Selective media plating
(Bouhnik et al., 1999)	OF	40 healthy individuals dose response 0 – 20g/day for 7 days	↑ bifidobacteria (10 g/ day and 20g/ day were optimal)	Selective media plating
(Beards et al., 2010a)	Maltitol	10 healthy individuals consumed 45.6 g in 100g chocolate	↑*Bifidobacterium* ↑*Bacteroides* ↑*Lactobacilli / enterococci* ↑ *F.prausnitzii* ↑Total bacteria	FISH
(Beards et al., 2010a)	Resistant starch	10 healthy individuals consumed 45.6 g in 100 g chocolate	↑*Bifidobacterium* ↑*Bacteroides* ↑*Lactobacilli / enterococci* ↑Total bacteria	FISH
(Walker et al., 2011)	Resistant starch	6 overweight individuals consuming 50-60 g/day	↑*Eubacterium retale* ↑*Ruminococcus bromii* ↑Roseburia NE Bacteroidetes, *Bifidobacterium* or *F.prausnitzii*	qPCR
(Martinez et al., 2010)	Type 2 resistant starch	10 healthy individuals 33 g/day for 3 weeks	NE Firmicutes NE Bacteroidetes NE Actinobacteria NE *Bifidobacterium*	16s rRNA pyrosequencing
(Martinez et al., 2010)	Type 4 resistant starch	10 healthy individuals 33 g/day for 3 weeks	↓ Firmicutes ↑ Bacteroidetes ↑ Actinobacteria ↑ *Bifidobacterium*	16s rRNA pyrosequencing

NE= no effect ↓ = decreased ↑ = increased, FISH – fluorescence *in situ* hybridisation. OF = oligofructose

1.9.5 Gut transit time

Whole gut transit time in healthy individuals is approximately 24 -72 hours although this is highly variable between individuals (Wang et al., 2015, Read et al., 1980). Gut transit time is associated with alterations in SCFA production and bacterial populations, and is also associated with different pH along the colon. In an investigation where transit time was altered by the intake of cisapride and loperamide, thus decreasing or increasing transit time in healthy individuals, it was found that reduced transit time (measured using radio-opaque pellets) increased total faecal SCFA production (El Oufir et al., 1996). Residence time has also been evaluated *in vitro* with continuous culture systems that allow control of the system retention time by changing dilution rate (Child et al., 2006). Child et al., (2006) used a retention time of 20 or 60 hours to mimic the role of gut transit time on SCFA production and bacterial populations. Differences in microbial composition and SCFA production were found to be related to different retention times. A reduced transit time decreased the proportion of acetate, whilst increasing the proportion of butyrate, although it had little effect on propionate production, which is in agreement with El Oufur et al., (1996). Although differences in total bacteria have not been identified as a consequence of altered transit time, differences in the bacterial compositions have been noted (Child et al., 2006, El Oufir et al., 1996, Rodes et al., 2011). El Oufir et al., (1996) observed altered bacterial populations due to increased transit times with the number of methanogens decreasing, and sulphate reducing bacteria increasing in prevalence, although total bacteria (measured using selective media) was not altered. This has also been demonstrated *in vitro* where transit time was reduced from 20 hours to 60 hours and FISH analysis was used to assess bacterial changes. The reduced transit time increased the proportion of *Faecalibacterium prausnitzii* (a butyrate producer), but reduced *Eubacterium recale* and *Clostridium coccoides*. *Bifidobacterium* spp however were able to withstand changes in transit time, suggesting that not all bacterial species were affected by changes in transit time (Child et al., 2006).

1.9.6 Colonic pH

Different locations in the colon have different pH with the proximal colon having a lower pH (as low as 4.5) than that of the distal colon (neutral)(Fallingborg et al., 1989). Low pH was associated with increased in acetate production, possibly due to reductions in lactic acid bacteria such as *Anaerosties caccae,* and *Eubacterium hallii*. As the pH increased to

~6.5 propionate and butyrate concentrations increased, likely due to increased populations of lactic acid bacteria (Belenguer et al., 2007).

1.9.6.1 *Effect of pH on SCFA production*

The distal colon frequently has a higher pH than the proximal colon because of a lack of fermentable substrate and improved colonic buffering. Slowly fermented substrates that reach the distal colon and undergo fermentation may be especially useful as most disease occurs in the distal colon (Edwards and Eastwood, 1995). *In vitro* investigations of the effect of pH on SCFA production are often carried out with the use of pH-controlled *in vitro* systems. For example, continuous culture studies conducted fermentations at a variety of pHs and clearly demonstrated an effect pH in each study but no overall pattern was established (Table 1-10) this may be due to the different bacterial profiles in each study. Walker et al., (2005) observed that after 200 hours increasing the pH from 5.5 to 6.5 led to changes in the bacterial composition using FISH analysis. A higher pH increased numbers of bacteria within the *Bacteroides- Prevotella* group, yet decreased *F.prausnitzi*i, and *Roseburia* spp both of which are butyrate producers. This is also supported by the changes in butyrate production where the low initial pH favoured butyrate production. In contrast, at the lower pH *Bacteroides*, and Clostridial cluster XVIa strains were reduced. As these bacteria yield propionate, this is supported by the observation that the higher pH favoured propionate production (Walker et al., 2005).

Table 1-10: SCFA proportion at different pH in controlled continuous fermentation systems

Reference	pH	Acetate %	Propionate %	Butyrate%
(Edwards et al., 1985)	5.0	54.8	37.0	8.2
(Edwards et al., 1985)	6.0	46.7	48.9	4.4
(Edwards et al., 1985)	7.0	67.4	29.8	2.8
(Walker et al., 2005)	5.5	70.0	4.8	25.3
(Walker et al., 2005)	6.5	69.3	15.4	15.4
(Belenguer et al., 2011)	5.5	50.7	23.0	19.3
(Belenguer et al., 2011)	6.0	53.4	29.8	16.9
(Child et al., 2006)	5.5	62.0	22.3	15.7
(Child et al., 2006)	6.2	61.4	23.5	15.1
(Child et al., 2006)	6.8	57.3	26.5	16.2
(Jiang and Savaiano, 1997)	5.7	42.4	52.1	5.4
(Jiang and Savaiano, 1997)	6.2	53.4	31.3	15.3
(Jiang and Savaiano, 1997)	6.7	61.4	38.5	0.1

Data presented are the molar percentage of the SCFA production

1.10 Enzymatic degradation of non-digestible carbohydrates

Colonic bacteria are responsible for catabolising the carbohydrates that have evaded digestion in the small intestine due to a lack of appropriate enzymes (CAZymes). For this carbohydrate catabolism, the colonic bacteria have developed a repertoire of saccharolytic enzymes that are upregulated when required. This enables the catabolism of a plethora of fibres and their constituent bonds, highlighting the symbiotic relationship that has developed between host and the gut microbiome. Different bacterial species have the ability to degrade different carbohydrates. For example, *Bacteroides thetaiotaomicrom* and *Bacteroides ovatus* are able to utilise a variety of different carbohydrate sources, with some common substrates but not all. *B.ovatus* could utilise hemicelluloses and beta-glucans, whereas *B. thetaiotaomicrom* could not (Martens et al., 2011). The importance of

this is demonstrated by metagenomic and population studies which have identified that after the house keeping genes, genes encoding the enzymes for the catabolism and utilisation of fibres and their sugars are predominant within the bacterial genome (Qin et al., 2010, Yatsunenko et al., 2012). To-date there are five classes of CAZymes; Glycoside hydrolases (GHs), Glycosyl Transferases (GTs), Polysaccharide Lyases (PLs), Carbohydrate esterases (CEs), Auxilary activities (AAs), and carbohydrate-binding molecules (CBMs), all of which are further described on the CAZyme database; CAZy (www.cazy.org)(Lombard et al., 2014). These classes of CAZymes consist of a wide variety of subfamilies, allowing a several carbohydrate structures and their linkages to be utilised (Lombard et al., 2014). CAZymes, along with other proteins are required for degrading polysaccharides and these genes are expressed on polysaccharide utilisation loci (PUL). PULs enable the utilisation of various substrates; an example is the starch utilisation system (SUS) enabling the degradation of starch (Martens et al., 2011, Rogers et al., 2013).

Due to the varied human diet, many different enzymes are required to utilise the different polysaccharides consumed. As it is metabolically expensive to express all the enzymes available, CAZyme expression is tightly regulated. The gut bacteria may be generalist or specialist, Bacteroidetes, a major phylum in the gut are generalists and able to utilise many different polysaccharides. However, *B. thetaiotaomicron* can catabolise pectin and starch, while *B. ovatus* utilises hemicelluloses and β-glucans. This difference even among generalists demonstrates the requirement for a selection of bacteria with different PULs and CAZymes (Martens et al., 2011, Rogers et al., 2013). Catabolism of dietary fibre to SCFA is determined by the colonic bacteria and the PULs present. The bacteria available determine the pathways used and as a result the SCFA that are produced. The bacteria may work in concert and crossfeed synergistically which means that changes in the population induced by dietary changes can alter SCFA pathways in complex interactions.

1.11 Pathways of SCFA production

Fermentation of fibres by colonic bacteria to acetate, propionate and butyrate requires a series of steps. The equation summarising SCFA production from glucose fermentation (Christian et al., 2003) is:

$$34.5\ C_6H_{12}O_6 \text{ --> } 48\ CH_3COOH + 11\ CH_3CH_2COOH + 5\ CH_3CH_2\ CH_2COOH + 23.75\ CH_4 + 34.2\ CO_2 + 10.5\ H_2O.$$

1.11.1 Acetate production

The main pathways of acetate production are the Embden-Meyerhof-Parnas (EMP) pathway (glycolysis) and Wood-ljungdahl pathway (reductive acetyl-CoA pathway) (Wolin et al., 1999). The EMP pathway produces acetate via pyruvate and acetyl-CoA using oxidative decarboxylation. This pathway utilises glucose, where one molecule of glucose leads to three molecules of acetate (Figure 1-6) (Wolin et al., 1999). The final step for acetate production requires acetate kinase and generates of one molecule of ATP (Munoz-Tamayo et al., 2011).

The Wood-ljungdahl pathway (reductive acetyl-CoA pathway) mobilises two molecules of carbon dioxide for the production of acetate. This pathway has two streams leading to the production of acetyl-CoA, these are the methyl and carbonyl (Figure 1-7). The methyl branch produces the methyl group of acetyl-CoA by forming a series of tetrahydrofolate molecules. The carbonyl end occurs by the reduction of carbon dioxide (CO_2) to carbon monoxide, a process which has been shown to be carried out by *Blautia hydrogenotrophica* (Louis et al., 2014).

For both pathways, acetyl-CoA is produced via acetyl-CoA synthetase, which then initiates a process leading to the production of acetate. Acetate then is either converted to butyrate or enters the colonic lumen and is then absorbed (Wolin et al., 1999).

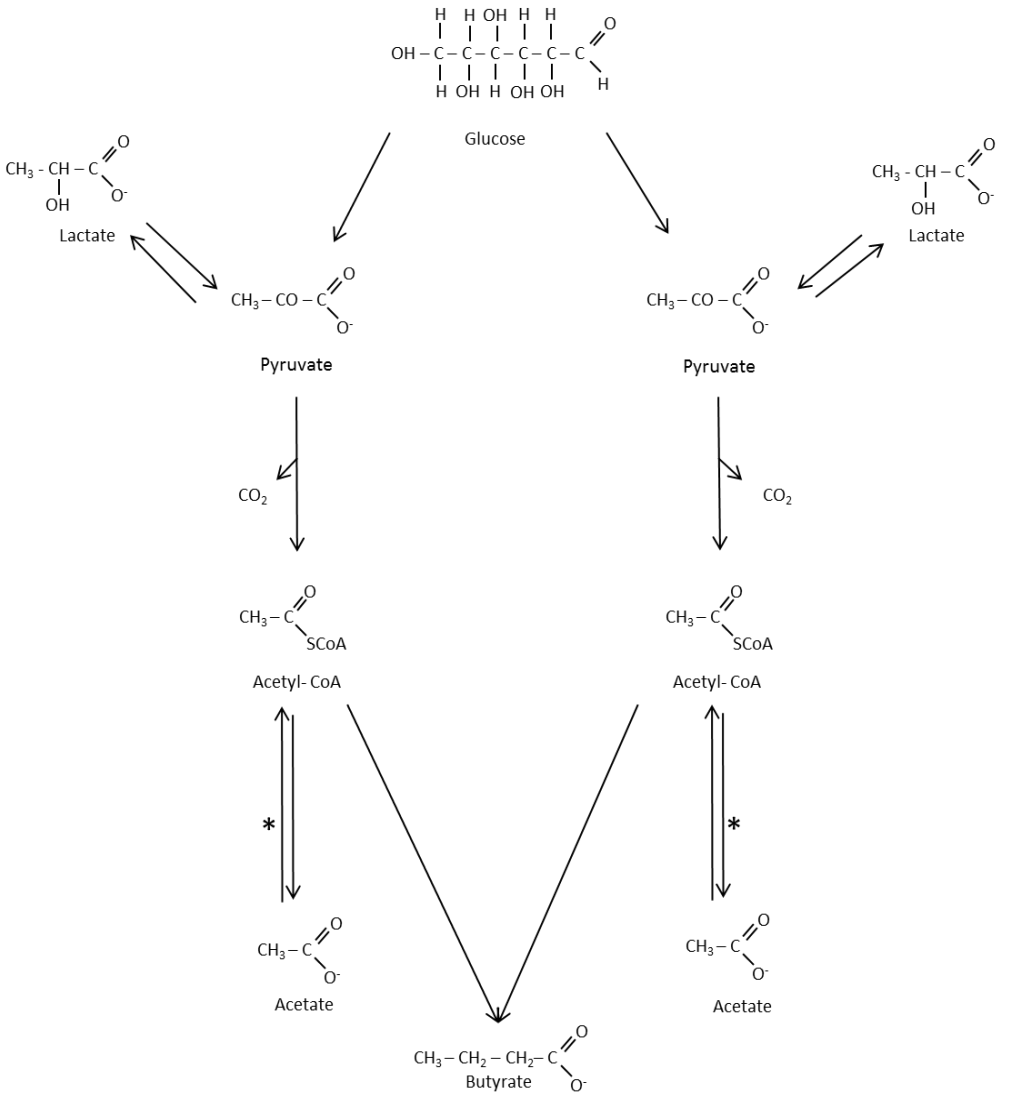

Figure 1-6: Embden-meyerhof-parnas pathway (glycolysis).

Acetate is production from glucose with the use of acetate kinase, and interconversion of acetate to butyrate occurs via acetyl-CoA. Asterix (*) indicates acetate kinase.

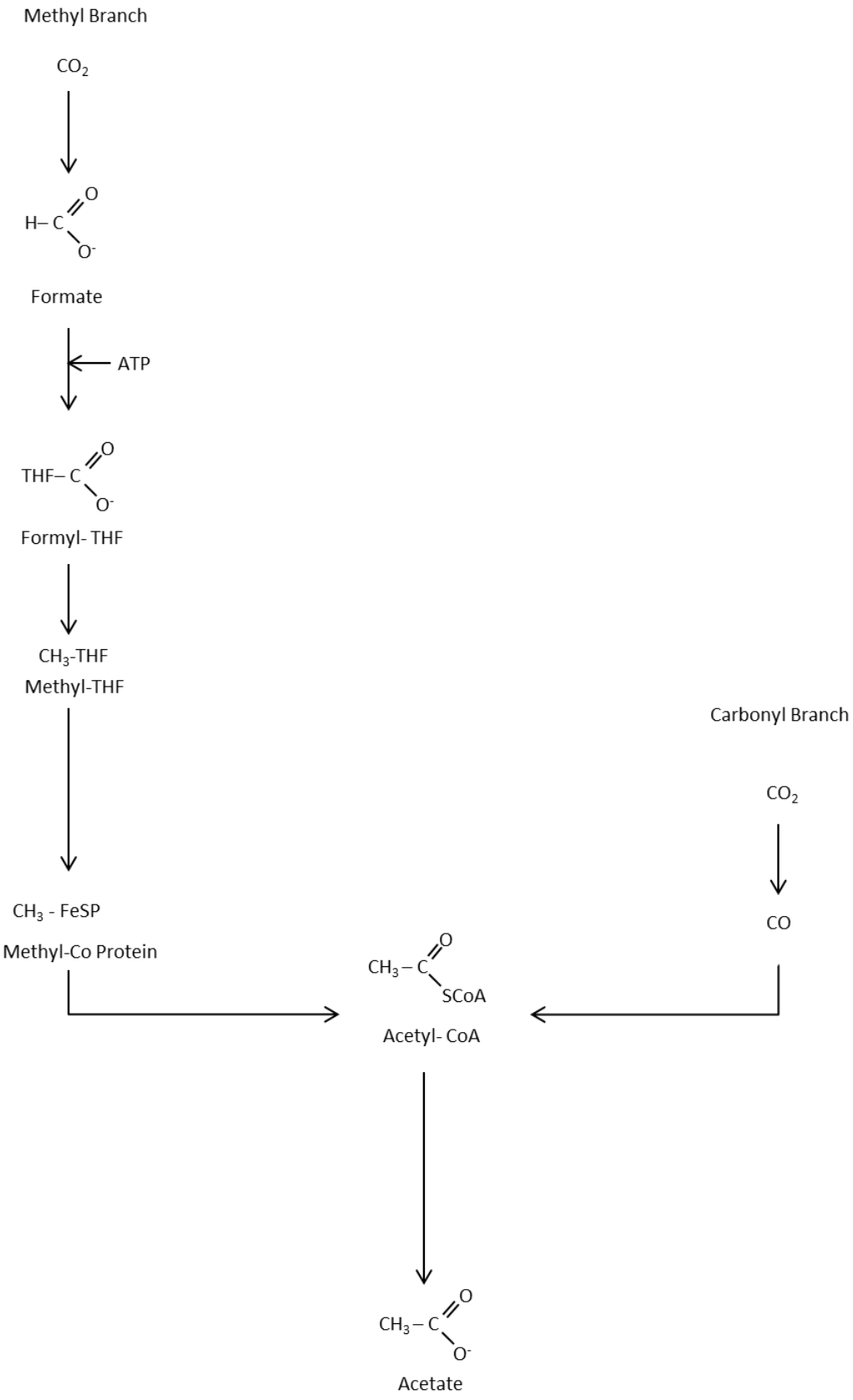

Figure 1-7: Wood Ljungdahl pathway (reductive acetyl-CoA pathway) of acetate production.

THF = tetrahydrofolate (FH_4)

1.11.2 Propionate production

1.11.2.1 *Succinate pathway*

The majority of propionate is produced via the succinate pathway (Wolin et al., 1999). This occurs by the conversion of oxaloacetate to succinate, then to propionyl-CoA then leading to the generation of propionate (Figure 1-8). When succinate is converted to propionyl-CoA one molecule of CO_2 is formed by the decarboxylation of methyl-malonyl-CoA. This is then further recycled for the re-carboxylation of oxaloacetate. Due to its symmetrical structure succinate is unique, this structure results in either of the two carboxylic acid ends being decarboxylated (Hosseini et al., 2011). Consequently, it is challenging to carry out pathway analysis of succinate. Using the isotopically labelled carbon, ^{13}C it was observed that after decarboxylation of succinate, 50% of the ^{13}C label was identified in methyl end and 50% was at the carboxyl end of propionate due to succinate being symmetrical (Wolin et al., 1999).

A variety of colonic bacteria produce propionate via the succinate pathway. *Bacteroides* strains such as *B.thetaiotaomicron* utilises methyl-malonyl-CoA in addition to a variety of polysaccharides and peptides to produce propionate (Reichardt et al., 2014). Bacteroidetes and *Veillonella parvula* (a type of negativicutes) produce succinate in the presence of lactate. *Phascolarctobacterium succinatutens* is a succinate utiliser and produces propionate from succinate (Reichardt et al., 2014). Other bacteria producing propionate via the succinate pathway include *Firmicutes,* Clostridial cluster IX (now called negativicutes) (which are present in high concentrations like *Bacteroides*) (Reichardt et al., 2014).

1.11.2.2 *Acrylate pathway*

The acrylate pathway produces propionate by forming acryl-CoA via lactate ultimately leading to propionate (Figure 1-8). There is a variety of different bacteria, which utilise the lactate pathway to produce propionate. These include *Eubcaterium hallii* and *Veillonella* spp*,* with the lactate being produced from pyruvate (Louis et al., 2014, Reichardt et al., 2014). *Coprococcus cactus* and *Megasphaera elsdenii* (negativicutes) also produce propionate via lactate (Louis et al., 2014, Reichardt et al., 2014).

1.11.2.3 *Propane-diol pathway*

The propane-diol pathway leads to the production of propionate with propanal being produced as a bi-product. *Salmonella enterica typhimurium* is an example of a bacterial species that has the ability to produce propionate via this pathway (Reichardt et al., 2014). *Roseburia inulinvorans* is also associated with propionate production when grown on fucose. *R.inulinvorans* it is associated with increased butyrate production when grown on glucose, demonstrating the role of substrate on the preference of SCFA production. Other ruminococcus species such as *Ruminococcus obeum* is also associated with producing propionate via this pathway, but also leads to butyrate production (Reichardt et al., 2014).

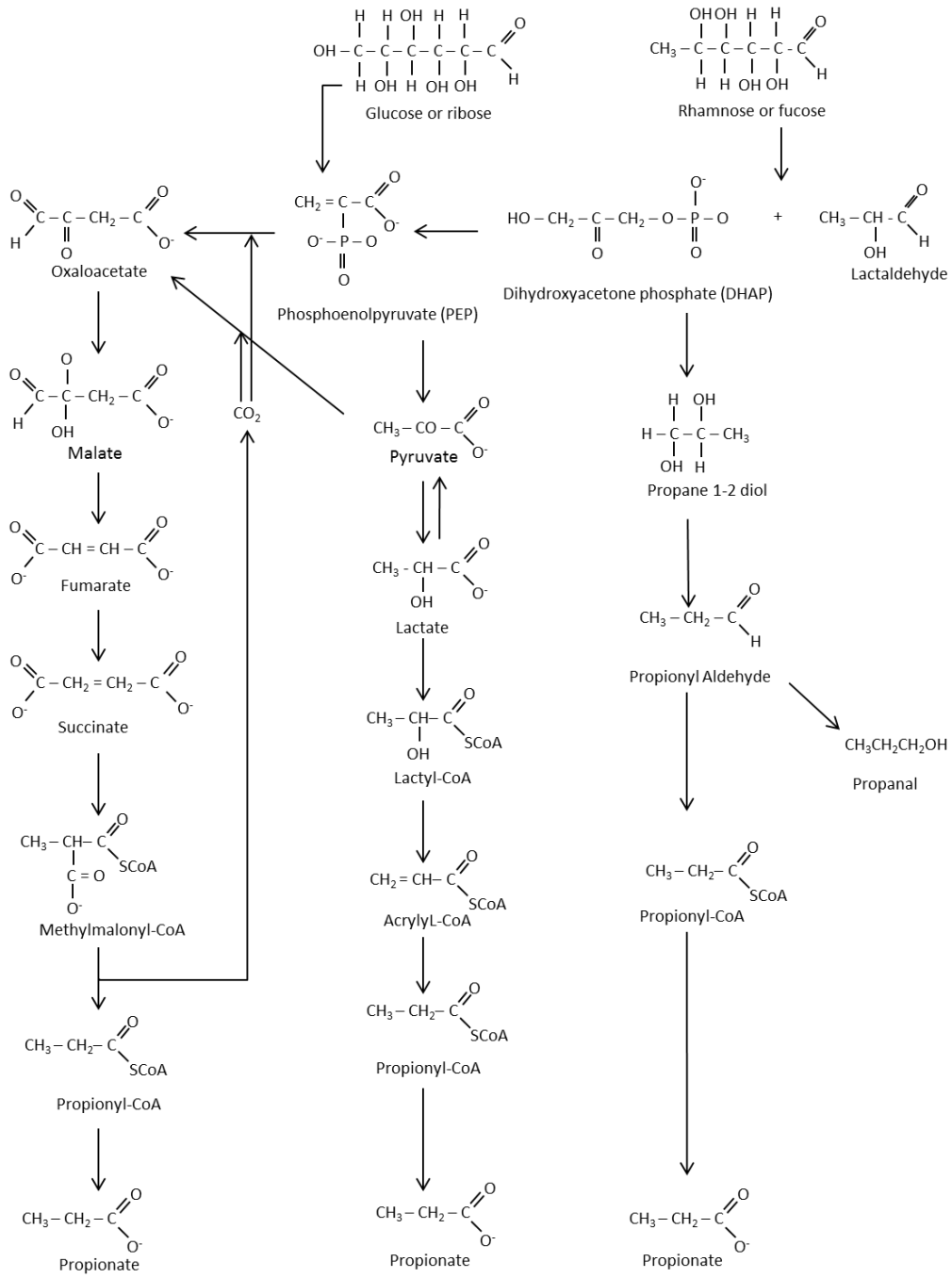

Figure 1-8: Pathways of propionate production,.

Left: Succinate pathway, Centre: Acrylate pathway, Right: Propane-diol-pathway based on (Reichardt et al., 2014)**.**

1.11.3 Butyrate production

Approximately 85% of all butyrate production occurs from the interconversion of acetate (Duncan et al., 2004) (Figure 1-6). Duncan et al., (2004) identified using *Roseburia intestinalis* and *F.prausntizii* that up to 90% of butyrate was produced from acetate, although this was not possible by all acetate producers (Duncan et al., 2004). For interconversion of acetate to butyrate to occur Butyryl CoA: Acetate CoA transferase is required and has been shown to be present in *F.prausnitzii*, *Eubacterium rectale, Roseburia faecis,* and *R.intestinalis* (Duncan et al., 2004, Reichardt et al., 2014, Louis et al., 2010).

About 15% of all butyrate is produced directly via the intermediates, butyryl-CoA and butyrate phosphate (Figure 1-9). For this to occur the bacteria possessing the enzyme, butyrate kinase is required. *B. thetaiotaomicron, B.fragilis, B. vulgatis, Coprococcus eutactus* as well as *Clostridium difficile* have the genes for butyrate kinase for direct butyrate production. Often bacteria that directly produce butyrate are unable to produce propionate suggesting that these bacteria only have the enzymes to allow for butyrate, or propionate production, but not both. There are exceptions to this rule, for example *C.eutactus* can produce propionate and butyrate, this occurs as it utilises lactate for propionate production and acetate for butyrate production (Reichardt et al., 2014).

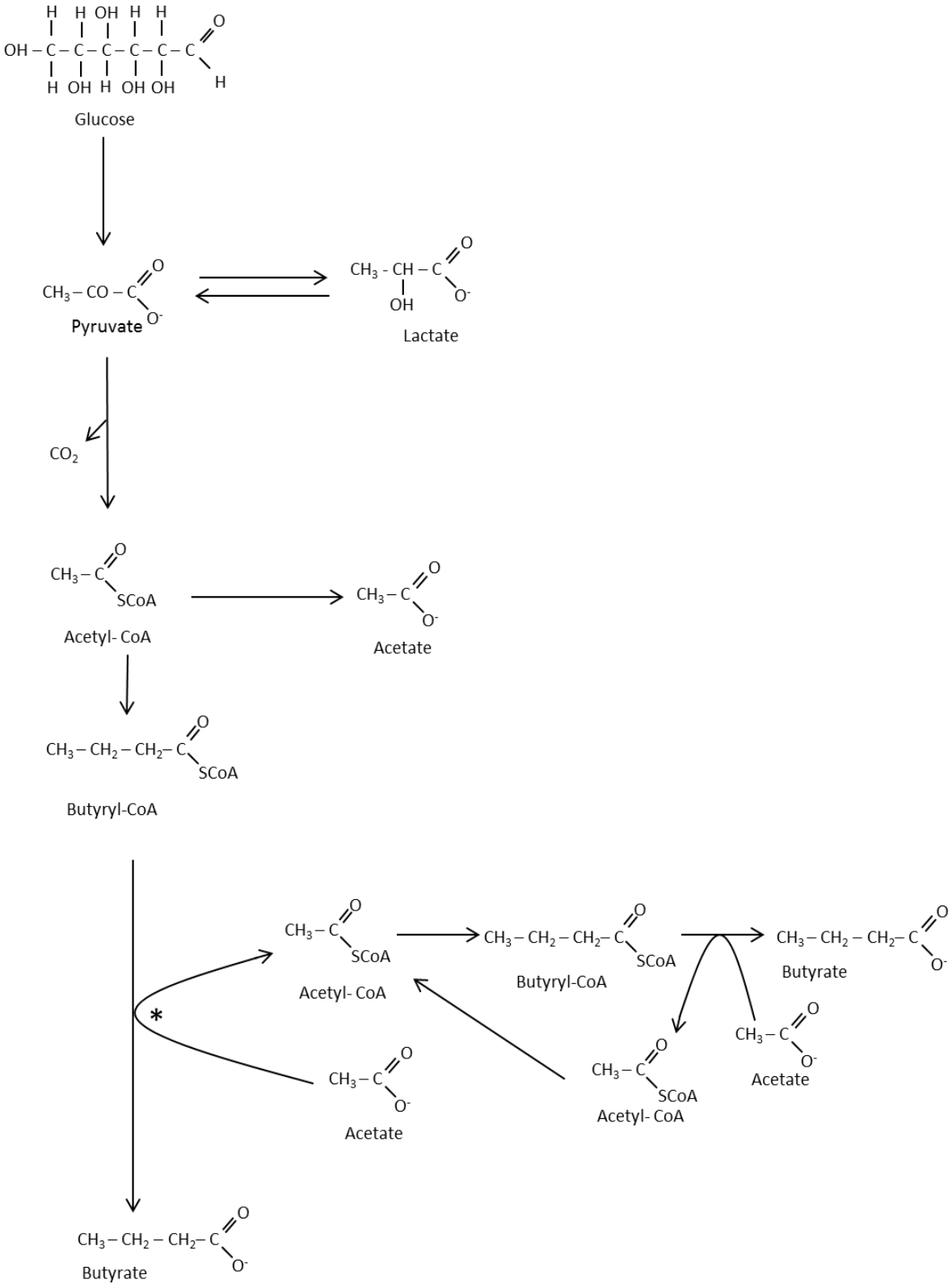

Figure 1-9: Direct Butyrate production and butyrate production by interconversion.

(*) indicates butyryl CoA: Acetate CoA transferase

This review of the potential roles of NDC and the production of propionate has highlighted many of the gaps in our knowledge, and in particular, the lack of a systematic approach in determining which physicochemical properties drive propionate production. Generally accepted assumptions about which substrates are propiogenic are not necessarily supported by the evidence and differences in methodology make comparisons of studies difficult. It is not yet fully understood if the type of NDC is the most important factor, or indeed what it is about an NDC that promotes different profiles of SCFA. It is not clear if the pH, bacterial profile or transit times are more influential.

1.12 Aims and objectives

What has been highlighted is the lack of a systematic approach for the assessment of which carbohydrates are best suited for the production of propionate. This PhD sought to elucidate some of the mechanisms that promote propionate production. This led to the following aims and objectives:

1. To carry out a systematic review of the investigations that have conducted *in vitro* batch fermentations of carbohydrate sources to identify propiogenic substrates.
2. To generate a new term to quantify SCFA production in cultures of faecal bacteria with different volumes and substrate concentrations.
3. To screen a selection of potentially propiogenic substrates identified within the systematic review, as well as other sources *in vitro* batch cultures using human faecal bacteria.
4. To explore the factors that increase propionate production such as the carbohydrate source, the role of bond anomer configuration and position using glucose-glucose disaccharides, initial culture pH.

Chapter 2 Materials and Methods

This chapter contains a discussion of the choice of model for the *in vitro* fermentation studies. It also describes the methods used in the experimental chapters but the rationale and overall design for each study are explained in the relevant chapters (Figure 2-1).

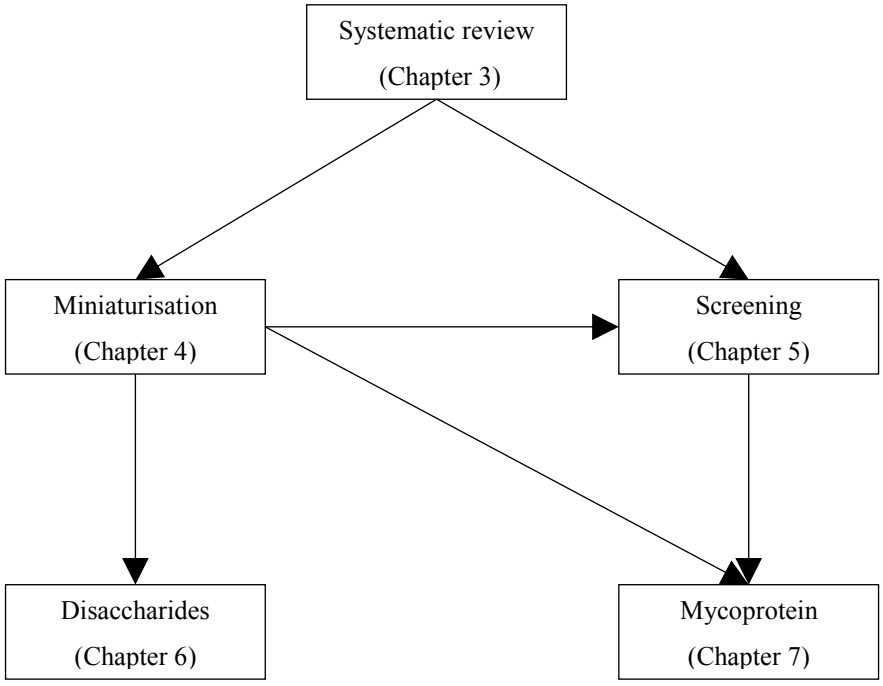

Figure 2-1: Flow chart of the experimental chapters within this thesis

2.1 Experimental models for assessment of the SCFA production of different substrates

The SCFA producing capabilities of different NDCs can be tested in a number of different ways in humans and animals. *In vitro* analysis has also been used to provide the information for *in silico* prediction of SCFA production (Kettle et al., 2015).

2.1.1 Human studies of SCFA production

Assessment of SCFA production in humans is difficult due to rapid absorption, and inaccessibility of the proximal colon. Few studies have adequately assessed the production in humans. Cummings et al., (1987) and Macfarlane et al., (1992) used samples from different parts of the colon of sudden death patients to assess SCFA concentrations as material moves along the large intestine. The same group also used sudden death victims to assess SCFA concentrations in the liver, and peripheral blood (Cummings et al., 1987).

Very few studies have assessed production itself. Bloemen et al., (2009) assessed production in surgery patients, but these were in the fasted state.

Assessing human SCFA production after consumption of a certain fibre is difficult as the proximal colon is so inaccessible. The impact of different foods on faecal SCFA (which is often presumed to be associated with production in the colon [Edwards and Eastwood., 1992]) has been estimated in human feeding studies (Table 1-5). These includes long term studies e.g. 28 days (Beards et al., 2010b) or for several months (Chambers et al., 2014) or acutely where the effect of a single meal is assessed (Tarini and Wolever, 2010). One of the main issues with these types of study is that it is difficult to control the habitual diet of the individuals tested.

The fermentability of a substrate can be estimated by measuring breath hydrogen, or measuring SCFA in blood samples (mostly acetate), urine (Verbeke et al., 2010) or in a faecal samples (Beards et al., 2010b). These methods have several issues when used as an index of SCFA production in the colon. For example, breath hydrogen is only an indicator of fermentation and does not give any information about the amount or types of SCFA production. Breath hydrogen can also be reduced if colonic hydrogen is used by methanogens in the colon, which are present in about one third to half of the population (Fernandes et al., 2000). Measuring SCFA in blood plasma is also fraught with difficulties as butyrate is extensively metabolised by the colonic enterocytes. The remainder of the butyrate and propionate are removed in the liver, and it is mainly acetate which reaches the peripheral blood (Bloemen et al., 2009). However, acetate is also made by human cells and fasting levels are likely to be of human rather than bacterial origin. Urinary SCFA are often at low concentrations and mostly consist of acetate with very low amounts of propionate and butyrate. Faecal SCFA can be misleading as 95% of the SCFA produced in the colonic lumen are absorbed (den Besten et al., 2013b). Assessment of colonic SCFA by measuring faecal samples is further hindered as each SCFA has been shown in a rectal infusion study, to be absorbed at different rates, with butyrate being preferentially absorbed over acetate (Vogt and Wolever, 2003).

An alternative is to use stable isotopes which could be employed as a direct measure of SCFA production by feeding a labelled NDC and measuring labelled SCFA, or indirectly by infusing labelled SCFA and noting the dilution by exogenous production. This method

was employed by Boets et al., (2015), by feeding 15g of ^{14}C-carboxylic acid inulin to healthy adults. This provided both assessment of breath hydrogen and breath $^{14}CO_2$ to provide a marker of fermentation and transit time (Boets et al., 2015). Assessment of acetate production, also used a similar method, where unlabelled lactulose was consumed and an infusion of ^{13}C- acetate given only. This enabled the analysis of breath hydrogen production and acetate concentrations in the peripheral blood, which enabled colonic acetate production to be calculated (Pouteau et al., 1998). An issues this this methodology is that to quantify SCFA production the concentration of splanchnic uptake of SCFA is required, which is variable between individuals, thus making it an unreliable method of assessing SCFA production.

2.1.2 Animal studies of SCFA production

Animal models are another way to assess the SCFA produced from different substrates. Animal models allow better regulation of the environment, food consumption and other aspects of control than is possible in human trials. The main advantage of animal studies is that the caecum and different regions of the colon can be accessed, which is not possible in healthy free-living humans. Animal studies can also provide direct access to the tissues and allow more mechanistic studies to be carried out. For example mouse models have shown an effect of diet on the bacterial profile (Ridaura et al., 2013), the effect of SCFA on induced pathologies, e.g. inflammation (Trompette et al., 2014), and the mechanisms by which some of the down-stream effects of SCFA such as food intake, body weight and hormone release (Lin et al., 2012).

There are also disadvantages in using animals to assess the production of SCFA by different substrates. Animal studies can be very expensive and different species have different physiological systems to humans, making it difficult translate findings. For example rats are coprophagic but are often used. Coprophagy may alter gut bacterial profiles and SCFA produced, causing further difficulties in the translation to the human gut but coprophagy can be reduced with suitable housing (Ridaura et al., 2013). Edwards and Eastwood (1992) fed rats ispaghula or wheat bran for 28 days. After sacrifice, the colon and cecum was removed and SCFA production within these regions, as well as in the faeces was measured. This led to the observation that SCFA concentrations reduced from the cecum to the faeces with a standard diet. It also showed that ispaghula consumption

increased SCFA concentrations in the whole of the large intestine (Edwards and Eastwood, 1992).

Assessment of SCFA production from different substrates has also been carried out in pigs that were cannulated via the cecum, giving access to the caecal contents. This model was used to assess the effect of 2 weeks of chicory or sugarbeet pulp in the animal diet on SCFA production. It showed that propionate increased by 28% with the diet containing sugarbeet pulp compared with chicory (Ivarsson et al., 2012). Animal models should be validated against human physiology where possible and any extrapolation of results should be made with caution (Sunvold et al., 1995, Edwards et al., 1992).

2.1.3 *In vitro* models of fermentation

Much of the assessment of the SCFA production from a dietary fibre is carried out using *in vitro* methodologies. Due to the location of the colon and the rapid absorption of SCFA from the colon, it is impractical to investigate colonic SCFA production directly in humans. Many different *in vitro* models mimicking the gut have been developed. These systems range from simple batch fermenters modelling only the large intestine, to those that aim to model the whole gut such as the simulator of the human intestinal microbial system (SHIME)(Molly et al., 1993), these are discussed below.

In vitro models can be inoculated by human or animal gut bacteria and from animals this can be from caecum or colonic contents as well as faeces. These are discussed below and Table 2-1 and are also reviewed in (Williams et al., 2015), (Payne et al., 2012) and (COST Action FA1005, 2015).

2.1.3.1 *Batch fermentations*

Batch fermentations are static fermenter systems that can provide different levels of control depending on the user's needs. At a basic level, these systems require a source of bacteria, often from a faecal slurry, some liquid such as phosphate buffer or a nutrient containing buffer a substrate to ferment (although a substrate free control is also needed), all within an anaerobic vessel. Additional controls can include temperature control (with a water bath or incubator) and movement (by a stirrer or shaking water bath). These systems

are effective for approximately 48 hours but have been used for up to 72 hours (Rasmussen et al., 1988). This method has been used on numerous occasions (Chapter 3) and various attempts have been made to standardise the protocol between different institutions (Barry et al., 1995, Edwards et al., 1996) (COST Action FA1005., 2015). A more controlled form of batch fermentation is also available where the pH is maintained at a chosen level for the duration of the fermentation by the addition of an acid or a base.

Batch fermentations were utilised by Sunvold et al., (1995) to assess the SCFA producing capabilities of four NDCs (cellulose, beet pulp, citrus pulp and citrus pectin) using faecal samples from humans, dogs, cats, pigs, horses and ruminal fluid of cattle for 48 hours. Patterns of SCFA production were similar for all (although concentrations were not), and that the substrate composition (e.g solubility) and fermentability was important in determining SCFA production in all of the inoculum tested (Sunvold et al., 1995).

Variations of batch systems

As these batch systems are quite simple and there are many ways to alter the system. For example, different investigations use different media. Adiotomre et al., (1990) used a media that comprised of a selection of trypticase, minerals and a carbonate-phosphate buffer, whereas Barry et al., (1995) utilised the same buffer but without the trypticase. In contrast, Edwards et al., (1996) did not use a complex media but carried out fermentations of starch with phosphate buffer and faecal inoculum alone. Other modifications of the media include the addition of an amino acid source such as yeast extract (Deville, 2007), casein hydrolysate (Belenguer et al., 2007), or brain heart infusion (Cherbut et al., 1991). Although complex media are not required *per se* for batch fermentations, complex media can be used in an attempt to promote bacterial growth by providing all of the substrates required. These media can also contain low concentrations of carbohydrate sugars for fermentation (e.g 0.015% w/vol arabinoxylan), and low concentrations of SCFA (35 mmol/l) (Belenguer et al., 2007). Additional bacteria, such as *B.thetaiotaomicron*, and *E.coli* have previously been added to the media to assess their role in SCFA production (Dongowski et al., 2000).

Fermentation systems are made anaerobic in a number of ways such as with the use of carbon dioxide, reducing agents (e.g sodium sulphide, cysteine hydrochloride) (Adiotomre

et al., 1990), oxygen free nitrogen (Edwards et al., 1996), anaerobic chamber (Cardelle-Cobas et al., 2009) and oxyrase for broth (Rumpagaporn et al., 2012). When the fermentation system is not in an anaerobic chamber, anaerobic conditions are maintained by sealing the bottle so it is airtight.

Fermentations inoculums can also consist of a single stool sample (non-pooled), or with multiple samples that have been combined and used as the inoculum (pooled) (Aguirre et al., 2014).

Substrates within the fermentation system can also differ where different quantities of substrates are added to the vials. For example, Khan and Edwards., (2002) increased the amount of substrate in the fermentation vials from 2.5 mg/ml to 10.0 mg/ml in 2.5 mg/ ml increments and found that higher substrate concentrations were inhibitory for SCFA production after 24 hours of fermentation. This was likely as a result of product inhibition due to increased SCFA in the fermentation vials, reducing further SCFA production (Khan and Edwards, 2002). As well as different substrate quantities differences in fermenter volume are used, for example Sanz., et al (2005) used a final volume of 1 ml, Edwards et al., (1996) conducted fermentations in 10 ml, and Adiotomore et al., (1990) had a final volume of 50 ml.

The fluid surface area to fluid volume ratio (SA:V) of the fermenter vial has also been demonstrated *in vitro* to alter total SCFA production after 24 hours (Stevenson et al., 1997). The SA:V from 1:1 to 1:4, was reduced by changing the position of the fermentation bottle within the incubator, which reduced the accessibility of the bacteria to the substrate. Pectin, starch and ispaghula were fermented and total SCFA production was reduced with SA:V ratio of 1:4 for pectin and starch, but not ispaghula and only at high substrate concentrations (10 g / L^{-1}). Given that the effect of SA:V was inconsistent across the substrates tested, it remains unclear whether variations in SA:V truly alters the rate and amount of substrate fermentation or whether this phenomenon is restricted to selected substrates.

Sampling time points also differ between studies. The ability to take samples can be limited by the volume in the fermentation vessel. Sanz et al., (2005) used one time point for SCFA analysis (12 hours) as fermentations were conducted with a final volume of 1ml.

Kaur et al., (2011) had four time points (4, 8, 24, and 48 hours) and removed the fermentation vial at each time point for analysis and Khan and Edwards., (2002) took samples from the same fermenter vial after 4, 8, and 24 hours. Therefore, SCFA production could seem to be different at each time point as different fibres are utilised at different rates. Pylkas et al., (2005) sampled at 0, 2, 4, 8, 12 and 24 hours and showed that SCFA production from cellulose was complete by 8 hours, production from polydextrose was complete by 12 hours and guar gum was not fully fermented after 24 hours (Pylkas et al., 2005). Batch cultures change in growth characteristics with time. They have an initial lag phase followed by exponential growth, a stationary phase and then death. In contrast, continuous or semi-continuous cultures are maintained at steady state, which better reflects conditions in the human colon.

2.1.3.2 *Continuous culture fermentations*

Continuous fermentations enable the *in vitro* assessment of different substrates at steady state but with more control, dilution of fermentation products, which reduces product inhibition, and often for a longer duration, and increases the complexity as the amount of regulation from the single stage to the multistage models increases. These systems vary in complexity with some having only one chamber and others mimicking the whole gut but also have pros and cons. These systems all generate data that can be easily compared to the human system, and can assess changes in bacterial profiles, without the requirement of metagenomic analysis such as those conducted by De Filippo et al., (2010) and David et al., (2014).

2.1.3.3 *Single stage fermenters*

These systems mimic the proximal colon only, and maintain the volume, pH, temperature, and mass of substrate within the system. These models can be used and sampled for a period of up to approximately 3 weeks.

This system has been adopted by Walker et al., (2005) and Edwards et al., (1985) where the ability to modify the pH during the fermentation was used to assess the effect of a shift in pH on the bacterial populations as well as SCFA production. For fermentation studies of food, a pre-digestion stage is often needed to remove material that would normally be digested and absorbed in the upper gut. This can be performed with *in vitro* models of

upper gut digestion but could also be done after consumption of foods by ileostomy patients and then their effluent used as a fermentation substrate. For example, this methodology was used by Fassler et al., (2007) who used the ileostomy effluent of ileostomy patients who consumed 40 g/day of resistant starch type 3 for three days, this pre-digestion step provided substrate to be fermented with batch and continuous culture (Fassler et al., 2007).

2.1.3.4 *Multi stage fermenters -Colonic systems*

The fermentation systems become much more complex with the multi-chamber systems. These systems can model different areas of the colon, such as the proximal, transverse, distal, and sigmoid colon/ rectum. These systems have a variety of different uses in studying the metabolism of substrates in the large intestine. For example, the three- stage continuous model, which has three vessels at different pH (proximal, transverse, and distal) can undergo the control that occurs with single stage fermenters but also allows the contents of each vessel to move into a different vessel, i.e. modelling the movement along the colon. The investigation by Child et al., (2006) used this system to assess the changes in bacterial and SCFA composition at different colonic pH for a period of 60 hours (Child et al., 2006).

2.1.3.5 *Multi stage fermenters –Gastrointestinal tract systems*

Gastrointestinal tract systems that mimic the whole gut have also been developed, examples include, the SHIME system (Molly et al., 1993) and the Toegepast Natuurwetenschappelijk Onderzoek (TNO) intestinal model (TIM) (Minekus et al., 1999) and are controlled via a computer. A further advantage of these systems is that they are both able to mimic 'absorption' by dialysis, in which the dialysate contains metabolites such as SCFA. The SHIME system models the stomach, SI, ascending, transverse and descending colon, this system can be maintained for many days and is highly controlled. Unlike other continuous models this also enables 'digestion' to be part of the assessment with the use of digestive enzymes and the absorption of the metabolites produced in the upper GI tract. This model requires approximately 14 days for stabilisation (Molly et al., 1993). There are two different TIM models, TIM-1 and TIM-2. TIM-1 models the upper gut whereas TIM-2 models the colon. TIM-2 consists of a loop of four different containers that are able to mimic peristaltic mixing and is able to function alongside the TIM-1

model, and enables the assessment of the absorption of metabolites up to 50kDa in size by dialysis (Minekus et al., 1999). Both of these systems have been utilised and compared in the investigation by Van den Abbeele et al., (2013) where the SHIME and the TIM-2 model were compared in terms of fermentation of long chain arabinoxylan and inulin. Both models demonstrated similar effects of the NDC on SCFA production and bacterial composition (Van den Abbeele et al., 2013).

Table 2-1: In vitro models for investigating SCFA production by different substrates

Model	Description	Advantages	Disadvantages
Batch[1]	Static fermenter system	• pH can be controlled • Temperature control • Allows screening of many samples • Inexpensive and does not require high level skills	• pH may change rapidly when not controlled • Build-up of potentially inhibitory products • Simplistic model of the colon • Short term
Single- chamber continuous[2]	Semi-continuous	• Steady state • Pulses of new media mimic ileal delivery • Provide pH, temperature, stirring control • Can use for longer durations • Effective for assessment of bacterial changes	• More expensive than batch techniques • Simplistic model of the colon • Low throughput
Three-stage continuous [3]	Three chambers at pH 5.5 (proximal), 6.2 (transverse) and 6.8 (distal)	• Allows different parts of the colon to be investigated • Can be long term • Provide pH control • Reliable • Inexpensive	• Does not account for absorption, or other secretions • Low throughput • Short term <1 week
EnteroMix® semicontinuous culture[4]	Four chambers at pH 5.0 (proximal), 6.0 (transverse), 6.5 (distal), and 7.0 (sigmoid colon/rectum)	• Allows different parts of the colon to be investigated • Many experiments can be conducted at the same time	• Expensive • Only short term experiments
SHIME[5]	5 reactors; Stomach, Small intestine, proximal, transverse and distal colon	• Digestion • Continuously stirred • Computer controlled, increasing reliability controlled • Continuous stirring	• Expensive • Requires 2 weeks of stabilisation. • Low through put
TIM-1[6]	8 reactors; stomach, Small intestine (duodenum, jejunum, ileum)	• Digestion • Computer controlled • SI peristaltic pump moving contents • Can alter gut transit time • Absorption • Allow dialysis of 5 kDa	• Expensive • Low throughput • Expertise required
TIM-2[7]	4 looping reactors as proximal colon	• Peristaltic pump for mixing • Membrane allows nutrient absorption • Allow dialysis of 50 kDa	• Expensive • Low throughput • Expertise required • Limited to three days

Table based on 1. (Edwards et al., 1996),(Khan and Edwards, 2005), 2. (Edwards et al., 1985), 3. (Macfarlane et al., 1998), 4. (Makivuokko et al., 2005), 5. (Molly et al., 1993), 6. (Minekus et al., 1995), 7. (Minekus et al., 1999, Williams et al., 2015, Payne et al., 2012)(COST Action FA1005., 2015).
Abbreviations: SHIME- Simulator of the human intestinal microbial ecosystem, TIM- TNO intestinal model, SI- Small intestine.

2.2 Methods used in this thesis

Due to the cost effectiveness and the ability to screen a large number of samples the *in vitro* batch system, without continuous pH control were used. These systems were inoculated with stool samples from healthy individuals in a system adapted from that of Adiotomre et al., (1990) and Laurentin and Edwards., (2004).

2.2.1 Batch fermentations

2.2.1.1 *Faecal sample collection and preparation*

Stool samples were obtained from healthy Caucasian individuals and prepared within 2 hours of passage. The samples were stored with an anaerobic sachet (Oxoid, Basingstoke, UK) within an airtight bag to facilitate an anaerobic environment, and placed within a bag containing a frozen icepack. Participants had not taken antibiotics within the previous 6 months and had no GI disease. Details of the number, and age and gender of individuals who provided stool samples are provided within each chapter.

Ethical permission was granted by the University of Glasgow, College of Medical, Veterinary and Life Sciences (MVLS) Ethics Committee, with the faecal donors giving informed written consent (information sheet in appendix 1 and 2).

Faecal samples were homogenised initially with a wooden tongue depressor, until the sample was uniform. An aliquot was combined with sodium phosphate buffer (Sorenson's buffer (0.133 M) 36 parts 0.066 M KH_2PO_4 and 61 parts 0.066 M Na_2HPO_4 at pH 7), to form a 32% faecal slurry and mixed with a blender until the sample was fully homogenised. Once homogenised, the faecal slurry was strained through a nylon stocking and immediately injected into the airtight fermentation vials. The bottle was then flushed with oxygen free nitrogen (OFN) to create an anaerobic environment.

2.2.1.2 *Fermentation Media*

Standard fermentation media

The fermentation medium as described in Adiotomre et al., (1990) was prepared 24 hours (or less) prior to the fermentation experiment and stored at 4ºC. To produce 1 L of fermentation medium; 2.25 g tryptone, 450 ml dH_2O, 112.5 µl micromineral solution, 225 ml bicarbonate buffer solution, 225 ml macromineral solution (Table 2-2) and 1.125 ml 0.1% resazurin to indicate anaerobic conditions. This then boiled on a heat pad for five minutes to sterilise and remove any dissolved oxygen and cooled under OFN until 37ºC. The fermentation medium was adjusted to pH 7 with 6 M HCl, added to the sterile, airtight fermentation vessels (no faecal slurry was present) and these were gassed with OFN until the vessel contents were anaerobic (based on the colour of resazurin). From this point onwards, the vessels remained airtight (further discussed below). Any alterations to this media are detailed in the chapter in which the relevant modification was introduced.

Impact of initial pH studies.

The experiments were based on the standard fermentation media method. The only modifications were the medium composition and the pH adjustment. All other processes were the same.

pH 6.8: Per 1 L of fermentation medium; 2.25 g tryptone, 112.5 µl micromineral solution, 225 ml bicarbonate buffer solution, 675ml macromineral solution (pH 6.8) (Table 2-2) and 1.125 ml 0.1% resazurin to indicate anaerobic conditions. pH was adjusted to 6.8 after boiling.

pH 5.4: Per 1 L of fermentation medium; 2.25 g tryptone, 112.5 µl micromineral solution, 225 ml bicarbonate buffer solution, 675 ml macromineral solution (pH 5.4) (Table 2-2) and 1.125 ml 0.1% resazurin to indicate anaerobic conditions. pH was adjusted to 5.4 after boiling.

Table 2-2: Composition of the solutions required to make 100ml of micromineral medium, bicarbonate butter and the standard, pH 6.8 and pH 5.4 macromedia media

Micromineral Medium	Bicarbonate Buffer	Macromineral Solution - Standard	Macromineral Solution - pH 6.8	Macromineral Solution - pH 5.4
13.2 g $CaCl_2.2H_2O$	0.4 g NH_4HCO_3	0.57 g Na_2HPO_4	0.3 g Na_2HPO_4	1.59 g Na_2HPO_4
10 g $MnCl_2.4H_2O$	3.5 g N_aHCO_3	0.62 g KH_2PO_4	0.28 g Na_2PO_4	0.85 g $C_6H_8O_7$
1 g $CoCl_2.6H_2O$	100 ml dH_2O	0.06 g $MgSO_4.7H_2O$	0.06 g $MgSO_4.7H_2O$	0.06 g $MgSO_4.7H_2O$
8 g $FeCl_3.6H_2O$		100 ml dH_2O	100 ml dH_2O	100 ml dH_2O
100 ml dH_2O				

All reagents were made prior to fermentation, dH_2O = distilled water

Table 2-3: Composition of the reducing solution per 100ml

Per 100ml	Reducing solution reagents
0.623g	$HSCH_2CH(NH_2)COOH$ HCl (Cysteine hydrochloride)
0.623g	$Na_2SO_4.9H_2O$ (Sodium sulphide non anhydrate)
4ml	1M NaOH
96ml	dH_2O

Solution was made just before use, dH_2O = distilled water

2.2.1.3 *Fermentation method*

Fermentations experiments were conducted with substrate sample sizes of 1g, 0.2g, 0.1g, and 0.05g. For all investigations the method was scaled proportionately (Table 2-4, Figure 2-2).

Table 2-4: Differences in quantities used between different vial sizes

	Standard	Mid-sized	Medium	Small
Vial Size	100ml	27ml	10ml	6ml
Substrate added	1g	0.2g	0.1g	0.05g
Fermentation medium	42ml	8.4ml	4.2ml	2.1ml
Reducing solution	2ml	400µl	200µl	100µl
Faecal slurry	5ml	1ml	500µl	250µl
Samples taken at each time point	3ml	1.6ml	800µl	400µl
NaOH	1ml	600µl	300µl	150µl

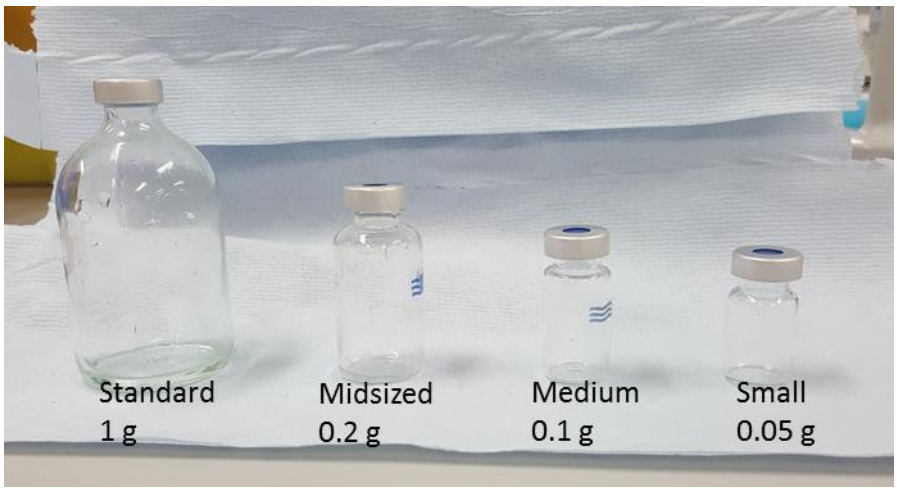

Figure 2-2: Vials used for the different sized fermentation systems:

Standard: Minaturisation (Chapter 4), Midsized: pH change, propiogenic substrates, starch fractions (Chapter 5), Medium: Miniaturisation (Chapter 4) beta glucans, legumes, starch fractions (Chapter 5), mycoptrotein (Chapter 7), Small: Miniaturisation (Chapter 4), Disaccharides (Chapter 6)

The 'standard' fermentation method is discussed here. On the day of the incubations the fermentation media was boiled for 5 minutes and cooled in OFN to 37°C, ensuring that all oxygen was removed from the medium, based on the colour change of resazurin (purple to clear). The pH was then readjusted to give a final pH of 7.0.

To each 100 ml bottle containing 1 g of each test substrate (Figure 2-2), 42 ml of reduced fermentation medium (Table 2-2) and 2 ml of reducing solution (Table 2-3) were added. Bottles were crimp sealed with silicon septa and aluminium caps (so gas tight) and degassed with OFN for one minute with the use of a needle (one for the OFN, another to allow oxygen to be displaced) ensuring media was clear/ pale pink, demonstrating the lack of oxygen in the vial. A 5 ml aliquot of filtered faecal slurry was injected into the bottles and degassed with OFN for a further minute. At 0 hours an aliquot of fermentation sample was obtained with a needle and syringe bottles were then incubated at 37^{o}C at 60 strokes/min. Aliquots of 3 ml were obtained with a needle and syringe at each time point and the pH measured with a (Mettler Toledo) pH meter, when required a semi-micro pH probe (Mettler Toledo) was used. To preserve and store the SCFAs, 1 ml of 1 M NaOH was added and then frozen at -20^{o}C until extraction. All investigations had aliquots taken at 0 and 24 hours, other time points used are discussed within each relevant chapter. The sample volume taken and therefore the amount of 1 M NaOH required was dependent on the size of fermentation vial, volumes are in shown in (Table 2-4, Figure 2-2).

2.3 SCFA Analysis

SCFAs were extracted from fermentation slurries at each time point and underwent analysis by GC:FID.

2.3.1 SCFA extraction

To allow quantitation of each SCFA, an external standard curve and internal standard were used to calculate SCFA concentrations within each sample. The external standard contained known concentrations of acetic acid (C2, 183.51 mM), propionic acid (C3, 132.52 mM), butyric acid (C4, 107.06 mM), valeric acid (C5, 88.63 mM), caproic acid (C6, 74.36 mM), enanthic acid (C7, 66.25 mM), caprylic acid (C8, 57.84 mM), isobutyric acid (iC3, 104.13 mM), isovaleric acid (iC5, 86.59 mM), and isocarpoic acid (iC6, 50.93 mM) all of which were contained in 2 M NaOH to prevent loss of SCFA. To account for losses of SCFA in the standards and samples an internal standard of 2-Ethyl butyric acid (73.8 mM, also in 2 M NaOH) was also added to all samples. SCFA were extracted from the external standard with the following method. Standards extracted by adding different concentrations of standard which was made up to 800 µl with distilled water, 100 µl internal standard, 100 µl orthophosphoric acid (OPA), and 3 ml diethyl-ether, per sample Table 2-5. This was then vortexed for 1 minute, the ether layer removed and 1 ml of diethyl-ether added and vortexed for 1 minute again, for a total of 3 times. For the samples, the same process was used with 800µl of slurry used in lieu of the 800 µl standard/water mix.

Due to a minimum volume of available slurry from the reduced size fermentation systems, this extraction method was reduced to 200 µl of sample. Here, 50 µl or 25 µl of OPA and 50 µl or 25 µl internal standard were added, and 1 ml of diethyl-ether was used (Table 2-5). This was then vortexed for 1 minute, the ether layer removed and 1ml of diethyl-ether added and vortexed for 1 minute again, for a total of 3 times. The external standards were also reduced so the final volume of standard/ water was 200 µl.

Table 2-5: Production of standards for a standard curve for a final volume of 1000µl or (300µl)

External standard volume (µl)	Water (µl)	Internal standard (µl)	OPA (µl)	Final volume (µl)
0	800 (200)	100 (50,25)	100 (50,25)	1000 (300,250)
10	790 (190)	100 (50,25)	100 (50,25)	1000 (300,250)
25	775 (175)	100 (50,25)	100 (50,25)	1000 (300,250)
50	750 (150)	100 (50,25)	100 (50,25)	1000 (300,250)
100	700 (100)	100 (50,25)	100 (50,25)	1000 (300,250)
200	600 (0)	100 (50,25)	100 (50,25)	1000 (300,250)

External standard volume of 100 µl discussed as the 'standard 100'. Values in brackets show the reduced volumes required for the miniaturised systems.

2.3.2 Gas chromatography with flame ionising detector (GC:FID)

A TRACE 2000 chromatograph (ThermoFisher Scientific, Loughborough, UK) was used to measure SCFA production. Peaks were identified and measured using Chrom-Card 22 bit data system (ThermoFisher Scientific) where the data was processed in Microsoft Excel 2013. The GC:FID measurement parameters and system are described in Table 2-6 and Figure 2-3.

Table 2-6: GC:FID measurement parameters used

Oven	
Initial temperature	80ºC for 1 minute
Ramp 1	Temp increasing by 15ºC/minute to 210ºC, held for 1 minute
Maximum oven temperature	260ºC for 10 minutes
Equilibration time	0.25 minutes
Total oven run time	10.67 minutes
Column Details	
Zebron ZB- Wax capillary column (catalogue no. 7EK-G007-22)	15 m (length) x 0.53 mm diameter (ID) x 1.0 µM film thickness. Polythene glycol phase. Manufactured by Phenomenex (Cheshire)
Splitless column	
Inlet Temperature	230ºC
Inlet Split Flow	36 ml/minute
Split-less time	0.5 minutes
Detector Details	
Base temperature	250 ºC
Air	350 ml/minute
Hydrogen	25 ml/minute
Nitrogen (carrier gas)	30 ml/minute
Injection parameters	
Sample injection	1 µl (hand injection 2 µl)
Air	1 µl

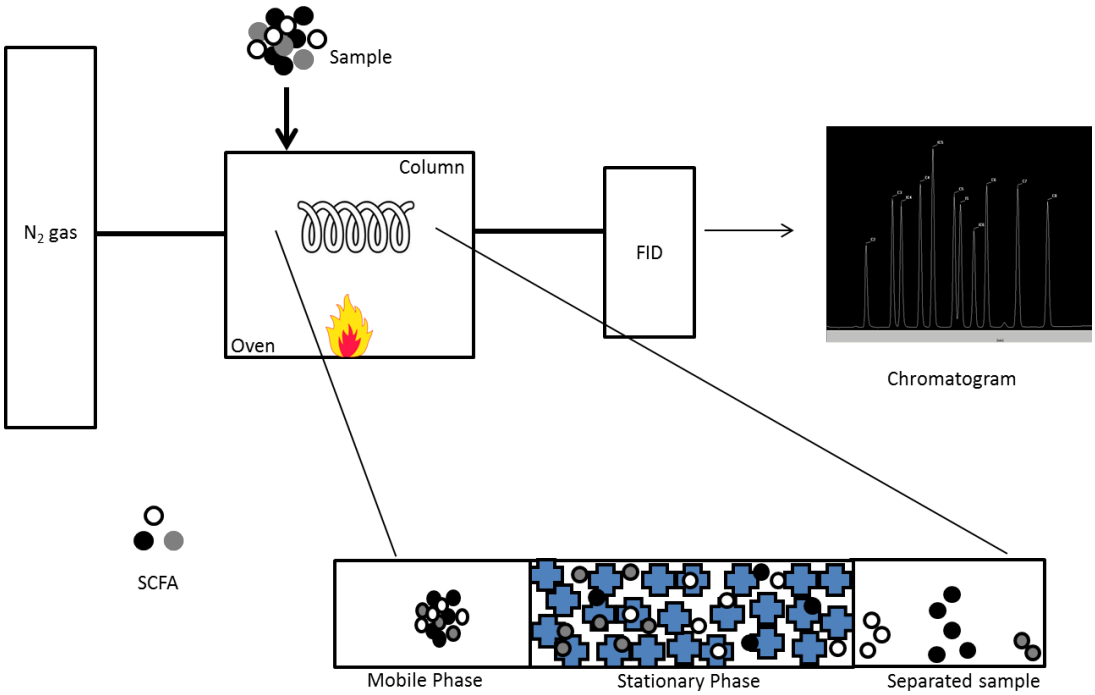

Figure 2-3: A schematic diagram of GC:FID
A sample is injected via the injector and becomes vaporised where they then reach the column. Samples encounter the mobile phase where it reaches the column and then the stationary phase, where the sample are separated based on volatility, boiling and polarity. The more volatile and less polar the sample is, the faster it travels through the column where they then reach the FID detector. The retention time is the time to reach the detector and the amount reaching the detector is expressed as millivolts to produce a chromatogram. FID – Flame ionisation detector

2.3.3 Calculations used to measure SCFA produced

When processing the data from the GC: FID, a number of quality controls were included. An initial quality control was carried out by assessing coefficient of variance (CV%) of the internal standard of all samples tested ensuring it was less than 10%. This enabled the identification of any samples which needed further attention as the peaks may not have been identified properly, the injection was poor, or it was erroneously extracted. A second quality control was a curve of the external standards to assess if they were properly extracted, by ensuring an R^2 value (minimum 0.95). A further control used to monitor the CV% of acetate observed by periodic injections of the 'standard 100' through the run of samples was less than 1%, as analysis of the SCFA produced by the samples fermented was based on the standard 100 (the sample containing 100 µl of external standard, Table 2-5).

To calculate the concentration of the unknown samples the response factor was identified by equation 1:

$$\text{Response Factor} = \left(\frac{\text{External Standard AUC}}{\text{Internal Standard AUC}}\right) \text{ x } \left(\frac{\text{Internal Standard Concentation (mM)}}{\text{External Standard Concentration (mM)}}\right) \quad \text{Equation 2.1}$$

The response factor was then used to calculate the sample concentration using the sample area ratio (sample AUC/ Internal standard AUC):.

$$\text{Sample Concentration (mM)} = \frac{\text{Sample Area ratio}}{\text{Response Factor}} \text{ x Internal standard concentration} \quad \text{Equation 2.2}$$

This information was used to present SCFA data as a concentration (mmol/l), ratio proportion (%), a rate term was also developed (equations 2.3 and 2.4).

$$\text{SCFA production per hour} = \frac{\text{Concentration (mM) * Volume (L)}}{\text{Mass (g) * Time (Hours)}} \quad \text{Equation 2.3}$$

Or

$$\text{SCFA production per day} = \frac{\text{Concentration (mM) * Volume (L)}}{\text{Mass (g)}} \quad \text{Equation 2.4}$$

2.3.4 Data handling and analysis

All statistical analysis and data handling was conducted using IBM SPSS Statistics version 22 and Microsoft Excel 2013. Total SCFA production was the sum of acetate, propionate and butyrate. Further information on statistical analysis used is located within each chapter.

Chapter 3 In vitro SCFA production by colonic bacteria from non-digestible carbohydrates– a systematic review

3.1 Introduction

As discussed in chapter one, increasing colonic propionate may have a role to play in the reduction of weight gain (Chambers et al., 2014). Propionate is beneficial as it has a several potential effects on satiety and metabolism (den Besten et al., 2013b) thought to be mediated by:

- Activation of FFAR2 and FFAR3, leading to the production of the anorexigenic gut hormones PYY and GLP-1
- Regulation of lipid and cholesterol synthesis
- Acting as a precursor for hepatic and intestinal gluconeogenesis, which in turn may influence energy balance

Therefore increasing colonic propionate should be beneficial for long-term health. It is believed that some carbohydrates are more propiogenic than others. Evaluating NDCs for their ability to increase propionate production could provide new opportunities for developing functional foods.

Individual investigations and reviews articles have identified a number of substrates that appear to selectively increase SCFA production, particularly propionate and butyrate. None of these assessments have been carried out as a systematic review.

Acetate, the most abundant SCFA produced, has been reported to be selectively increased by oligofructose fermentation (Macfarlane and Macfarlane, 2003, Wang and Gibson, 1993, Khan and Edwards, 2005). Several substrates including psyllium (Wong et al., 2006, Kaur et al., 2011, Edwards and Eastwood, 1992), rhamnose (Gietl et al., 2012, Vogt et al., 2004b), and β-glucans (Hosseini et al., 2011, Hughes et al., 2008, Queenan et al., 2007) have been associated with selectively increased propionate. Proportional increases in butyrate have also been linked with the fermentation of resistant starch, inulin, (Kolida et al., 2002, Hosseini et al., 2011, Laurentin and Edwards, 2004) and β-glucans (Kaur et al., 2011). These associations were not reproducible in all studies, however, and have not been established on the basis of a systematic review of the evidence, making substrate selection to increase propionate or butyrate rather subjective.

Moreover, the available evidence does not identify the main drivers of propionate production or whether a single substrate is preferential. From these papers, it is not clear if simply increasing the dietary fibre content is more effective at increasing propionate production than using a supposedly propiogenic NDC.

Epidemiological evidence, such as that summarised in Table 1-2 and in the systematic review by Wanders et al., (2011) on dietary fibre feeding studies, suggest that increasing dietary fibre intake is beneficial to health. It is still unclear if the type of fibre consumed is important in terms of increasing propionate production.

Assessment of the ability of NDCs to selectively increase propionate *in vivo* is problematic and impractical due a lack of appropriate methods providing access to the proximal colon. Approximately 95% of colonic SCFA formed are absorbed (Verbeke et al., 2015, Bloemen et al., 2009) and so faecal SCFA may be misleading. Studies evaluating SCFA production are further hindered by differential SCFA uptake in tissues which may alter the molar proportions excreted (Vogt and Wolever, 2003).

The most relevant measurements of SCFA production are those using stable isotopes as described in Chapter 2 (Pouteau et al., 1998), but currently this methodology is not well validated nor in widespread use. Instead, assessment of SCFA production is often undertaken using *in vitro* techniques. As discussed in Chapter 2 there are a variety of *in vitro* techniques including continuous and batch culture experiments with some modelling the whole GI tract and others modelling only the colon. Batch fermentation techniques are very simple and can provide high throughput screening of SCFA production from different carbohydrates (Williams et al., 2015) (Chapter 2). A drawback of most of these batch fermentation techniques is that they lack several of the physiological activities in the colon including pH control (beyond simple buffers) and removal of bacterial products by absorption. pH control is possible with the right equipment but is not often used. The exact models used vary considerably in different studies which makes direct comparison difficult. These differences include the amount of substrate added, culture volume, and the composition of inoculum used in the system. Differences in sampling times and presentation of the SCFA data can also limit comparisons. For example, across a selection of studies, SCFA data were presented in units including; mmol/l (Laurentin and Edwards,

2004), mmol per 50mg CHO (Kaur et al., 2011), mmol/g substrate (Bourquin et al., 1992), and as a ratio of SCFA produced (Wang and Gibson, 1993).

As a result, it is challenging to select substrates which preferentially produce propionate. This is particularly apparent for substrate mass as increased substrate dose is not linearly associated with increased SCFA production (Khan and Edwards, 2002), making assessment of SCFA production between studies using different methods onerous. This lack of consistency in methods used also compounds the lack of reproducibility and consistency when assessing SCFA production. When comparing the fermentation of ispaghula in different studies, propionate production was shown to range from 0.72 mmol/g substrate (Bliss et al., 2013), to 26.67 mmol/l (Mortensen et al., 1991). This, along with a lack of a systematic approach in determining SCFA production, hinders the development of functional foods that could be used to increase propionate production. It is further very difficult to translate these results to the human colon as there is so little understanding of the determinants and influences *in vivo*.

As there is an apparent lack of reproducible evidence in the literature, the aim of the study discussed in this chapter was to:

1. Conduct a systematic review to identify and evaluate articles using *in vitro* batch fermentations to assess the SCFA producing capabilities of different substrates.
2. Develop a unit quantifying the rate of individual SCFA production which normalises for substrate quantity and fermenter volume in order to compare the different batch fermentation methods used.
3. To use both the systematic review, and the rate unit to identify substrates that are propiogenic i.e. selectively increase the amount of propionate produced.

3.2 Methods

3.2.1 Information sources

Search engines used to identify publications were; PubMed, SCOPUS, Web Of Science and Medline Ovid. Searches were carried out to include investigations from the earliest possible year (1900, 1960, 1864 and 1947 for each database respectively), to 14-05-2014.

3.2.2 Data collection

The search terms used were ‘in vitro colonic fermentation’, ‘in vitro batch fermentation’, ‘in vitro human fermentation’, ‘in vitro carbohydrate fermentation’, ‘human carbohydrate fermentation’, ‘faecal fibre fermentation’, ‘in vitro fibre fermentation’, ‘in vitro short chain fatty acid production’, ‘in vitro faecal fibre fermentation’, ‘short chain fatty acid fermentation’ and, ‘in vitro volatile fatty acid production’. All variations of spelling (British, and American English) and truncations of words using Boolean Operators where appropriate were used. Journal articles selected were limited to those using human adults in the English language. Reference lists from identified articles and reviews were used to gain additional articles. The process of elimination of articles was based on the PRISMA guidelines described in (Moher et al., 2009). To reduce potential bias predetermined inclusion and exclusion criteria (Table 3-1) were used to evaluate the quality of studies to be included for further analysis.

Table 3-1: Inclusion and exclusion criteria for analysis of articles identified from abstracts

Inclusion Criteria	Exclusion criteria
• Batch fermentation	• Not a batch fermentation (i.e. continuous culture)
• Initial pH 6 ≤ 8	• pH stat controlled experiment[1]
• 24 hour time point	• Initial pH <6, >8
• Use of a fresh faecal slurry only	• additional bacteria added (i.e. use of bacterial pellet, probiotic)
• Samples from healthy adults	• Samples from children, infants, disease states or animals
• Data on acetate, propionate and butyrate[4]	• Gastrointestinal disorders
• Volume of fermentation system provided[4]	• Use of antibiotics (within the study or participants within the previous fortnight)
• Mass of substrate fermented given[4]	• Manipulation of the diet[3]

1. Articles where the fermentation system is conducted with the use of an acid or a base to keep it at a constant pH
2. If SCFA production was presented as a ratio only, total SCFA concentration must be provided
3. Includes; supplements provided to the donor, no exclusion or inclusion of foods from the diet of the sample donor
4. To produce a rate term of SCFA production, the minimum information required was: mass of substrate fermented, final volume and amount of each SCFA produced

3.2.3 Criteria for analysis

The data from each paper required standardisation to properly compare the SCFA production between studies. Each selected paper required a minimum information set including fermentation volume, substrate mass and SCFA production (Table 3-1). Papers were further excluded if insufficient methodological information was provided and the authors were not contactable. Of four authors approached, only one (for two papers) answered to provide more data when approached. Examples of insufficient details included; not providing details of the amount of substrate added to each vial, the presence of a pre-digestion step without providing the mass of fibre undergoing fermentation, and no information on the volumes in each fermenter vial.

3.2.4 Data Analysis

Initially, SCFA production data were grouped by substrate type fermented. The studies fell into two separate groups; those that used pooled faecal samples (more than one individual donor) and those that used a single donor (non-pooled) for the inoculum. These two types of study were considered separately. Many of the authors presented their SCFA production using different units of measurement, such as mmol/l, and mmol/100 mg carbohydrate, as well as a molar ratio. Production of SCFA is dependent of the quantity of substrate in the vial and the culture volume. To correct for these incongruities, a rate term was developed (see section 3.3.2) with 24-hour SCFA production data presented as mmol/g carbohydrate/ day and to normalise the variability in sampling time points as mmol/g carbohydrate/ time fermented (hours). Other time points were grouped as follows: 1-5 hours early fermentation, 6-9 hours mid fermentation, and 10-23 hours mid/late fermentation, which took into account the sigmoid pattern of SCFA production by fermentation.
Once the data obtained with the different methods were standardised using the rate term, the mean and standard deviations (SD) of the rates of production and the molar ratios of SCFA were calculated and compared between papers. For inclusion of a substrate, three separate mean values for fermentation were needed. This could have been from three separate studies but if more than one very similar substrate was used in one study this could be used as a second mean value. However, at least two independent studies were required for each substrate.

3.2.4.1 *Grouping substrates*

For comparison of substrates from the different studies, substrates were grouped based on similar chemistry, similar physicochemical properties and the same sources. Thus, data on the fermentation of sugars and disaccharides were grouped based on what they were; i.e glucoses were grouped together, and different studies fermenting lactulose were grouped together. The grouping became more complex with the dietary fibres. Substrates were initially grouped broadly, e.g. β-glucans, pectin, guar gum, starch, and derivatives of seaweed. If it became apparent that there were subgroups with sufficient replicate data, e.g raw starch and pre-digested starch, oligofructose and inulin, they were separated and assessed independently. Different celluloses have different SCFA producing capabilities, often only the manufacturer (which produce different celluloses) was provided. Due to this, celluloses were grouped together. If multiple fibres were assessed in the same fermentation bottle (e.g. guar and oligofructose together), these were grouped separately to the individual components. If the substrate identified did not fit into a group of substrates, it was set as its own group where it then had to fulfil the criteria to be included for analysis (Section 3.2.3).

3.2.4.2 *Assumptions made in the development of the rate term*

Although the rate term allowed the standardisation of the unit of SCFA production from different studies, two assumptions were required.

Assumption 1: SCFA production is linear

This is not the case, in the study by Aura et al., (2005) who carried out *in vitro* batch fermentations of various fractions of rye brans with multiple time points (0, 1, 2, 4, 6, 8, and 24 hours). The majority of SCFA production occurred between 1 and 8 hours of fermentation, and only minor increases in total SCFA production occurred between 8 and 24 hours for some substrates indicating that the substrate was rapidly fermented, and exhausted within 8 hours (Aura et al., 2005). Another observation with this study was that SCFA production for all substrates tested plateaued between 6 and 8 hours (Aura et al., 2005, Bliss et al., 2013, Khan and Edwards, 2005). The plateau value for 24 hours was not used but was included for the time point at which the plateau was achieved.

Not all the individual SCFA were produced at the same rate, for example butyrate production with xylanase treated rye bran and rye bran extract increased no further after 8 hours whereas for rye bran, rye bran residue and extruded rye bran the butyrate production continued to increase after 8 hours (Aura et al., 2005).

When studies used different actual time points they were grouped into early, mid, mid late fermentation and per day. This had less of an effect when the system was compared per day as this only took the production after 24 hours into account, at which point many of the sugars within the substrate being tested had been used, and no further SCFA production occurred (Khan and Edwards, 2002, Aura et al., 2005, Salvador et al., 1993)

Assumption 2: Altering the substrate concentration in the incubation proportionally increases SCFA production.

A number of studies have assessed the role of substrate dose on *in vitro* SCFA production. It was found that production was not linear and doubling the substrate mass did not double the total SCFA produced (Mortensen et al., 1991, Stevenson et al., 1997, Khan and Edwards, 2002). Mortensen et al., (1991) fermented different masses of substrates (Figure 3-1) and found a non linear relationship with SCFA (Mortensen et al., 1991). Similar effects were also identified in fermentations by Khan and Edwards, (2002) where increasing lactulose dose from 2.5 to 10 mg/ml in 2.5 increments was also not associated with a dose dependent increase in total SCFA production, but when the actual production was compared with predicted production, there was a non-linear dose dependent decrease in total SCFA production.

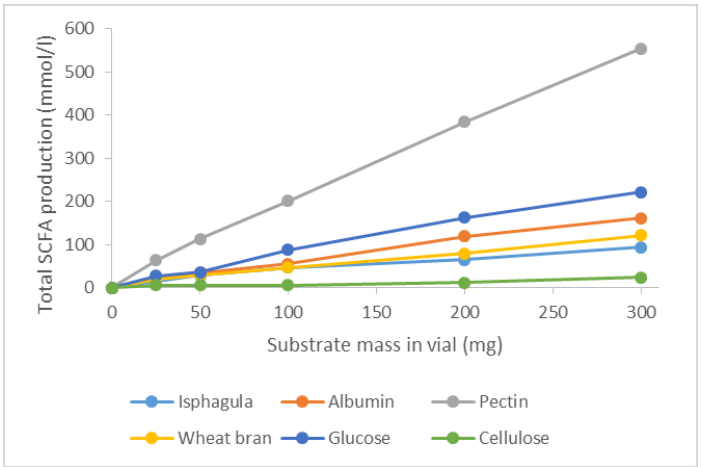

Figure 3-1: The effect of increasing substrate mass in fermentation vial on total SCFA production.

Data from *in vitro* batch fermentations performed by (Mortensen et al., 1991) with a selection of substrates total SCFA production after 24 hours of fermentation with the pooled inoculum of three individuals is shown.

3.3 Results

3.3.1 Identification of articles for the systematic review.

Figure 3-1 shows the PRISMA flowchart selection process for assessing papers fulfilling the inclusion/ exclusion criteria (Table 3-1, Section 3.2.2). Search terms and checking reference lists generated 15,541 abstracts for screening, 14,226 articles did not undergo further assessment and were rejected based on the information within the title and abstract, 1315 articles had their full texts assessed. Texts were scrutinised to identify studies fulfilling the criteria required for inclusion, resulting in 114 articles in this systematic review.

During evaluation of the articles it became apparent that the *in vitro* batch fermentation methodologies and the presentation of SCFA production by different substrates varied substantially, making direct comparison challenging. A rate term was developed to overcome this (Section 3.3.2). One of the main differences in experimental design was whether the faecal inoculum used to seed the cultures was from one donor, or was pooled from several donors. Fifty-one papers used pooled faecal samples and 65 used single faecal samples (two papers conducted fermentations using both pooled and non-pooled stool samples).

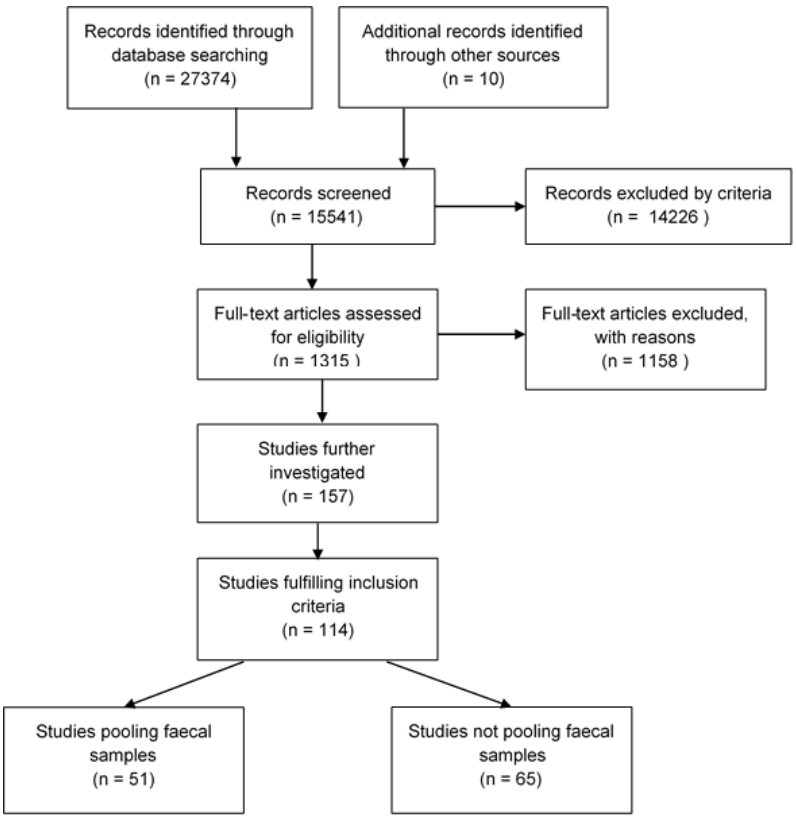

Figure 3-2: Flow diagram based on PRISMA guidelines outlining the process of elimination of articles identified

The process of elimination adapted from PRISMA resulted in 114 articles undergoing further scrutiny. Two articles reported both, pooled and non-pooled data.

3.3.2 Rate unit

A rate term was developed for inter-study comparison. This rate term required four pieces of information:

1. Time (hours) at which the sample was taken for SCFA analysis
2. Mass of substrate (grams) added to the fermentation vessels
3. Total inoculum volume (L) added to the fermentation vessels
4. Measured SCFA concentration (mmol/l)

Equation 3.1 outlines the formula to calculate SCFA production as mmol/g carbohydrate/hour. This equation was most commonly used for intermediary time points; a simple modification allowed it to be utilised for 24-hour data, Equation 3.2.

$$\text{SCFA Production (mmol/g CHO/hr)} = \frac{\text{Concentration (mM)} \times \text{Fermenter Volume (L)}}{\text{Substrate amount (grams)} \times \text{Time (hours)}} \quad \text{Equation 3.1}$$

$$\text{SCFA Production (mmol/g CHO/day)} = \frac{\text{Concentration (mM)} \times \text{Fermenter Volume (L)}}{\text{Substrate amount (grams)}} \quad \text{Equation 3.2}$$

3.3.2.1 *Reduction in SCFA variability with the rate unit*

The use of the rate term enabled comparison of different investigations and reduced the inter-study variability in measured SCFA production (Table 3-2, Figure 3-3). However, this worked better for some substrates than others. The coefficient of variance (CV %) of propionate production increased by 13% for inulin when presented as a rate term (93.33% vs 106.47%), but it decreased for glucose (176.63% vs 101.08%), pectin (285.91% vs 46.34%), control (blank) (150.81% vs 121.15%) and cellulose (7793% vs 144.74%) (Table 3-2). Figure 3-3 also highlights that when the data was transformed to the rate unit, the data was less variable. Therefore, the rate allows comparison of investigations with more accuracy, making them more comparable.

3.3.3 Pooled vs non-pooled

A key methodological difference observed was the decision to seed the fermenters with stool samples from a single donor (non-pooled) or from combined stools from multiple donors (pooled). Variability of SCFA production was compared for a selection of the substrates identified during paper analysis (Table 3-3, Table 3-4). The interquartile range (IQR) of the rate of SCFA production was generally lower, or there was little effect when stools in non-pooled faeces studies were compared to those using pooled samples (Table 3-3). For example, variation in propionate and butyrate production for cellulose were similar, but for the non-pooled samples was higher for acetate. The IQR however was lower for all of the non-pooled samples when compared to the pooled samples for guar. This suggests that the inter-study variability was not equal between substrates, however differences due to study methodology may have had an effect on the measured SCFA production (Table 3-3).

Table 3-2: Coefficient of variance (CV %) of acetate, propionate, butyrate, and total SCFA production after comparison of data provided within the articles and when converted to a rate

CV%	Inulin n=20		Glucose n=22		Pectin n=20		Cellulose n=13		Control (blank) n=34	
	Given	As rate	Given	As rate	Given	As rate	Given	As rate	Given	As rate
Acetate	74.98	80.14	176.58	80.14	279.86	42.85	20480.3	463.24	169.22	97.83
Propionate	93.33	106.47	176.63	101.08	285.91	46.34	7793	144.74	150.81	121.15
Butyrate	89.42	81.39	194.56	166.53	254.88	41.12	7529.41	235.82	153.32	130.46
Total	68.45	79.38	169.67	91.39	276.59	38.61	15361.23	330.76	155.71	100.45

Both pooled and non-pooled data were included. N= number of studies providing data. CV% is the variation of all of the studies together as given within text and as the rate term (mmol/g CHO/day), n= number of investigations.

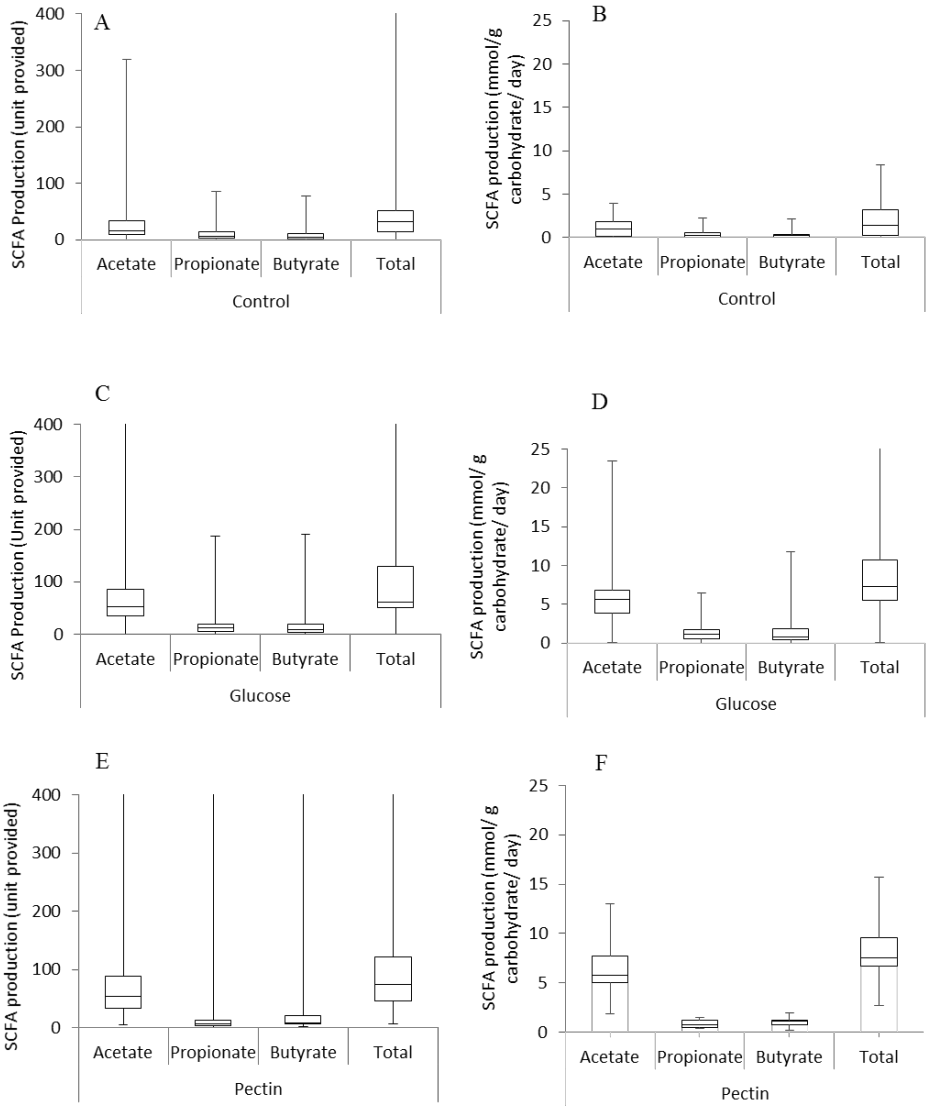

Figure 3-3: Variability observed between individual investigations SCFA observations presented as a box plot.

A, C, E provides information on control (blank), glucose and pectin when compared from the value given in the publication. B, D, F provides information on control (blank), glucose and pectin when compared as a rate. This demonstrates that when the data was transformed into a rate, the variability was reduced.

Differences in the IQR between pooled and non-pooled samples were less clear when comparing the proportions of acetate, propionate, and butyrate. For example, the IQR for cellulose (n=13) was equal for acetate production (~17.5%); non-pooled data had a larger IQR for propionate but a smaller IQR for butyrate production. In contrast, for ispaghula

(n=15) and pectin (n=20) the IQRs were similar for all SCFA for pooled and non-pooled studies (Table 3-4).

These findings are in contrast to previous studies which found that pooling (or not pooling) of the stool samples did not affect the overall production of SCFA (Aguirre et al., 2014, Mortensen et al., 1991). These investigations like those of others such as Stewart and Slavin., (2006) and Ferguson and Jones., (2000) who pooled the stool samples only did one technical repeat. The lack of technical repeats may mask experimental variability as well as potential actual variability in SCFA production that can occur with and between individuals.

Pooling samples may alter the sample bacteria, as the bacteria may interact/ compete in the lag phase of the culture, as one dominant/ established microbiota ecosystem tries to integrate with another. This was seen in mice (coprophagic animals) where combining two different bacterial profiles, led to one dominating over the other (Ridaura et al., 2013). Faecal transplants in humans have been shown to alter the bacterial population with the population of the recipient becoming that of the donor for 10 weeks (Fuentes et al., 2014). Based on this, pooling samples *in vitro* could well affect the bacterial population, which may also affect SCFA production, and may negate the effect of inter-individual variation. As a result, the decision was made to assess SCFA production from the pooled and non-pooled studies separately.

Table 3-3: IQR of rate data from pooled and non-pooled stool samples

	Number of studies		**Acetate**		**Propionate**		**Butyrate**	
	non-pooled	pooled	non-pooled	pooled	non-pooled	pooled	non-pooled	pooled
Cellulose	6	7	1.1	0.2	0.3	0.2	0.2	0.2
Pectin	10	10	1.7	3.0	0.8	0.3	0.8	0.3
Glucose	13	9	3.0	5.6	1.1	1.1	1.4	0.6
Ispaghula	7	8	3.7	5.2	0.6	2.2	0.8	0.3
Inulin	8	12	4.1	2.4	1.5	1.0	0.7	1.7
Guar	6	6	1.2	2.4	0.5	1.0	0.3	0.5
Control	19	15	1.1	2.6	0.3	0.7	0.3	0.6

Rate data was calculated to mmol/g carbohydrate/ day

Table 3-4: IQR of molar ratios from pooled and non-pooled stool samples

	Number of studies		Acetate		Propionate		Butyrate	
	non-pooled	pooled	non-pooled	pooled	non-pooled	pooled	non-pooled	pooled
Cellulose	6	7	17.7	17.4	12.4	4.2	5.2	10.5
Pectin	10	10	6.3	8.7	4.0	3.5	5.3	7.3
Glucose	13	9	13.2	17.0	9.4	8.5	8.9	9.7
Ispaghula	7	8	7.6	5.7	7.3	8.0	4.9	6.4
Inulin	8	12	6.2	0.6	6.2	7.6	9.8	14.3
Guar	6	6	2.3	3.4	5.0	1.4	3.5	1.7
Control	19	15	19.9	7.1	8.0	6.7	10.6	2.4

3.3.4 Analysis of Non-pooled SCFA data

SCFA production was grouped by early, mid, late and 24 hours of fermentation. This was to reduce the effect of the assumption of the rate term that production is linear, as well as for comparison of the substrates at different stages within the fermentation system.

3.3.5 SCFA production from 1-9 hours (early to mid-fermentation)

Between 1 and 5 hours of fermentation, three substrates were identified, and not included for analysis.

Between 6 and 9 hours of fermentation, 13 substrates had sufficient data for further analysis (Table 3-5, Table 3-6). Lactulose fermentation generated the highest rate of total SCFA (1.17 [0.6] mmol/g CHO/hour) and acetate production (0.97 [0.5] mmol/ g CHO/ hour), as well as high butyrate production (0.93 [0.1] mmol/ g CHO/ hour). Highest rates of propionate and butyrate production occurred with guar gum yielding 0.18 (0.1) and 0.12 (0.0) mmol/g CHO/ hour respectively. Glucose, guar gum, and raw starch ranked in the top five for acetate, propionate, butyrate and total SCFA production. Cellulose, and heat-treated sugarbeet fibre both ranked in the bottom 5 for production of all SCFA (Table 3-5).

Proportions of SCFA production did not always reflect the rate of production. Cellulose fermentation led to the lowest rate (0.04 [0.0] mmol/g CHO/ hour), but the highest proportion of propionate (25.9 [5.5] %, Table 3-6). Resistant starch followed a similar pattern in which fermentation produced low rates, but high proportions of butyrate (0.64 [0.1] mmol/g CHO/ hour, 15.2 [8.2] %, Table 3-6).

On occasion, rates did translate to proportions of SCFA production. This is exemplified with guar gum and ispaghula which both had high rates and ratios of propionate production (Guar: 0.18 [0.1] mmol/g CHO/ hour, 22.6 [6.8] %, ispaghula: 0.12 [0.0] mmol/g CHO/ hour, 24.3 [7.1] %). This was also seen with FOS and oat fibre, both of which produced low rates and proportions of propionate.

3.3.6 Mid to late fermentation - SCFA production between 10 and 23 hours

Nine substrates were compared between 10 and 23 hours of fermentation, and as a result, the top and bottom 4 substrate identified are presented in Table 3-7.
Acetate and total rates of production were the highest with pectin, generating 0.37 (0.2) and 0.47 (0.3) mmol/g CHO/ hour. In contrast, pectin produced low rates of propionate and butyrate yielding 0.06 (0.1) and 0.04 (0.1) mmol/ g CHO/ hour. This was also translated to molar proportions of SCFA production with pectin leading to the highest ratio of acetate (83.3 [7.7] %) and the lowest of propionate (10.6 [2.6] %) and butyrate (6.2 [5.1] %). Sugarbeet fibre gave rise to high rates of production, ranking first for propionate (0.10 [0.1], mmol/g CHO/hour) and ranking second for acetate (0.28 [0.1] mmol/g CHO/ hour) and total production (0.44 [0.2] mmol/ g CHO/ hour, Table 3-7). This was translated to increased proportions of acetate but not propionate (Table 3-8). Butyrate production occurred the most with both, raw and resistant starch yielding 0.09 (0.1) and 0.09 (0.0) mmol/g CHO/hour respectively. High butyrate proportions were also associated with resistant starch, which led to 29.3 (8.0) % of total SCFA produced forming butyrate (Table 3-7, Table 3-8).

Pea and maize fibre consistently had the lowest rates of SCFA production, but differed in the proportions of acetate, propionate and butyrate formed. Pea fibre generated high ratios of acetate (71.5 [16.1] %), whereas maize fibre produced high proportions of propionate (27 [0.7] %), and butyrate (16.6 [1.9] %, Table 3-8)

Table 3-5: The top and bottom five ranked producers of acetate, propionate, butyrate, and total SCFA at 6-9 hours of fermentation (mmol/g carbohydrate/hour)

Top 5	Ranked on Acetate		Ranked on propionate		Ranked on butyrate		Ranked on total	
1	Lactulose (1-3)**	0.97 (0.5)*	Guar gum (7, 10, 11)	0.18 (0.1)	Guar gum (8, 10, 11)	0.12 (0.0)	Lactulose (1-3)	1.17 (0.6)
2	Glucose (1,3-7)	0.84 (0.6)	Glucose (1,3-7)	0.14 (0.1)	Lactulose (1-3)	0.93 (0.1)	Glucose (1,3-7)	1.01 (0.7)
3	Pectin (6-9)	0.52 (0.3)	Sugarbeet fibre (9, 14-17)	0.12 (0.1)	Glucose (1,3-7)	0.86 (0.1)	Guar gum (8, 10, 11)	0.78 (0.1)
4	Guar gum (7, 10, 11)	0.48 (0.2)	Ispaghula (6-8)	0.12 (0.0)	Raw starch (12-13)	0.85 (0.1)	Pectin (6-90	0.66 (0.5)
5	Raw starch (12-13)	0.44 (0.4)	Raw starch (12-13)	0.11 (0.1)	Oligofructose (18-22)	0.83 (0.1)	Raw starch (12-13)	0.64 (0.4)
Bottom 5								
5	Ispaghula (6-8)	0.34 (0.2)	Wheat bran (8, 6, 23, 24)	0.08 (0.0)	Sugarbeet fibre (9, 14-17)	0.06 (0.1)	Wheat bran (8, 6, 23, 24)	0.50 (0.3)
4	Oat fibre (9,23, 24)	0.27 (0.2)	Oat fibre (9,23, 24)	0.08(0.1)	Ispaghula (6-8)	0.06 (0.0)	Oat fibre (9,23, 24)	0.42 (0.3)
3	Heat-treated sugarbeet fibre (14, 25)	0.22 (0.4)	Heat-treated sugarbeet fibre (14, 25)	0.07 (0.1)	Pectin (6-9)	0.06 (0.1)	Resistant starch (5,6,26,27)	0.37 (0.2)
2	Resistant starch (5,6,26,27)	0.21 (0.1)	Oligofructose 1(8-22)	0.06 (0.1)	Heat-treated sugarbeet fibre (14,25)	0.04 (0.1)	Heat-treated sugarbeet fibre (14,25)	0.32 (0.5)
1	Cellulose (2,6-8)	0.11 (0.1)	Cellulose (2,6-8)	0.04 (0.0)	Cellulose (2,6-8)	0.02 (0.0)	Cellulose (2,6-8)	0.18 (0.1)

Mean *(standard deviation) of the data obtained within articles fulfilling the inclusion criteria.. Top 5, 1-5 = high to low, Bottom 5, 1-5 low to high . **References:1- (Mortensen et al., 1988) 2- (Mortensen et al., 1990), 3- (Cardelle-Cobas et al., 2009), 4- (Olano-Martin et al., 2000), 5- (Zhu et al., 2013), 6- (Mortensen and Nordgaard-Andersen, 1993), 7-(McBurney, 1989), 8- (Mortensen et al., 1991), 9-(Titgemeyer et al., 1991), 10-(Khan and Edwards, 2005), 11- (McBurney and Thompson, 1989), 12-(McBurney et al., 1990), 13- (Weaver et al., 1989), 14- (Guillon et al., 1992), 15- (Oufir et al., 2000), 16- (Fardet et al., 1997), 17- (Barry et al., 1995), 18- (Yang et al., 2014), 19- (Stewart et al., 2008), 20- (Chen et al., 2013),21, (Kaur et al., 2011), 22- (Rumpagaporn et al., 2012), 23- (Bourquin et al., 1992), 24- (McBurney and Thompson, 1990), 25- (Cherbut et al., 1991), 26- (Zhao and Lin, 2009), 27- (Thompson et al., 2011)

Table 3-6: The top and bottom five ranked producers of acetate, propionate, and butyrate at 6-9 hours of fermentation based on the molar proportion (%)

Top 5		Ranked on acetate		Ranked on propionate		Ranked on butyrate	
	1	Lactulose (1-3)**	83.0 (3.4)*	Cellulose (2, 6-8)*	25.9 (5.5)	Resistant starch (5, 6, 26, 27)	18.3 (8.2)
	2	Pectin (6-9)	81.8 (7.0)	Ispaghula (6-8)	24.3 (7.1)	Oligofructose (8-22)	16.8 (2.2)
	3	Glucose (1, 3-7)	77.0 (13.9)	Resistant starch (5,6,26,27)	23.0 (10.6)	Guar gum (8, 10, 11)	16.2 (8.8)
	4	Oat fibre (9, 23, 24)	74.5 (13.0)	Heat-treated sugarbeet fibre (14, 25)	22.7 (2.0)	Raw starch (11-12)	15.2 (10.5)
	5	Oligofructose (8-22)	73.04 (11.3)	Guar gum (8, 10, 11)	22.6 (6.8)	Wheat bran (8, 6, 23, 24)	14.2 (5.4)
Bottom 5							
	5	Heat-treated sugarbeet fibre (14, 25)	66.2 (3.2)	Glucose (1, 3-7)	15.3 (11.9)	Ispaghula (6-8)	10.4 (3.7)
	4	Ispaghula (6-8)	65.3 (5.8)	Oat fibre (9, 23, 24)	14.0 (5.8)	Sugarbeet fibre (9, 14-17)	10.2 (7.7)
	3	Cellulose (2, 6-8)	63.5 (4.3)	Pectin (6-9)	11.7 (3.6)	Glucose (1, 3-7)	7.7 (5.6)
	2	Guar gum (8, 10, 11)	61.2 (10.6)	Oligofructose (8-22)	10.2 (9.4)	Lactulose (1-3)	7.30 (2.2)
	1	Resistant starch (5, 6, 26, 27)	58.7 (18.5)	Lactulose (1-3)	9.5 (2.2)	Pectin (6-9)	6.5 (4.3)

Mean *(standard deviation) of the data obtained within articles fulfilling the inclusion criteria. Top 5, 1-5 = high to low, Bottom 5, 1-5 low to high. **Reference numbers correspond to those in the legend of Table 3-5

Table 3-7: The top and bottom four producers of acetate, propionate, butyrate and total SCFA from 10-23 hours of fermentation (mmol/g carbohydrate/hour)

Top 5	Ranked on acetate		Ranked on propionate		Ranked on butyrate		Ranked on total	
1	Pectin (7, 9)**	0.37 (0.2)*	Sugarbeet fibre (9, 14- 17, 28)	0.10 (0.1)	Resistant starch (26, 27)	0.09 (0.0)	Pectin (7, 9)	0.47 (0.3)
2	Sugarbeet fibre (9, 14- 17, 28)	0.28 (0.1)	Resistant starch (26, 27)	0.08 (0.1)	Raw starch (5, 12, 13)	0.08 (0.1)	Sugarbeet fibre (9, 14- 17, 28)	0.44 (0.2)
3	Raw starch (5, 12, 13)	0.21 (0.1)	Raw starch (5, 12, 13)	0.08 (0.1)	Wheat bran (24-25, 28)	0.07 (0.1)	Raw starch (5, 12, 13)	0.37 (0.2)
4	Heat treated sugarbeet fibre (14, 25)	0.20 (0.1)	Heat-treated sugarbeet fibre (14, 25)	0.07 (0.0)	Oat fibre (9, 23, 24)	0.07 (0.1)	Resistant starch (26, 27)	0.33 (0.2)
Bottom 5								
4	Oat fibre (9, 23, 24)	0.18 (0.1)	Oat fibre (9, 23, 24)	0.06 (0.0)	Pectin (7, 9)	0.04 (0.1)	Oat fibre (9, 23, 24)	0.31 (0.2)
3	Resistant starch (26, 27)	0.17 (0.1)	Pectin (7, 9)	0.06 (0.1)	Heat-treated sugarbeet fibre (14, 25)	0.04 (0.0)	Heat-treated sugarbeet fibre (14, 25)	0.31 (0.1)
2	Maize fibre (25, 28, 29)	0.09 (0.0)	Maize fibre (25, 28, 29)	0.04 (0.0)	Maize fibre (25, 28, 29)	0.03 (0.0)	Maize fibre (25, 28, 29)	0.16 (0.0)
1	Pea fibre (9, 25, 28)	0.08 (0.0)	Pea fibre (9, 25, 28)	0.03 (0.0)	Pea fibre (9, 25, 28)	0.02 (0.0)	Pea fibre (9, 25, 28)	0.13 (0.1)

Mean *(standard deviation) of the data obtained within articles fulfilling the inclusion criteria. Top 5, 1-4 = high to low, Bottom 5, 1-4 low to high. ** References: (number order follows that of Table 3-6: 5- (Zhu et al., 2013), 7- (McBurney, 1989), 9-(Titgemeyer et al., 1991), 12-(McBurney et al., 1990), 13- (Weaver et al., 1989), 14- (Guillon et al., 1992), 15- (Oufir et al., 2000), 16- (Fardet et al., 1997), 17- (Barry et al., 1995),23- (Bourquin et al., 1992), 24- (McBurney and Thompson, 1990), 25- (Cherbut et al., 1991), 26- (Zhao and Lin, 2009), 27- (Thompson et al., 2011), 28-(Salvador et al., 1993), 29- (Cherbut et al., 1997)

Table 3-8: The ranked molar proportion (%) of acetate, propionate, and butyrate after 10-23 hours of fermentation

Top 5	Ranked on acetate		Ranked on propionate		Ranked of butyrate	
1	Pectin (7, 9)**	83.3 (7.7)*	Maize fibre (25, 28, 29)	27.0 (0.7)	Resistant starch (26, 27)	29.3 (8.0)
2	Pea fibre (9, 25, 28)	71.5 (16.1)	Resistant starch (26, 27)	25.9 (6.3)	Wheat bran (24-25, 28)	20.6 (3.0)
3	Sugarbeet fibre (9, 14-17, 28)	69.8 (14.5)	Heat treated sugarbeet fibre (14, 25)	22.7 (1.62)	Raw starch (5,12,13)	18.4 (9.1)
4	Oat fibre (9,23, 24)	66.3 (11.2)	Wheat bran (24-25, 28)	21.1 (4.8)	Maize fibre (25, 28, 29)	16.6 (1.9)
Bottom 5						
4	Raw starch (5,12,13)	63.4 (16.0)	Oat fibre (9,23, 24)	18.8 (3.2)	Heat treated sugarbeet fibre (14, 25)	13.5 (1.97)
3	Wheat bran (24-25, 28)	58.3 (5.9)	Pea fibre (9, 25, 28)	18.5 (9.4)	Sugarbeet fibre (9, 14-17, 28)	10.8 (6.3)
2	Maize fibre (25, 28, 29)	56.4 (2.3)	Raw starch (5, 12, 13)	18.2 (8.1)	Pea fibre (9, 25, 28)	10.0 (7.1)
1	Resistant starch (26, 27)	44.8 (13.9)	Pectin (7,9)	10.6 (2.6)	Pectin (7,9)	6.2 (5.1)

Mean *(standard deviation) of the data obtained within articles fulfilling the inclusion criteria. Top 5, 1-4 = high to low, Bottom 5, 1-4 low to high. **Reference numbers correspond to those in the legend of Table 3-8.

3.3.7 SCFA Production per day (24 hour SCFA production)

A total of 27 different fermentable substrates were compared for analysis at 24 hours of fermentation.

Lactulose generated the highest rate of total, acetate and butyrate generating 19.70 (22.5), 13.68 (13.3) and 2.49 (4.0) mmol/g CHO/ day, and ranked second for propionate production yielding 3.49 (5.5) mmol/g CHO/ day. GOS ranked second for the rate of acetate, butyrate and total, but did not rank in the top five for propionate production (Table 3-9 - Table 3-11, Figure 3-4 - Figure 3-7). Rhamnose was ranked highest for propionate production and proportion, producing 4.51 (0.5) mmol/ g CHO/ day and 40.1(8.3) % (Table 3-9, Table 3-10). Interestingly, rhamnose was the only substrate that did not rank in the top 5 for acetate production, but ranked in the top 5 for total SCFA production generating a total SCFA production rate of 11.76 (2.3) mmol/ g CHO/ day. The lowest production for all SCFA occurred with green kiwi fibre fermentation which yielded 0.76 (0.0) mmol/g CHO/ day of total SCFA (Table 3-9, Figure 3-4 - Figure 3-7).

Proportionally, β- glucan led to the highest percentage of butyrate (34.3 [0.6] %), and consequently this led to the lowest proportion of acetate (42.3[0.3] %, Table 3-10, Figure 3-4). Rate and ratio of production was similar in few of the substrates tested. Both, proportion and rate of propionate production ranked highest with rhamnose fermentation. Pectin, lactulose, xylose and lactose were all in the top 5 for acetate rate and ratio. Similarities in rate and ratio of butyrate production were only seen for inulin (top 5) and xylose (bottom 5). The opposite of this also occurred, such as with green kiwi fibre and cellulose where the ratio of production ranked in the top five but the proportion ranked in the bottom 5 (Table 3-9, Table 3-10, Table 3-11, Figure 3-5).

It can be seen from Figure 3-7, Table 3-11 that there was little difference between the top and bottom propionate producers, with approximately a 5.0 mmol/ g CHO/ day difference between substrates were observed, similar effects were also seen for total production (Table 3-11, Figure 3-4 - Figure 3-7). This indicates that the type of fibre may not be the most influential factor when determining individual SCFA production.

Table 3-9: Top and bottom five ranked producers of acetate, propionate, butyrate and total SCFA at 24 hours fermentation (mmol/g carbohydrate/day)

Top 5	Ranked on acetate		Ranked on propionate		Ranked on butyrate		Ranked on total	
1	Lactulose (1-3)**	13.68 (13.3)*	Rhamnose (1, 30, 43)	4.51 (0.5)	Lactulose (1-3)	2.49 (4.0)	Lactulose (1-3)	19.7 (22.5)
2	GOS (3, 30-32)	10.0 (6.9)	Lactulose (1-3)	3.49 (5.5)	GOS (3, 30-32)	1.74 (1.2)	GOS (3, 30-32)	13.77 (8.3)
3	Xylose (1, 30)	8.71 (6.8)	Arabinose (1, 30, 33)	3.01 (1.2)	Sugarbeet fibre (9, 15-17, 28,45)	1.53 (1.4)	Rhamnose (1, 30, 43)	11.76 (2.3)
4	Lactose (1, 3, 30, 33)	8.55 (3.9)	Guar gum (7, 10, 23, 24, 35, 43)	2.78 (0.5)	Inulin (31, 33, 42, 46-48)	1.29 (1.5)	Xylose (1, 30)	11.29 (7.6)
5	Pectin (6,-9, 33-37)	6.54 (2.8)	Xylose (1,30)	2.22 (1.2)	Rhamnose (1, 30, 43)	1.16 (0.7)	Lactose (1, 3, 30, 33)	10.75 (4.5)
Bottom 5								
5	Maize fibre (25, 28, 29)	1.53 (0.04)	Oat bran (9, 23, 24, 44)	0.55 (0.6)	Maize fibre (25, 28, 29)	0.40 (0.1)	Maize fibre (25, 28, 29)	2.58 (0.3)
4	Corn bran (9, 23, 24)	1.34 (0.7)	Pea fibre (9, 25, 28)	0.46 (0.3)	Xylose (1,30)	0.35 (0.4)	Pea fibre (9, 25, 28)	2.35 (1.3)
3	Beta-glucan (38, 39)	1.30 (0.0)	Corn bran (9, 23, 24)	0.43 (0.2)	Pea fibre (9, 25, 28)	0.34 (0.3)	Corn bran (9, 23, 24)	2.22 (1.1)
2	Cellulose (6,8, 23, 24, 35, 40)	0.87 (0.7)	Cellulose (6, 8, 23, 24, 35, 40)	0.38 (0.3)	Cellulose (6, 8, 23, 24, 35, 40)	0.25 (0.2)	Cellulose (6,8 , 23,24, 35, 40)	1.50 (1.1)
1	Green kiwi fibre (41, 42)	0.49 (0.0)	Green kiwi fibre (41, 42)	0.13 (0.0)	Green kiwi fibre (41, 42)	0.14 (0.0))	Green kiwi fibre (41, 42)	0.76 (0.0)

Mean *(standard deviation) of data obtained within articles fulfilling inclusion criteria. GOS: Galactoooligosaccharide. Top 5, 1-5 = high to low, Bottom 5, 1-5 low to high: **References 1 (numbers follow on from Table 3-8)- (Mortensen et al., 1988) 2- (Mortensen et al., 1990), 3- (Cardelle-Cobas et al., 2009), 6-(Mortensen and Nordgaard-Andersen, 1993) , 8- (Mortensen et al., 1991), 9-(Titgemeyer et al., 1991), 10-(Khan and Edwards, 2005), 15- (Oufir et al., 2000), 16- (Fardet et al., 1997), 17- (Barry et al., 1995), 23- (Bourquin et al., 1992), 24-(McBurney and Thompson, 1990), 25- (Cherbut et al., 1991), 26- (Zhao and Lin, 2009), 27- (Thomson et al., 2011), 28-(Salvador et al., 1993), 29- (Cherbut et al., 1997), 30- (Gietl et al., 2012), 31- (Rycroft et al., 2001a), 32- (Rycroft et al., 2001b), 33- (Wang and Gibson, 1993), 34-(Gelissen and Eastwood, 1995), 35- (Adiotomre et al., 1990), 36- (Waldecker et al., 2008b), 37- (Bourquin et al., 1996), 38- (Kim and White, 2009), 39- (Kim and White, 2010), 40-(Yu et al., 2013), 41- (Rosendale et al., 2012). 42- (Parkar et al., 2012), 43- (Fernandes et al., 2000), 44-(Kedia et al., 2009), 45-(Michel et al., 1996), 46- (Parkar et al., 2013), 47- (Salazar et al., 2008), 48- (Hughes et al., 2007)

Table 3-10: Top and bottom five ranked acetate, propionate, butyrate and total SCFA producers based on molar proportion (%) at 24 hours of fermentation

Top 5		Ranked on acetate		Ranked on propionate		Ranked on butyrate	
	1	Lactose (1, 3, 30, 33)**	78.0 (10.1)*	Rhamnose (1, 30, 43)	40.1 (8.3)	Beta-glucan (38, 39)	34.3 (0.64)
	2	Pectin (6,-9, 33-37)	77.5 (5.8)	Modified pectin (48, 49)	36.0 (25.6)	Inulin (31, 33, 42,46-48)	24.2 (5.3)
	3	Lactulose (1-3)	75.6 (12.6)	Guar gum (7, 10, 23, 24, 35, 43)	32.5 (3.2)	Green kiwi fibre (41, 42)	18.9 (0.1)
	4	Glucose (1-6, 8, 13, 30, 33, 34, 44, 47)	73.9 (31.4)	Arabinose (1, 30, 33)	30.6 (9.8)	Raw starch (5, 12, 13, 33, 43, 52)	18.2 (5.6)
	5	Xylose (1,30)	73.7 (13.9)	Cellulose (6,8, 23, 24, 35, 40)	27.9 (8.6)	Oat bran (9, 23, 24, 44)	17.4 (5.3)
Bottom 5							
	5	Modified pectin (48, 49)	55.2 (22.6)	Oligofructose (4, 10, 31-33, 44, 50)	15.9 (7.3)	Gum Arabic (9, 23, 35)	9.4 (1.7)
	4	Guar gum (7, 10,23,24, 35, 43)	55.1 (3.6)	Glucose (1-6, 8, 13, 30, 33, 34, 44, 47)	15.5 (15.6)	Laminarin/ seaweed derivatives (45, 55)	9.2 (4.7)
	3	Cellulose (6,8, 23, 24, 35, 40)	54.9 (13.4)	Lactulose (1-3)	13.3 (5.1)	Modified pectin (48, 49)	8.8 (2.3)
	2	Rhamnose (1, 30, 43)	50.2 (8.0)	Pectin (6,-9, 33-37)	11.3 (3.3)	Arabinose 1, 30, 33)	4.1 (4.1)
	1	Beta- glucan (38, 39)	42.3 (0.3)	Lactose (1,3,30, 33)	10.2 (2.9)	Xylose (1,30)	2.7 (1.9)

Mean *(standard deviation) of the data obtained within articles fulfilling the inclusion criteria. Top 5, 1-5 = high to low, Bottom 5, 1-5 low to high. References (based on above): **References 1- (Mortensen et al., 1988) 2- (Mortensen et al., 1990), 3- (Cardelle-Cobas et al., 2009), 4- (Olano-Martin et al., 2000), 5- (Zhu et al., 2013), 6- (Mortensen and Nordgaard-Andersen, 1993), 7-(McBurney, 1989), 8- (Mortensen et al., 1991), 9-(Titgemeyer et al., 1991), 10-(Khan and Edwards, 2005), 12-(McBurney et al., 1990), 13- (Weaver et al., 1989), 23-(Bourquin et al., 1992), 24- (McBurney and Thompson, 1990), 30- (Gietl et al., 2012), 31- (Rycroft et al., 2001a), 32- (Rycroft et al., 2001b), 33- (Wang and Gibson, 1993), 34-(Gelissen and Eastwood, 1995), 35- (Adiotomre et al., 1990), 36- (Waldecker et al., 2008b), 37- (Bourquin et al., 1996), 38- (Kim and White, 2009), 39- (Kim and White, 2010), 40-(Yu et al., 2013), 41- (Rosendale et al., 2012), 42-{Parkar, 2012 #297} 43- (Fernandes et al., 2000), 44-(Kedia et al., 2009), 45-(Michel et al., 1996), 46- (Parkar et al., 2013), 47- (Salazar et al., 2008), 48-(Hughes et al., 2007), 48-(Dongowski and Lorenz, 1998), 49- (Gulfi et al., 2007), 50- (Zhang et al., 2013), 51- (Michel et al., 1998), 52-(Khalil et al., 2014),53- (Weaver et al., 1992), 54-(Christian et al., 2003), 55- (Kuda et al., 2005)

Table 3-11: Comparison of ranked acetate, propionate, and butyrate at 24 hours of fermentation

	Ranked on acetate		Ranked on propionate		Ranked on butyrate	
Top 5	**Rate**	**%**	**Rate**	**%**	**Rate**	**%**
1	Lactulose	Lactose	Rhamnose	Rhamnose	Lactulose	Beta-glucan
2	GOS	Pectin	Lactulose	Modified pectin	GOS	Inulin
3	Xylose	Lactulose	Arabinose	Guar gum	Sugarbeet fibre	Green kiwi fibre
4	Lactose	Glucose	Guar gum	Arabinose	Inulin	Raw starch
5	Pectin	Xylose	Xylose	Cellulose	Rhamnose	Oat bran
Bottom 5						
5	Maize fibre	Modified pectin	Oat bran	Oligofructose	Maize fibre	Gum arabic
4	Corn bran	Guar gum	Pea fibre	Glucose	Xylose	Laminarin/ seaweed derivatives
3	Beta-glucan	Cellulose	Corn bran	Lactulose	Pea fibre	Modified pectin
2	Cellulose	Rhamnose	Cellulose	Pectin	Cellulose	Arabinose
1	Green kiwi fibre	Beta-glucan	Green kiwi fibre	Lactose	Green kiwi fibre	Xylose

Top 5, 1-5 = high to low, Bottom 5, 1-5 low to high

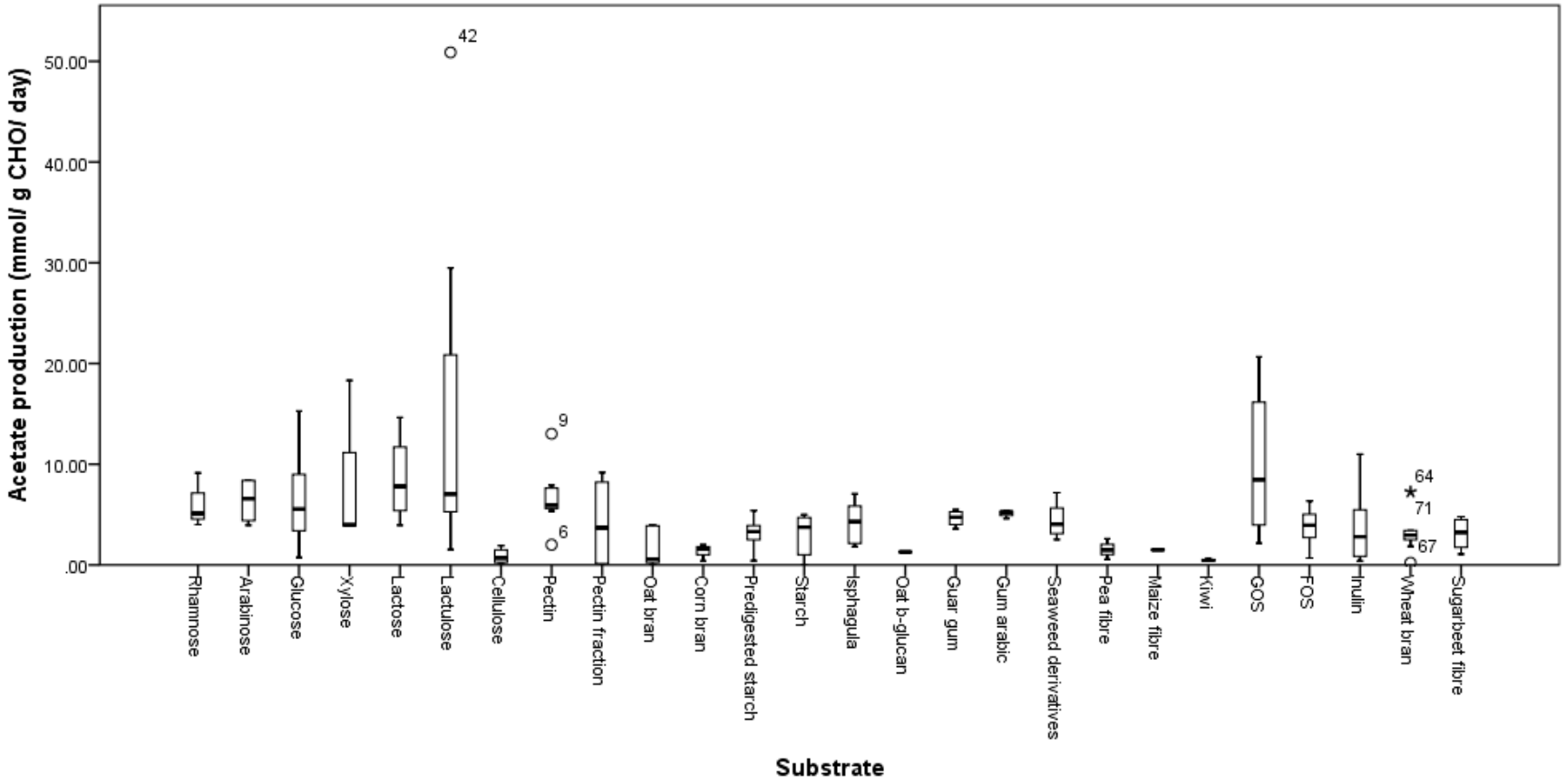

Figure 3-4: Box plots to show the 24-hour acetate production of all of the substrates assessed within the systematic review (mmol/g CHO/ day).

FOS= oligofructose, GOS= galactooligosaccharide. Outliers are signified by a circle, and extreme outliers with a *

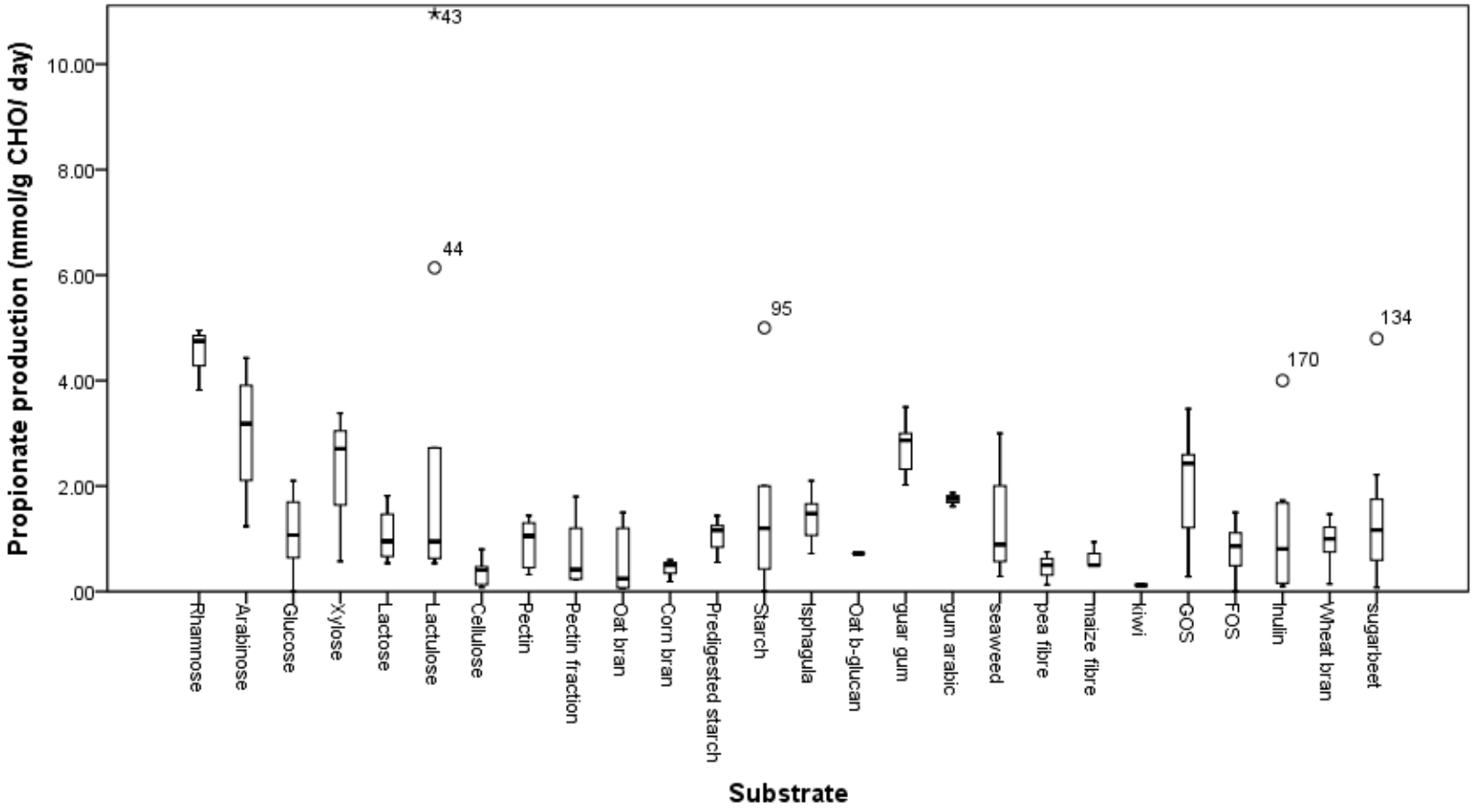

Figure 3-5: Box plots show the 24-hour propionate production of all of the substrates assessed within the systematic review (mmol/g CHO/ day).

FOS= oligofructose, GOS= Galactoooligosaccharide. Outliers signified by a circle, extreme outliers with a *

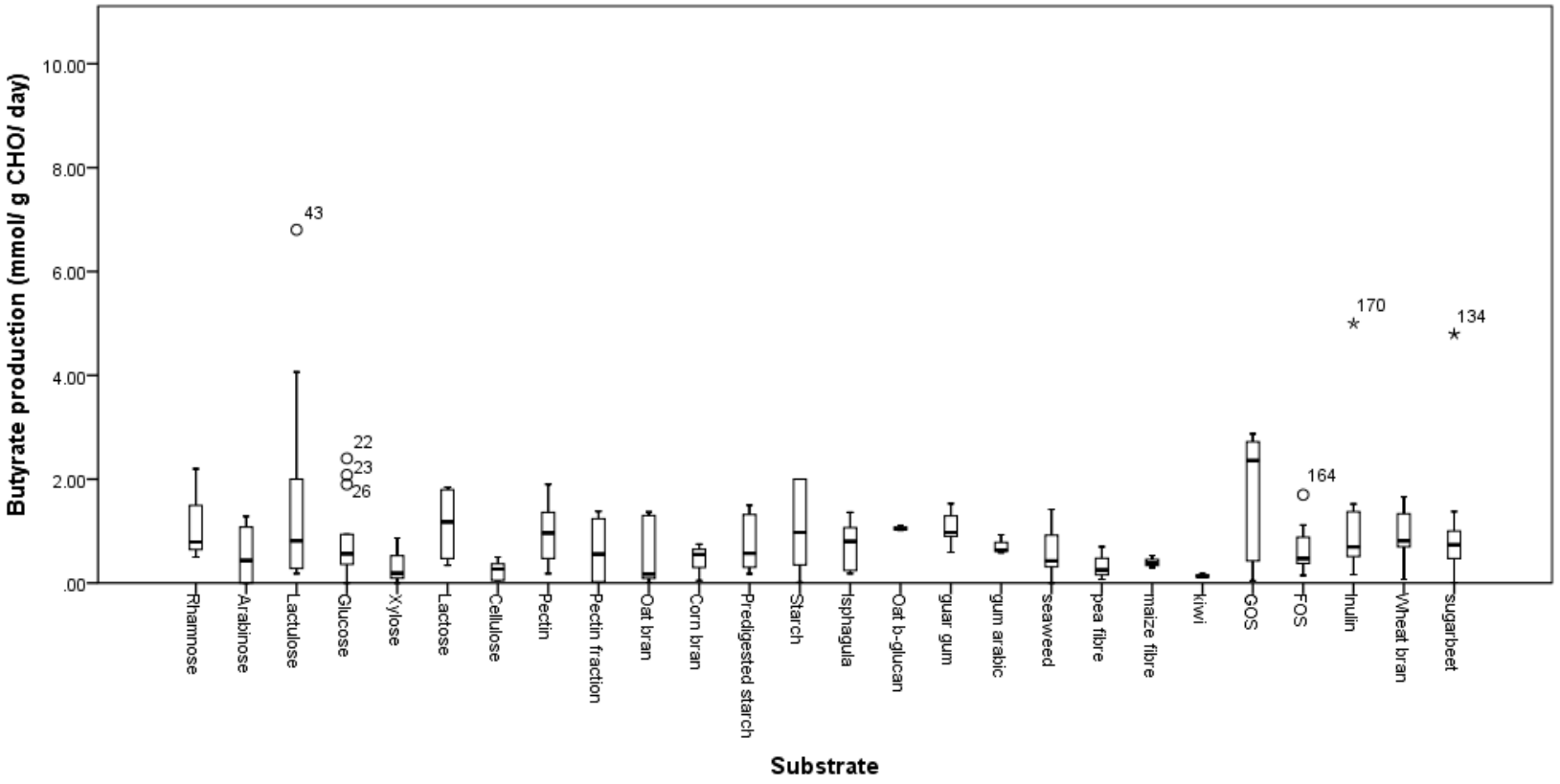

Figure 3-6: Box plots to show the 24-hour butyrate production of all of the substrates assessed within the systematic review (mmol/g CHO/ day)

FOS= oligofructose, GOS= Galactoooligosaccharide. Outliers are signified by a circle, and extreme outliers with a *

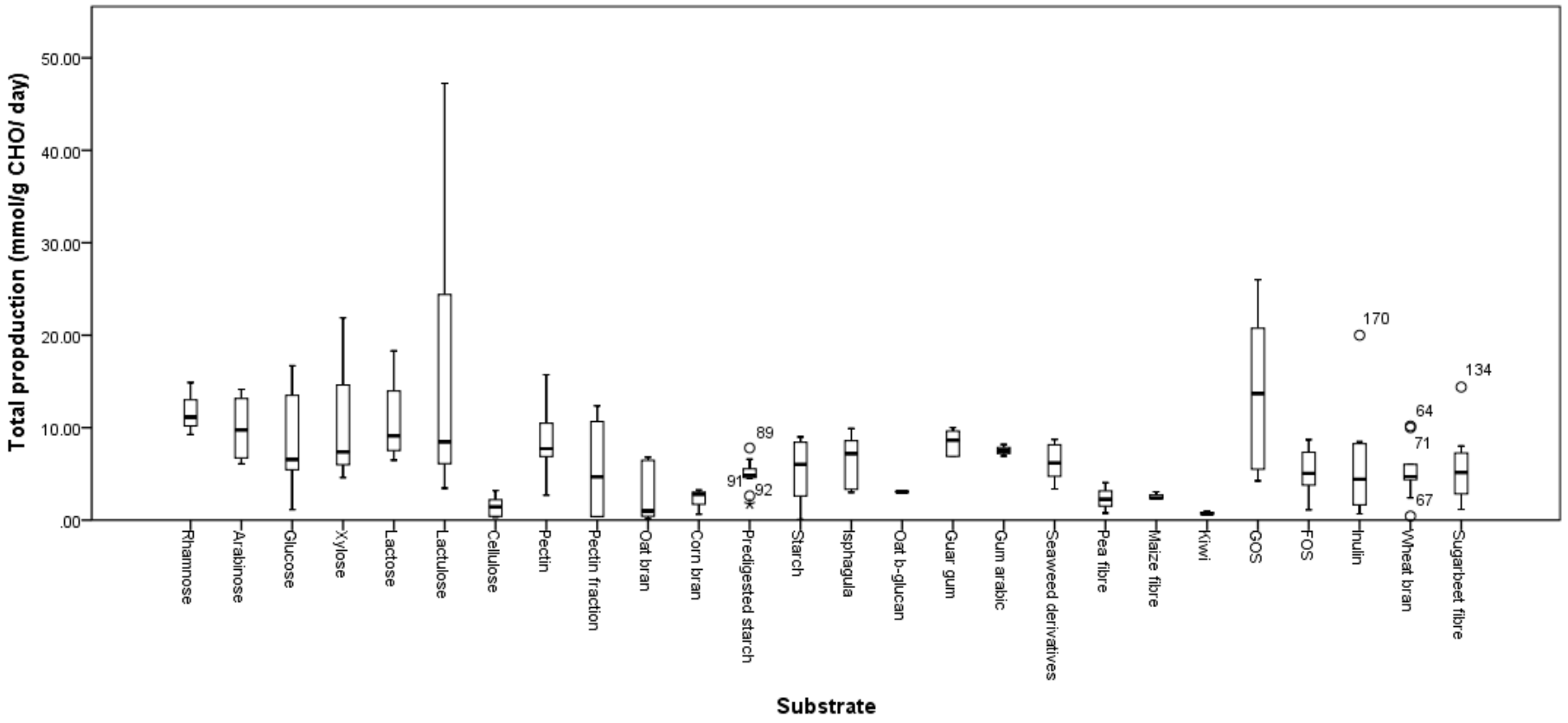

Figure 3-7: Box plots show the 24-hour total SCFA production of all substrates assessed within the systematic review (mmol/g CHO/ day)

FOS= oligofructose, GOS= Galactoooligosaccharide. Outliers signified by a circle, and extreme outliers with a *

3.3.8 Pooled SCFA production per day (24 hour SCFA production)

After screening the studies with a 24 hour time point, sufficient data were available for comparison of 18 substrates. There was no substrate which selectively increased a specific SCFA (Table 3-13). Beta-glucan and lactulose fermentation generated the highest rate of acetate, propionate and butyrate.

Fermentation of cellulose consistently resulted in low production of SCFAs generating a total rate of 0.9 mmol/g CHO/ day. Proportionally, the ability of different substrates to selectively produce SCFA was more varied. For example, pectin resulted in the highest proportion of acetate (90 [3.20 %) and the lowest proportion of propionate (8.0 [0.4] %) and butyrate (2.2 [0.5] %). Guar gum generated the highest proportions of propionate with 39.0 (6.2) % of total SCFA (Table 3-13, Table 3-14).

3.3.9 Additional articles which are not attached to tables

Table 3-12: Articles fulfilling all criteria but information was not provided in the tables as they are not in the top or bottom ten at any stage.

Non-pooled	(Lei et al., 2012, Rasmussen et al., 1988, Kotchariana, 2004, Pickardt et al., 2004, Trinidad et al., 1996, De Preter et al., 2010, McBurney et al., 1988, Casiraghi et al., 2011, Bourquin et al., 1993, Gullón et al., 2011)
Pooled	(Granito et al., 2001, Amrein et al., 2003, Rose et al., 2010, Stewart and Slavin, 2009, Lebet et al., 1998, Kim and White, 2011a, Niemi et al., 2013, Mallillin et al., 2008, Veeriah et al., 2007, Nordlund et al., 2013)

Table 3-13: The top and bottom five ranked producers of acetate, propionate, butyrate and total SCFA at 24 hours based on rate of production when samples are pooled (mmol/g carbohydrate/day)

Top 5	Ranked on acetate		Ranked on propionate		Ranked on butyrate		Ranked on total	
1	β-glucan (1-6)**	22.04 (30.5)*	β-glucan (1-6)	8.95 (13.7)	β-glucan (1-6)	12.95 (18.9)	β-glucan (1-6)	43.94 (62.2)
2	Lactulose (1, 4- 9)	16.92 (34.9)	Lactulose (1, 4- 9)	3.68 (8.7)	Lactulose (1, 4- 9)	7.81 (18.1)	Lactulose (1, 4- 9)	28.45 (61.7)
3	Glucose (10-14)	7.05 (6.5)	Resistant starch (3-5, 15-18)	2.46 (7.7)	Oligofructose (3,10,19-25)	2.46 (3.8)	Resistant starch (3-5, 15-18)	11.92 (35.5)
4	Resistant starch (3-5, 15-18)	6.92 (20.7)	Guar gum (2, 22, 37)	2.42 (0.8)	Resistant starch (3-5, 15-18)	2.53 (7.3)	Glucose (10-14)	11.06 (11.9)
5	Oligofructose (3, 10, 19-25)	5.84 (6.7)	Arabinoxylan (23, 33, 38)	2.30 (2.5)	Glucose (10-14)	2.3 (3.6)	Oligofructose (3, 10, 19-25)	9.50 (11.6)
Bottom 5								
5	Ispaghula (3, 13, 14, 26)	2.34 (1.5)	Inulin (2, 3, 8, 10, 21, 29, 30, 39, 40)	0.81 (0.8)	Arabinoxylan (23, 33, 38)	0.61 (0.6)	Ispaghula (3, 13, 14, 26)	4.31 (2.1)
4	laminarin/ seaweed derivatives (12, 27, 28)	2.32 (1.9)	Raw starch (15, 22, 11)	0.72 (0.4)	Ispaghula (3,13, 14, 26)	0.55 (0.4)	Laminarin/ seaweed derivatives (12, 27, 28)	3.56 (2.5)
3	Partially hydrolysed guar gum (13, 29, 30)	1.25 (1.1)	Pectin (11, 14, 20, 22)	0.65 (0.3)	Laminarin/ seaweed derivatives (12, 27, 28)	0.38 (0.1)	Partially hydrolysed guar gum (13, 29, 30)	3.49 (3.3)
2	Rye bran (31-35)	1.14 (2.1)	Rye bran (31-35)	0.40 (0.7)	Rye bran (31-35)	0.36 (0.7)	Rye bran (31-35)	1.90 (3.5)
1	Cellulose (14,22, 36)	0.57 (0.3)	Cellulose (14, 22, 36)	0.19 (0.1)	Cellulose (14,22, 36)	0.16 (0.1)	Cellulose (14, 22, 36)	0.92 (0.6)

Mean *(standard deviation) of the data obtained in articles fulfilling the inclusion criteria. Top 5, 1-5 = high to low, Bottom 5, 1-5 low to high **References1: 1-(Kim and White, 2011c), 2-(Queenan et al., 2007), 3(Kaur et al., 2011), 4- (Sayar et al., 2007), 5 (Kim and White, 2010), 6-(Wood et al., 2002), , 7-(Arrigoni et al., 2005), 8-(Arrigoni et al., 2002), 9- (Gulfi et al., 2005), 10- (Jenkins et al., 2011), 11-(Nordgaard et al., 1995), 12- (Deville, 2007), 13-(Pylkas et al., 2005), 14-(Mortensen et al., 1991), 15- (Martín Bernabé et al., 2011), 16-(Fassler et al., 2007), 17-(Zhou et al., 2013), 18- (Zhang et al., 2012), 19-(Yu et al., 2013), 20-(Chen et al., 2013), 21- (Queenan et al., 2007), 22-(Ferguson and Jones, 2000), 23-(Rumpagaporn et al., 2012), 24- (Munjal et al., 2009), 25- (Yang et al., 2014), 26-(Bliss et al., 2013), 27- (Bobin-Dubigeon et al., 1997), 28-(Lahaye et al., 1993), 29 - (Noack et al., 2013), 30- (Ohashi Y, 2012), 31-(Karppinen et al., 2000), 32-(Nordlund et al., 2012), 33- (Karppinen et al., 2001), 34- (Aura et al., 2005), 35-(Aura et al., 2006), 36- (Wong et al., 2005), 37- (Stewart and Slavin, 2006), 38- (Glei et al., 2006), 39- (Hartzell et al., 2013), 40-(Beyer-Sehlmeyer et al., 2003), 41 - (Barry et al., 1995).

Table 3-14: The top and bottom five ranked SCFA producers based on molar proportion (%) at 24 hours when samples are pooled

Top 5	Ranked on acetate		Ranked on propionate		Ranked on butyrate	
1	Pectin (11, 14, 20, 22)**	75.0 (6.7)*	Guar gum (2, 22, 37)	39.0 (6.2)	Partially hydrolysed guar gum (13, 29, 30)	46.0 (25.3)
2	Glucose (10-14)	71.9 (13.7)	Ispaghula (3, 13, 14, 26)	31.5 (12.5)	β-glucan (1-6)	30.6 (5.4)
3	Raw starch (15, 22, 11)	64.1 (19.8)	Arabinoxylan (23, 33, 38)	28.6 (11.4)	Inulin (2, 3, 8, 10, 21, 29, 30, 39, 40)	30.0 (13.7)
4	Cellulose (14, 22, 36)	63.3 (12.5)	Laminarin/ seaweed derivatives (12, 27, 28)	26.5 (2.8)	Oligofructose (3, 10, 19-25)	27.0 (7.5)
5	Lactulose (1, 4- 9)	63.1 (13.5)	Resistant starch (3-5, 15-18)	23.9 (11.6)	Lactulose (1, 4- 9)	25.1 (10.0)
Bottom 5						
5	Ispaghula (3, 13, 14, 26)	56.2 (22.4)	Raw starch (15, 22, 11)	13.1 (11.0)	Glucose (10-14)	15.1 (9.2)
4	Resistant starch (3-5, 15-18)	54.6 (14.7)	Glucose (10-14)	13.0 (5.4)	Arabinoxylan (23, 33, 38)	14.5 (5.8)
3	β-glucan (1-6)	50.18 (6.8)	Inulin (2, 3, 8, 10, 21, 29, 30, 39, 40)	13.0 (8.3)	Laminarin/ seaweed derivatives (12, 27, 28)	14.1 (5.0)
2	Guar gum (2, 22, 37)	49.1 (3.2)	Lactulose (1, 4- 9)	11.4 (4.1)	Ispaghula (3,13, 14, 26)	12.3 (11.2)
1	Partially hydrolysed guar gum (13, 29, 30)	35.8 (17.2)	Pectin (11, 14, 20, 22)	9.6 (3.0)	Guar gum (2, 22, 37)	11.9 (8.0)

Mean (standard deviation)* of the data obtained within articles fulfilling the inclusion criteria. Top 5, 1-5 = high to low, Bottom 5, 1-5 low to high: **References- numbers are based on those in the legend of Table 3-13

3.4 Discussion

The factors influencing the propensity of a particular NDC to selectively increase the production of a specific SCFA is not fully understood. This gives rise to challenges in decision making when selecting NDCs for intervention studies designed to test the effects of increased propionate production. To identify potential propiogenic substrates a systematic review was conducted to evaluate the SCFA producing capabilities of a variety of different substrates, determined using *in vitro* batch fermentations. Searches generated 114 acceptable articles and permitted comprehensive analysis of the role of NDC on propionate production *in vitro* to be carried out (Figure 3-2).

Many issues were identified when comparing the studies for example a lack of a standardised methodology for *in vitro* batch fermentation setup and analysis. Unfortunately, attempts to agree a standardised model to reduce inter-study method variations have been unsuccessful and are not standard practice (Edwards et al., 1996, Barry et al., 1995),(COST Action FA1005, 2015). The published literature therefore is based on a range of methodologies, with variability in substrate quantity, inoculum volume and composition, and fermenter size which made direct comparisons difficult. Different fermentation protocols may alter the rate, ratio and extent of SCFA produced. The amount of substrate added to the fermentation vial has been shown to alter SCFA production with 10 mg/ml lactulose producing less total SCFA than 7.5 mg/ml lactulose. This was probably due to end product inhibition occurring through a lack of absorption which would naturally occur in the colon (Khan and Edwards, 2002).

Another major issue identified when processing the data was the variety of units used to present the SCFA production. On some occasions, inadequate information on the unit used to present SCFA production was given. For example, Jonathan et al., (2012) presented SCFA production as mmol/g organic matter, without providing information on the substrate (e.g water content), so the fibre content could not be calculated (Jonathan et al., 2012). This prevented calculation of the rate term, so the study was not included within the review

Units used to describe SCFA production ranged from mmol/l, (Laurentin and Edwards, 2004, Adiotomre et al., 1990) µmol/ g substrate, (Titgemeyer et al., 1991) and µmol per

50mg carbohydrate (Kaur et al., 2011). A rate unit for the standardisation of SCFA data, was therefore developed to enable direct comparisons. This rate term (mmol/g CHO/day, or mmol/g CHO/ hour) took into account the mass of substrate, volume added, concentration of SCFA produced, and the duration of fermentation. It became clear although using the molar ratios of SCFA production reduced the variation between investigations it was potentially misleading. Molar proportions did not take into account the total production, and therefore a substrate with a high percentage of an individual SCFA, may have not resulted in the highest final concentrations of each SCFA. This was demonstrated by green kiwi fruit fibre at 24 hours (Table 3-9, Table 3-10), which was ranked fourth for butyrate proportion (19 [0.1] %), but yielded the lowest production (0.14 [0.0] mmol/g CHO/ day). In addition, maize fibre produced the highest proportion of propionate (27 [0.7] %), but was the fourth lowest for rate (0.04 [0.0] mmol/ g CHO/ hour) between 10 and 23 hours (Table 3-7, Table 3-8).

There was also substantial inter-individual variation in SCFA within investigations, which is supported by previous *in vitro* studies (Salazar et al., 2008, Casiraghi et al., 2011, McBurney and Thompson, 1989, Carlson et al., 2016). However this inter-individual variation was shown not to inhibit the assessment of effects of dietary fibre on SCFA production in studies comparing pooled (inter-individual variation is negated by mixing samples) and non-pooled samples (Mortensen et al., 1991, Aguirre et al., 2014). Mortensen et al., (1991) concluded that variation was the same on all occasions whereas (Aguirre et al., 2014) concluded that pooling the stool samples reduced overall variability. Pooling of the stool samples however does not take into account the fact that not all individuals have the same colonic microbiota, and cannot ferment all NDCs equally. Differences in the bacterial composition of cultures with pooling have also been identified by Aguirre et al., (2014) where fermentation by pooled bacteria were different to that of each individual donor, in some cases but not others. For example, for one of the individual's faecal samples, which was later pooled, had a 35 fold increase in *Prevotella,* and a 19 fold increase in *Roseburia* compared to the final pooled inoculum. It was also identified that the response of the bacteria to pooling was not equal for all individuals and that some form of competition for bacterial dominance was occurring (Aguirre et al., 2014). The ability of some bacteria to dominate over existing bacterial populations has also been seen in faecal transplants of patients with *Clostridium difficile* infection. For these recipients, the bacterial population of the donor becomes that of the recipient, and

this remained at day 70 post-transplant (Fuentes et al., 2014). Animal studies have also shown the dominance of a bacterial population using mouse models. Ridaura et al., (2013) demonstrated that when obese mice were housed with lean mice that the obese mice became lean, and had the 'lean' bacterial profile, whereas the lean mice remained lean, suggesting a dominant set of bacteria. The obese mice housed with the lean bacteria also produced comparable SCFA to the lean mice, but not the obese mice with the obese bacteria, suggesting that pooling the bacteria may alter the SCFA production capabilities. However, housing mice with different bacteria, albeit with coprophagy to aid mixing, does not really mimic mixing different faecal samples in incubations (Ridaura et al., 2013).

As the strategy to pool or not pool stool samples was one of the main technical differences in papers used for the systematic review, later analysis was split into pooled and non-pooled data. After separating papers based on methodology, 51 articles were found to pool the faecal samples and 65 articles did not pool stool samples, (two articles (Barry et al., 1995, Mortensen et al., 1991) reported both pooled and non-pooled investigations. An observation with the pooled samples was that although multiple stool donors were used, only one biological repeat was carried out. This occurred with 32 of 51 (63%) articles using one biological repeat, which was higher than that of the non-pooled samples 12 of the 65 (32%), indicating that the non-pooled experiments had increased statistical power.

Studies not pooling the samples often conducted fermentations on three separate occasions; this is more appropriate as this means there are three biological repeats as opposed to one, further increasing statistical power. Although pooling stool samples is likely to reduce the variability within an investigation, it led to increased inter-study variation on some occasions (Table 3-3, Table 3-4). This may be due to individuals having different levels of colonic microbiota diversity. Greater diversity may aid rapid adaptation to the NDC present, producing different SCFA profiles. However, this may vary when stool samples are pooled and a dominant ecosystem is not established.

SCFA production during the intermediary time points, such as 10 - 23 hours were also considered (Table 3-5 - Table 3-8). This later phase of the batch culture indicates if the NDC is no longer being fermented (for a rapidly fermented NDC) or for slowly fermented NDC this may be the fastest period of SCFA production. Moreover, some SCFA are not greatly produced until later in the fermentation. Butyrate is often produced in greater

amounts after 8 hours as it may be formed by conversion from other SCFA (Morrison et al., 2006, Khan and Edwards, 2005). In the studies from the systematic review, between 6 and 9 hours, cellulose generated the highest proportion of propionate and was the lowest producer of all the SCFA, further demonstrating how using a simple ratio can be misleading when evaluating SCFA production by NDC. This increased proportion of propionate from cellulose may be attributed to the presence of glycosidic β-bonding which is associated with increased propionate production (Arora et al., 2012). SCFA production from glucose was the highest of the substrates between 6 and 9 hours. This may be because glucose is the most easily fermented. Between 10 and 23 hours, pea and maize fibre resulted in the lowest SCFA production; unfortunately, the data describing the SCFA production of these fibres prior to 10 hours did not fulfil the criteria for consideration in this review. When SCFA were compared as daily production, pea and maize fibre also ranked in the bottom 5 suggesting that they are poorly fermented substrates. Maize fibre between 10 and 23 hours led to a high proportion of propionate (27%), but ranked second from bottom for rate of production yielding 0.03 (0.0) mmol/g CHO/ hour further demonstrating how the ratio can be misleading.

After 24 hours of fermentation (Table 3-9, Table 3-10), it was found that rhamnose generated the highest proportion and rate of propionate production in the studies with individual faecal samples. Rhamnose has also been associated with increased propionate *in vivo*, where consumption as part of a meal increased levels of serum propionate (Vogt et al., 2004a, Vogt et al., 2004b).

Lactulose yielded high rates of SCFA production for the pooled and non-pooled samples This was demonstrated when 20g of lactulose was consumed by healthy individuals along with an infusion of labelled acetate where the fermentation of lactulose increased concentrations of exogenous acetate (Pouteau et al., 1998). At 24 hours cellulose yielded low rates of SCFA production for the pooled and non-pooled, this is supported by *in vivo* studies that did not identify cellulose as producing high faecal SCFA concentrations (Spiller et al., 1980).

When comparing the 24 hour non-pooled data with the pooled data, there were differences in the top and bottom SCFA producers. Pooled analysis identified that β-glucans generated the highest rate total SCFA, acetate, propionate and butyrate, whereas in the non-pooled

studies production from β-glucan was unremarkable; ranking 20th, 24th, 19th and 9th (out of 27) for total, acetate, propionate, and butyrate production.

One issue that affected the differences in the ranks of the NDC for SCFA between studies that pooled or did not pool faecal samples was that some of the substrates were not included due to lack of studies which met the inclusion criteria. Thus, the ranking of the substrates should not be directly compared between studies with these two methodologies.

The main key finding of this review was that there was no substrate which particularly stood out in terms of propionate production, although rhamnose and β-glucan showed promise. This also indicates that if a fibre is proportionally propiogenic but does not have high total SCFA production it may not always be the best option for selection. Therefore it may be beneficial to maximise the total intake of dietary fibre as increasing total SCFA production also increases propionate production. The quantity of propionate in the colon is likely to be more important than the proportion of SCFA when considering the physiological effects.

The differences between propionate production from different NDCs were relatively limited and increasing propionate production in the colon, on the basis of this data, could easily be achieved by increasing intake of almost any fibre studied. When comparing guar gum and pre-digested starch (i.e. resistant starch), increasing the dose of resistant starch by 60% could yield a similar amount of propionate as guar although guar has the higher molar proportion of propionate. However, the ratio may be important for some physiological functions where SCFA compete for effects. Wolever and colleagues found that the acetate/ propionate ratio determined impact on lipogenesis (Wolever et al., 1995, Wolever et al., 1991).

Also highlighted within this review was the lack of a systematic approach to screen the drivers of propionate production. The following chapters within this thesis aim to do this.

Chapter 4 Miniaturisation Validation

Some substrates tested within this thesis were too expensive to run in the standard large (1g) scale fermentation system. Therefore, a study was undertaken to miniaturise and validate reduced volume systems to enable increased numbers of both, biological and technical replicates. Miniaturised systems could potentially enable fermentations with 20x less substrate than previously required. The substrates oligofructose, pectin, guar and a blank (control) were used for this validation, as they are commonly used as control substrates in the laboratory.

4.1 Materials and Methods

Miniaturisation systems requiring 1 g, 100 mg, or 50 mg of substrate were compared (Table 4-1). These systems were used to ferment a selection of different substrates using the standard fermentation method in anaerobic conditions as described in Capter 2- Section 2.2.

Table 4-1: Changes to the fermentation composition because of the reduction in the fermentation size

	Standard	Medium	Small
Vial Size	100 ml	10 ml	6 ml
Substrate added	1 g	0.1 g	0.05 g
Fermentation medium	42 ml	4.2 ml	2.1 ml
Reducing solution	2000 µl	200 µl	100 µl
Faecal slurry	5000 µl	500 µl	250 µl
Final volume of fermentation	49 ml	4.9 ml	2.45 ml
Sample taken per time point	3000 µl	800 µl	400 µl

4.1.1 Stool donors

4.1.1.1 *Standard fermentation system*

M/F 3/2, aged 22-52, mean age 33.4 years (median age 25 years), n= 5
These fermentations were conducted independently of the medium and small fermentation systems.

4.1.1.2 *Medium and small fermentation system*

M/F 2/2, aged 22-52, mean age 35.5 years (median age 34 years), n= 4.
These fermentations were conducted the same faecal samples. The majority of donors provided a second sample for these systems, only one individual did not provide a second sample (male, 25 years).

4.1.2 Substrates tested

- Oligofructose (Beneo 95, Mannheim, Germany)
- Pectin (from apple, Sigma, Poole, UK)
- Guar gum (Sigma, Poole, UK)

A substrate free control was also used

4.1.3 Time points

- 1g fermentation system – 0, 2, 4, 8, and 24 hours
- 100 mg and 50 mg system – 0, 8, and 24 hours. Sampling was limited to three time points due to a reduced fermenter volume.

4.2 SCFA assessment

The SCFA in the fermenter supernatant were measured as described in Chapter 2- Section 2.2.1 with 100 µl (for standard system) and 25 µl (for medium and small systems) of 73.8 mM 2-ethyl-butyric acid as internal standard.

4.2.1 Statistical analysis and data presentation

Production of SCFA was made comparable by the use of the rate term μmol/g CHO/ hour. This rate unit is further discussed in Chapter 3- section 3.3.2. Molar proportions were also used to compare SCFA production. Data is presented as Mean (SEM).

All comparisons of the different vial sizes were conducted using ANOVA with post hoc Bonferroni analysis using SPSS version 22 (IBM, New York, USA). Graphs were produced on Microsoft Excel 2013.

4.3 Results

4.3.1 Process of 'miniaturisation'

A series of issues were identified whilst miniaturising the fermentation system. At each time point with the 'large' 1 g system 3000 μl of sample was taken. This was not possible in the smaller vessel sizes as this is 61% and more than the total of the fermentation volume of the medium and small systems. If 3000 μl of the fermentation slurry was required for the medium and small systems, a single fermentation vessel containing 100mg or 50 mg of substrate could be used and the vial removed at each time point, i.e. three time points = three vials per substrate. Due to this, the amount of slurry that was taken at each time point was reduced, and the number of time points used was limited to three (including the final time point). This had subsequent down-stream effects such as the requirement of a pH meter that was narrower, and able to measure the pH of smaller volumes. SCFA extraction was also modified due to the requirement of 800 μl of slurry with the standard 'in house' fermentation system, which was reduced requiring only 200 μl of slurry per extraction (Chapter 2 – Section 2.2.1). In essence, the process of fermentation and subsequent SCFA analysis was proportionally decreased by a factor of 20, achieving similar final concentrations in all vessels used.

4.3.2 Comparison of fermentations

The rate of SCFA production of the different models were compared to observe whether the volume had any impact. There were no significant differences in each SCFA produced for any of the substrates tested or for the blank control at 8 or 24 hours (Figure 4-1, Figure 4-2). The fermentation systems all resulted in the same pattern of SCFA production. At 8 and 24 hours the propionate production was in the order: control < oligofructose < pectin < guar for all vial sizes (Figure 4-1, Figure 4-2). Similar effects to the concentration were also seen when the variability of the molar proportion of propionate were assessed. Decreasing vial size, increased variability in propionate proportion this was clear for guar where the SEM for percentage propionate the large vial was 3.6%, the medium vial, 4.48%, and the small vial was 7.71% (Figure 4-4).

Although no significant differences in SCFA production between the vial sizes were observed, the variability for total SCFA production differed between vessel. As the amount of substrate used was decreased, the more variable the total SCFA production became. This was likely due to the propagation of errors, occurring when reducing the fermentation volume. In most cases, the equipment used could be reduced to accommodate the difference in volumes, but not all errors in equipment were the same (Table 4-2). This did not take into account difficulties encountered when adding the faecal slurries, which were increasingly difficult to measure as the volume reduced. The weighing scale also had an error of 1 mg resulting in the error in mass of substrate for the small system being 20x that of the large system (Table 4-2). This propagation of effects likely occurred and each time point, where the viscosity could have also played a role in the increasing error, particularly with the 50 mg system. The reduced fermentation size was challenging with use of the viscous fibres, such as guar, which produce a viscous supernatant, which reduced the volume of fermentation slurry available for sampling. Increased errors were also observed for oligofructose, as the propagation of errors could possibly have non-significantly altered the production of SCFA, which would be more apparent in a highly propiogenic substrate (Figure 4-3).

Table 4-2: Experimental error of different fermentation vessels used

	Standard	Error	Medium	Error	Small	Error
Scales error (1 mg)	1 g	0.1%	100 mg	1%	50 mg	2%
Fermentation medium	42 ml	1.84%	4.2 ml	1.45%	2.1 ml	0.6%
Reducing solution	2000 µl	1.92%	200 µl	1.9%	100 µl	2.4%
Faecal slurry	5000 µl	0.64%	500 µl	3.08%	250 µl	0.48%
Error % per vessel		4.50%		7.43%		5.48%

Errors were calculated by the average of five weighed measurements of water for each volume required.

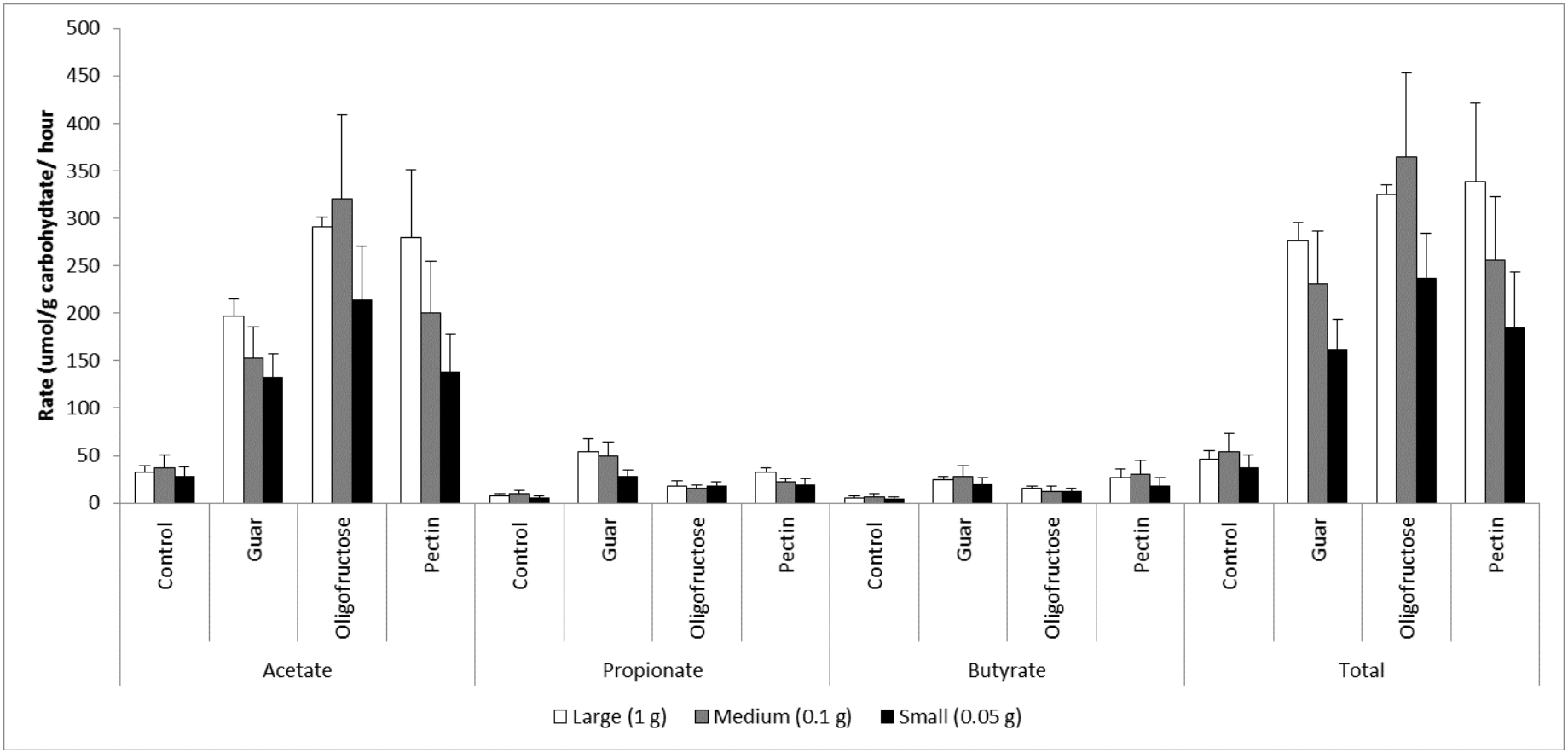

Figure 4-1: Rate of SCFA production after 8 hours of fermentation (µmol/ g carbohydrate/ hour).

Miniaturised fermenters containing 50 mg, 100mg (n=4) and 1000 mg (n=5), of guar gum, oligofructose or pectin were compared. Presented are mean + SEM. No significant differences as a result of vial size were observed.

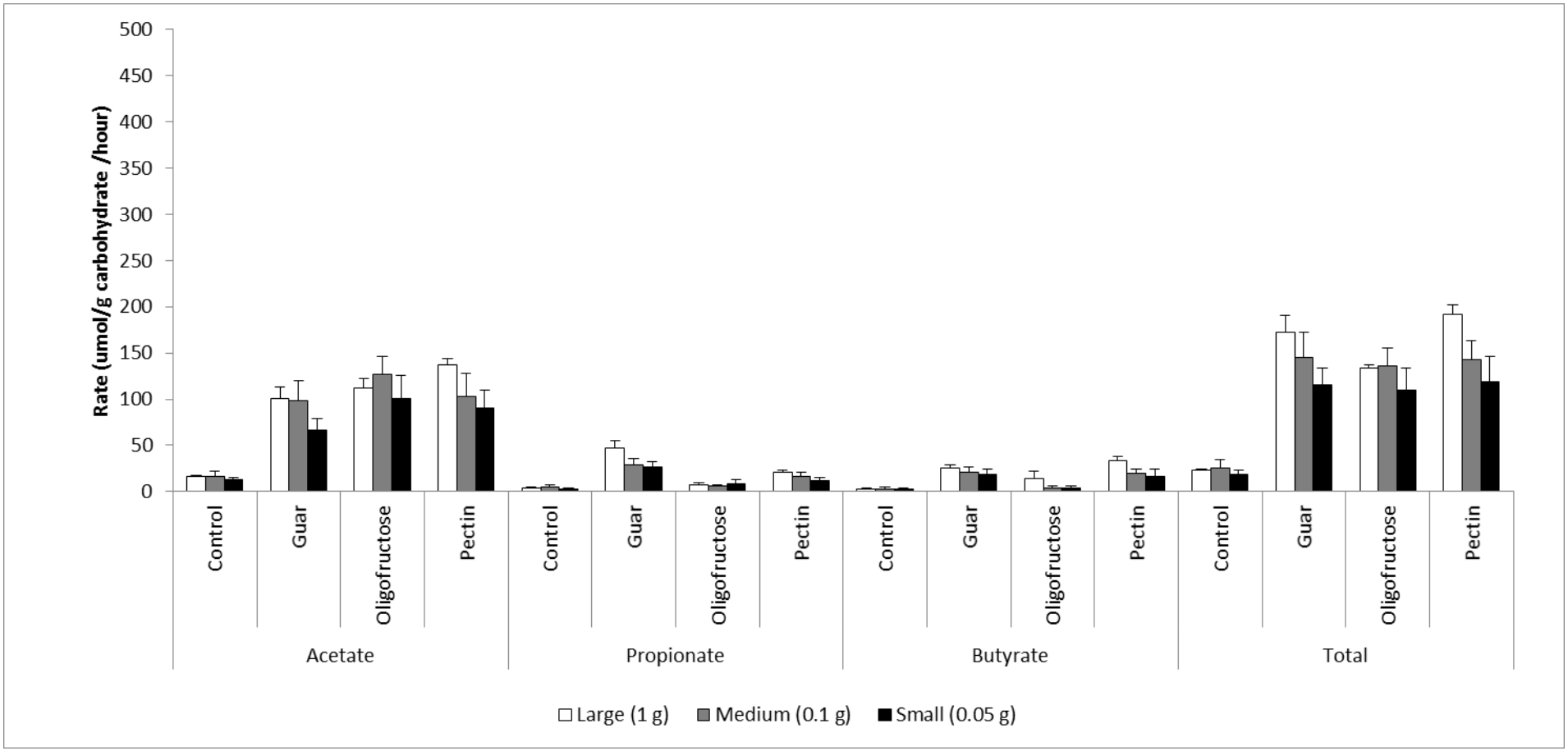

Figure 4-2: Rate of SCFA production after 24 hours of fermentation (µmol/ g carbohydrate/ hour).

Miniaturised fermenters containing 50 mg, 100mg (n=4) and 1000 mg (n=5), of guar gum, oligofructose or pectin were compared. Presented are mean + SEM. No significant differences as a result of vial size were observed..

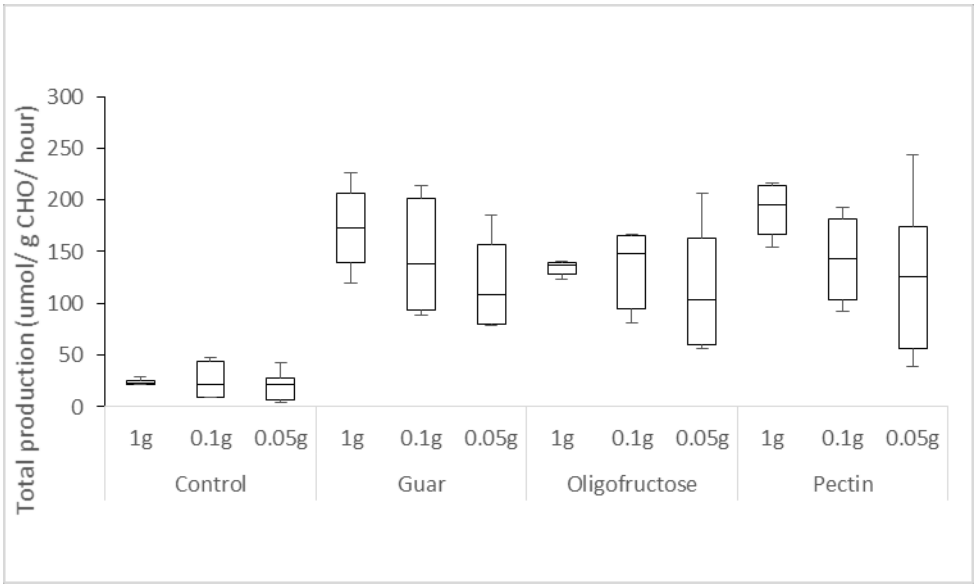

Figure 4-3 : Total rate of SCFA production after 24 hours of fermentation.

Fermentation with miniaturised fermenters containing 1000 mg (n=5), 100 mg and 50 mg (n=4) of guar gum, oligofructose or pectin. No significant differences were observed because of substrate amount.

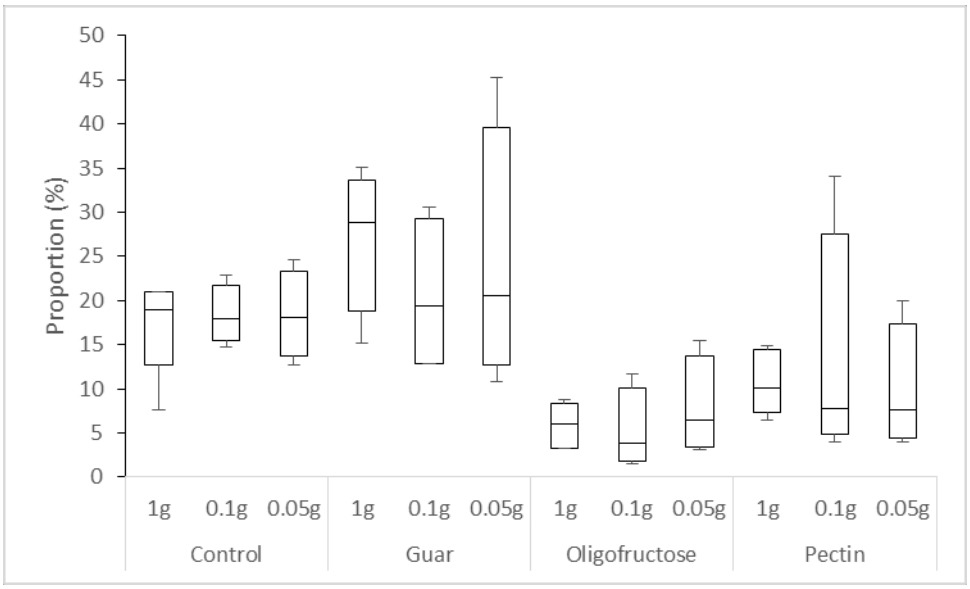

Figure 4-4: Propionate proportion after 24 hours of fermentation

Fermentation with miniaturised fermenters containing 1000 mg (n=5), 100 mg and 50 mg (n=4) of guar gum, oligofructose or pectin. No significant differences were observed because of substrate amount.

.

4.4 Discussion

It was possible to miniaturise the fermentation system without detrimental effects to the production of SCFA. This paves the way for screening expensive and less abundant substrates in a way that is more commercially viable, and enabled more biological replicates of costly substrates, as less substrate is required for each vessel. This provides increased power for the assessment of the SCFA producing capabilities of different substrates. Although the systems were reduced, proportions of the components of each fermenter were maintained, i.e. 2.0% w/v of substrate, the less substrate added to the vial, the less SCFA and increased variability in the SCFA production occurred.

Some of this variability would likely have occurred due to inter-individual variability in the SCFA production, as seen in previous studies (Carlson et al., 2016). The effect of this variability was limited in this miniaturisation by using stool samples from the same individuals. As different stool samples were used to seed the large vessels compared to the medium and small fermentation vials intra-individual variability in SCFA production may have occurred. This is as the diets were not controlled or replicated, and diet has been shown quickly alters the gut bacteria (David et al., 2014). For the medium and small vessel sizes, where the same stool sample was used to seed the vessels, the natural biological inter-donor variability would have also occurred, but to a similar extent for both sample sizes (Figure 4-1, and Figure 4-2).

This suggests that some of the differences variability occurring between the medium and small vessel sizes may have been because of experimental error. This was particularly likely in the small system, during the setup of the fermentation and with the subsequent time points. For example, the medium and small systems, each sampling removed 16% of the slurry (for further SCFA analysis). This was greater that the large system (6.0%), but does not explain the difference in the variability between the medium and small systems. Differences between the medium and small systems, in particular may have been because of propagation of error occurring during the miniaturisation of the system. These errors were reduced by using different equipment where the sizes used were reduced accordingly, but was not always possible and may have resulted in the differences seen. This was apparent, in particular when weighing out the initial mass of substrate. The scale used had an error of 1 mg, which for the large fermentation system translates to 0.1% of mass added,

but when considering the small fermentation system this is a 2.0% difference in the mass added, thus leading to the further error, when reducing the fermentation system (Table 4-2).

Based on this the 'small system' was only subsequently used when many biological replicates of an expensive substrate was required, such as those within Chapter 6. Other studies have used similar fermentation systems that have been reduced to have a total volume of 700 µl, and limited sampling to one time point only (Sanz et al., 2005c, Hernandez-Hernandez et al., 2011) Small-scale fermenters with a 5 ml working volume and 50 mg of substrate can also be used in a pH-controlled system (Hernandez-Hernandez et al., 2011). An advantage of the system within this chapter is that the reduction in substrate quantity, along with a reduced volume, did not alter the SCFA production profiles of the substrates tested. It has been previously demonstrated in the *in vitro* investigation by Khan and Edwards., (2002) where the amount of substrate was reduced from 10 mg/ml to 2.5 mg/ml, differences in SCFA production of lactulose were observed (Khan and Edwards, 2002).

Another advantage of this system was that a smaller amount of stool sample was required to provide sufficient slurry for multiple fermentations. This was particularly beneficial when there was many different substrates for fermentation, or many different types of analysis conducted with one stool sample. A disadvantage of these miniaturised systems is that the number of sampling times and volumes taken needs to be reduced for enough fermentation media to remain for the whole duration of the experiment. This required down-stream analysis to be modified and was taken into account when planning experiments that required slurry for different forms of analysis.

Overall, it has been demonstrated that the 1g in house system could be reduced without detrimental effects to SCFA production. However, although not significant, differences in the variability were seen, this was likely due to the increased experimental error, occurring when reducing the size of the fermentation vessel. This indicates that due to this error, the main benefit of the small system is when there the substrate being tested is scarce, and that the using the medium and large systems are more beneficial for screening of substrates in which are more abundant or less costly.

Chapter 5 Identification of substrates that selectively increase propionate production *in vitro*

5.1 Introduction

The potential importance of colonic propionate and its role in contributing to the health-benefits of non-digestible carbohydrates (NDC) were discussed in Chapter 1. Increasing the amount and proportion of propionate produced from the fermentation of carbohydrates by colonic bacteria may affect: satiety and therefore eating behaviour (Chambers et al., 2014, Byrne et al., 2016), liver metabolism of lipids and carbohydrates improving lipoprotein and cholesterol levels in the plasma (Berggren et al., 1996, Heimann et al., 2015). An understanding of which carbohydrates preferentially increase propionate production would enable selection of NDCs for dietary manipulation to improve health.

In Chapter 3 (systematic review) which considered which substrates promote the generation of propionate it was concluded that the evidence predicting NDCs that are 'propionate' producers was not clear-cut. This was despite perceived evidence of some carbohydrates (such as β-glucans) being more propiogenic than others. The systematic review showed that carbohydrates yielding high proportions of propionate did not necessarily produce greater amounts of propionate when compared *in vitro*. Comparisons between carbohydrates were also hampered by the vast variation in the methodologies used between studies. Therefore, a more thorough and standardised comparison of carbohydrates is required to identify which NDC selectively increase propionate production in the human colon. In this chapter, a selection of potentially propiogenic substrates were considered and screened *in vitro* for their propiogenic potential. The substrates were chosen based on the data acquired within the systematic review and other sources. The substrates tested are discussed below:

5.1.1 B-glucans

Beta (β) – glucans are polymers of glucose linked with beta glycosidic bonding and are associated with the increased production of propionate. Queenan et al., (2007) showed *in vitro* that oat and barley β-glucans (consisting of β[1-3] and β[1-4] linkages) increased propionate production compared to controls (Queenan et al., 2007). Increased *in vitro* propionate production has also been seen with more uncommon β-glucans such as those in

seaweeds such as laminarin (brown algae, β[1-3], β[1-6] bonding) and Gelidium (red algae, β [1-4] bonding) (Ramnani et al., 2012, Deville, 2007).

Beta glucans have also been shown to increase colonic propionate and have beneficial health outcomes after consumption. When hypercholesteraemic individuals consumed 6 g/ day of oat β-glucan by for 6 weeks benefits such as reduced total and LDL cholesterol were observed (Queenan et al., 2007). Kuda et al., (2005) fed rats a diet containing 2% laminarin for 14 days, and observed a 66% increase in caecal propionate compared to controls and high molecular weight alginate (Kuda et al., 2005). Feeding of laminarin (from *laminara hyperborea*) to pigs for 14 days also altered propionate production and bacterial composition. There was a dose dependent increased in the proportion of propionate, and decrease in caecal *Enterobacterium* spp (6.94- 6.7cfu /g digesta), and *Bifidobacterium* spp (8.33- 7.86 cfu /g digesta) numbers with β-glucan dose. These effects however were not associated with differences in caecal pH (Lynch et al., 2010).

5.1.2 Legumes

The fibre content of legumes is between 8 and 32% dietary fibre with, 30-75% being insoluble. Colonic fermentation of legumes may have beneficial down-stream effects on satiety (McCrory et al., 2010, Guillon and Champ, 2002). *In vitro* fermentation, using human faecal bacteria, of the dietary fibre content of a selection of legumes and root crops including kidney bean, mung bean, lima bean, and peanut (as extracted using the (AOAC) 991.43 method) was assessed by Mallillin et al., (2008). It was identified that legumes produced high concentrations and proportions of propionate (Mallillin et al., 2008). For example, 65% of the total SCFA production was formed as propionate from kidney bean fibre fermentation. Data from this investigation was difficult to extrapolate to the general population as only a single fermentation consisting of one stool donor was used (Mallillin et al., 2008). In another *in vitro* investigation, fermentation of lentil and chickpea fibre, pre-digested using an *in vitro* digestion model, resulted in SCFA with 26% and 19% propionate and 7% and 16% of butyrate respectively. This suggested that legumes may be candidates for propionate production (Hernandez-Salazar et al., 2010).

The impact of legumes on satiety and weight management has been previously reviewed (Guillon and Champ, 2002, McCrory et al., 2010, Barrett and Udani, 2011). Consumption

of a low dose (2.6 g per day) or a high dose (5.8 g per day) of peanut sprout extract via a capsule for 4 weeks by 15 overweight and obese individuals showed several beneficial effects. The low dose decreased waist circumference and plasma TAG concentrations. The high dose also decreased waist circumference, had no effect of TAG concentrations but decreased blood LDL cholesterol compared to dextrin (control) (Ha et al., 2015). Consumption of *Phaseolus vulgaris* (kidney bean, Beanblock®) extract by 12 individuals as a tablet before a meal decreased postprandial insulin, and a reduced change in glucose compared to the placebo. The experimental group also experienced greater feelings of satiety, and a reduced desire to eat compared to the placebo. Along with changes in perceived satiety, the experimental group also had significantly reduced plasma ghrelin production compared to the placebo group, which were associated by decreased β-cell activity (Spadafranca et al., 2013).

Propionate is associated with improved insulin sensitivity, and β cell function (Tang et al., 2015) and *in vitro* increases in propionate demonstrate that the soluble fibre of legumes may yield high concentrations of propionate.

5.1.3 Resistant starch and starch derivatives

Starch is a glucose polymer consisting of amylose (α[1-4] bonds) and amylopectin (α[1-4] and α[1-6] bonds). These bonds are broken in the upper gut by salivary and pancreatic amylase and brush border enzymes. Approximately 10% of dietary starch resists digestion in the SI and reaches the colon; this is termed resistant starch (RS). RS is fermented in the colon, and has been subcategorised to RS1-RS5, each of which has different chemical and physical properties. RS1 is inaccessible to digestion enzymes, such as within a grain, and is heat resistant. RS2 is starch is tightly stored in granules, is often found in bananas and is comparatively dehydrated compared to other RS types. RS3 (retrograded) has undergone some form of processing, often cooked, then cooled like cooked and cooled potatoes in potato salad. RS4 is starch that has been chemically modified, such as by esterification (Topping and Clifton, 2001). RS5 is starch formed of an amylose-lipid complex (Thompson et al., 2011).

RS increases SCFA production in *in vitro* fermentation investigations and increased faecal SCFA in feeding trials. In batch cultures of faecal bacteria RS selectively increased the

production of butyrate (Kaur et al., 2011, Weaver et al., 1989)(Chapter 3). Pyrodextrinised (RS4) forms of potato, lentil and cocoyam starch generated significantly higher amounts of propionate (at the cost of acetate) compared to the native forms of these starches, *in vitro* (Laurentin and Edwards, 2004). Ye et al., (2015) fed 19 individuals up to 10 g of Fibersol-2 (pyrodextrinised corn starch) in a test drink (peach flavoured ice tea, 0, 5 or 10 g) along with a meal. Feelings of satiety were increased, and hunger was decreased for up to two hours postprandially after the 10 g dose of Fibersol-2. This was related to increased concentrations of PYY and GLP-1 (Ye et al., 2015), both of which have been associated with SCFA production (Lin et al., 2012).

Altered eating behaviour has also been seen with other types of RS. Willis et al., (2009) observed when feeding 20 individuals a muffin containing approximately 8 g of fibre (muffin total weight - 92 g), including Hi-Maize (RS2) that there was decreased hunger and food intake (Willis et al., 2009). The Hi-maize muffins were associated with low palatability compared to other muffins tested, which may have altered feelings of hunger and satiety. Differences in satiety hormones by Hi-maize were also observed by Bodinham et al., (2013) where individuals (n= 30) were fed 80 g of Hi-maize (48 g of resistant starch), spread over breakfast and lunch. After breakfast, there was a reduction in GLP-1, and after lunch, there was no difference in GLP-1, however feelings of hunger and satiety were not measured. This was thought to be a result of the lack of change in concentration of GLP-1 from that produced at the breakfast, not necessarily the base-line (Bodinham et al., 2013).

5.2 Objectives

The aim of the work in this chapter was to identify substrates which may increase the production or proportion of propionate. This would enable further mechanistic analysis of why they increase yields of propionate, as opposed to other dietary fibres.

The objective was to screen a range of potential substrates using *in vitro* batch cultures and to assess SCFA production. Along with the substrate, the role of initial pH and its effects on propionate production were also assessed.

5.3 Materials and Methods

The screening of a selection of substrates to identify those that are propiogenic were grouped into a series of different experiments. These experiments were grouped based on substrate type or methodology. These experimental groups were:

Experiment 1: Beta glucans
Experiment 2: Legumes
Experiment 3: Starch (and modified starch)
Experiment 4: Modified barley starches
Experiment 5: Starch fermentation at two initial pHs (6.8 & 5.4)
Experiment 6: Guar, Trehalose dihydrate, Cellobiose and Glucagel fermentation at two initial pHs (6.8 & 5.4)

5.3.1 *In vitro* fermentation of the different substrates

Section 2.1.3 outlines the protocols used for the *in vitro* fermentations used for the screening of the substrates. This *in vitro* method is similar to those compared within the systematic review (Chapter 3) and is high throughput, enabling a number of different substrates to be screened.

Chapter 2 and Chapter 4 outline the differences, and the validation of the fermentation models utilised for the screening of substrates. Two different fermentation sizes were used within this chapter, these were:

1. Medium sized vessel: Substrate added 100 mg; total volume 4.9 ml
2. Mid-sized vessel: Substrate added; 200 mg, total volume 9.8 ml

As shown in Chapter 2- Section 2.2.1 there were three different fermentation buffers used all of which differed in composition:

1. Standard – has an initial pH of 7.0
2. pH 6.8 buffer – has an initial pH of 6.8
3. pH 5.4 buffer – has an initial pH of 5.4

5.3.2 Experimental Controls

All fermentation experiments included a positive control. Positive controls for each experiment were:

- High performance inulin (HPI, Beneo HP, Mannheim, Germany)
- Oligofructose (OF, Beneo P95, Mannheim, Germany).

A negative control (blank) was also included

5.3.3 Individual experiment details

5.3.3.1 *Experiment 1: Beta-glucans*

Substrates: The substrates were commercially available from different sources, with variable levels of purity and variations in β-glucan bonding.

- Oatwell 22: 22% oat β-glucan, bonding β(1-3), β(1-4) (DSM, Heerlen, Netherlands)
- Oatwell 28: 28% oat β-glucan, bonding β(1-3), β(1-4) (DSM; Heerlen, Netherlands)
- Promoat oat β-glucans 35% β-glucan, bonding β(1-3), β(1-4) (Tate and Lyle; Kimstad, Sweden
- Glucagel: a barley β-glucan consisting of 75% β-glucan, bonding β(1-3), β(1-4) (DKSH; London, UK)
- Laminarin: a polysaccharide extracted from *Laminaria digitata* consisting only of glucoses bonding β(1-3), β(1-6) i.e. 100% β-glucan (Sigma; Poole, UK)

Positive control: High performance inulin (Beneo; Mannheim, Germany)

Fermentation model used: 100 mg substrate in medium sized vial. Standard media

Stool donors: M/F, 2/1, aged 23-26 years, mean: 25 years, (median: 25 years), n = 3
Sampling time points: 0 and 24 hours (the 6 hour time point was omitted due to culture viscosity which would result in sampling difficulties)

5.3.3.2 *Experiment 2: Legumes*

Substrates: The soluble fibre fraction of the legumes was extracted by Dr Douglas Morrison by hot water extraction and air dried (50°C). Native dried beans were purchased from a local supermarket (Sainsbury's). Legumes tested were:

- Peanut
- Mung bean
- Kidney bean

Positive control: Oligofructose (Beneo P95; Mannheim, Germany)
Fermentation model used: 100 mg substrate medium sized vial. Standard media
Stool donors: M/F, 2/1, aged 23-52 years, mean: 33 years, (median: 24 years), n = 3 (duplicate vials)
Time points: 0, 6, and 24 hours

5.3.3.3 *Experiment 3: Starch (and modified starch) fermentation*

Substrates: A selection of different starches were fermented, to identify any potential role in the selective production of propionate. These were:

- Potato starch (Sigma; Poole, UK),
- Hi-Maize starch, resistant starch (Ingredion; Manchester, UK),
- Fibersol-2, resistant maltodextrin (Matsutani Chemical Industry Co., Ltd; Hyogo, Japan)

Positive control: Oligofructose (OF, Beneo P95, Mannheim, Germany)
Fermentation model used: 100 mg substrate, medium sized vessel, pH 6.8 media
Stool donors M/F, 2/1, aged 23-25 years, mean: 24 years (median: 24 years), n = 3 (duplicate vials)
Time points: 0, 6, and 24 hours

5.3.3.4 *Experiment 4: Modified barley starches*

Substrates: Barley derived starches outlined by (Carciofi et al., 2012) were fermented. The starches used were; Amylose only (AO) and wild type (WT) starch. The starches were processed resulting in:

- Whole grain
- Milled
- Pure starch (Starch that has been purified)

Fermentation model used: Whole-grain and milled - 200mg (mid-sized) vessel. Pure starch – 100 mg (medium) vessel. All fermentations used pH 6.8 media
Positive control: Oligofructose (OF, Beneo P95; Mannheim, Germany)
Stool donors: M/F, 2/1, aged 23-25 years, mean: 24 years, (median: 24 years), n = 3 (duplicate vials)
Time points: 0, 6, and 24 hours

5.3.3.5 *Experiment 5: Starch fermentation at two initial pHs (6.8 & 5.4)*

Substrates: A selection of different starches previously tested in Experiment 3: Starch (and modified starch) fermentation, Experiment 4: Modified barley starches were fermented with two different initial pHs. This was used to identify any potential role of initial in the production of propionate. These were:

- From Experiment 3
    - Potato starch
    - Hi-maize starch
    - Fibersol-2
- From Experiment 4
    - Pure starch (of amylose only and wild type barley)

Positive control: Oligofructose (OF, Beneo P95, Mannheim, Germany)
Fermentation model used: 100 mg substrate, medium vessel, pH 6.8 and pH 5.4 media
Stool donors: M/F, 2/1, aged 23-25 years, mean 24 years, (median: 24 years), n = 3 (duplicate vials)
Time points: 0, 6, and 24 hours

5.3.3.6 *Experiment 6: Guar, Trehalose dihydrate, Cellobiose and Glucagel fermentation at two initial pHs (6.8 & 5.4)*

Substrates: A selection of different substrates associated with increased propionate were fermented at different pHs to identify any potential role in the production of propionate. These were:

- Guar gum (Sigma, Poole, UK)
- Glucagel (DKSH, London, UK)
- Trehalose dihydrate (Carbosynth, Berkshire, UK)
- Cellobiose (Carbosynth, Berkshire, UK)

Positive control: Oligofructose (OF, Beneo P95, Mannheim, Germany)
Fermentation model used: 200 mg (mid- sized) vessel, pH 6.8 and pH 5.4 media
Stool donors: M/F 1/2, aged 23-24 years, mean: 23.7 years (median: 24 years), n = 3
Time points: 0, 6, and 24 hours

5.3.4 pH measurements

At each sampling time point, the pH was measured with
the use of a pH meter (HANNA). This was carried out before the addition of NaOH.

5.3.5 SCFA analysis

The SCFA production was analysed by GC: FID, after undergoing ether extractions. This was conducted as described in Chapter2- Section 2.3.

5.3.6 Data presentation and statistical analysis

Statistical analysis was conducted using IBM SPSS version 22. Tests of normality was analysed by the Shapiro Wilk test. Statistical analysis was as follows. Data for pH, SCFA concentration, ratio and rate unit between fibres or initial pH cultures were compared using ANOVA and post hoc Bonferroni. Where data were not normally distributed, log transformation was used before statistical analysis. Data comparing two time points (6 and 24 hours) were compared by Mann Whitney U test.

5.4 Specific details of statistical analysis for each experiment

Experiment 1: Beta- glucans

SCFA concentration: ANOVA and post hoc Bonferroni
pH and SCFA ratio: Data was $\log_{(10)}$ transformed and then analysed with ANOVA and post hoc Bonferroni

Experiment 2: Legumes

All data was $\log_{(10)}$ transformed and then analysed with ANOVA and post hoc Bonferroni. Differences between times were measured with Mann Whitney U test.

Experiment 3: Starch (and modified starch) fermentation

pH: data was $\log_{(10)}$ transformed and then analysed with ANOVA and post hoc Bonferroni.
SCFA concentration and ratio: ANOVA and post hoc Bonferroni.

Experiment 4: Modified barley starches

All data was $\log_{(10)}$ transformed and then analysed with ANOVA and post hoc Bonferroni.

Experiment 5: Starch fermentation at two initial pHs (6.8 & 5.4)

pH and SCFA ratio: data was $\log_{(10)}$ transformed and then analysed with ANOVA and post hoc Bonferroni
Rate: ANOVA and post hoc Bonferroni.

Experiment 6: Guar, Trehalose dihydrate, Cellobiose and Glucagel fermentation at two initial pHs (6.8 & 5.4)

pH and rate: Data was $\log_{(10)}$ transformed and an ANOVA was conducted with post hoc Bonferroni
Ratio: ANOVA and post hoc Bonferroni

5.5 Results

5.5.1 Experiment 1: Beta- glucans

The β-glucans tested were fermented and produced significant amounts SCFA. Due to fermentation being a closed system, the pH reduced for all substrates from pH 7.0 to a pH between 4.0 and 5.0. For the blank the pH reduced by only 0.03 units. The greatest reduction in pH was identified with Laminarin and Promoat fermentation falling from pH 7.06 (0.35) to 4.15 (0.08), and pH 7.08 (0.27) vs 4.28 (0.26) and were significantly lower that all other substrates and the blank tested $p<0.05$. As well as having the lowest pH, Promoat and Laminarin also generated the highest concentrations of total SCFA (91.7 [15.4] mmol/l and 147.9 [31.4] mmol/l, Figure 5-1).

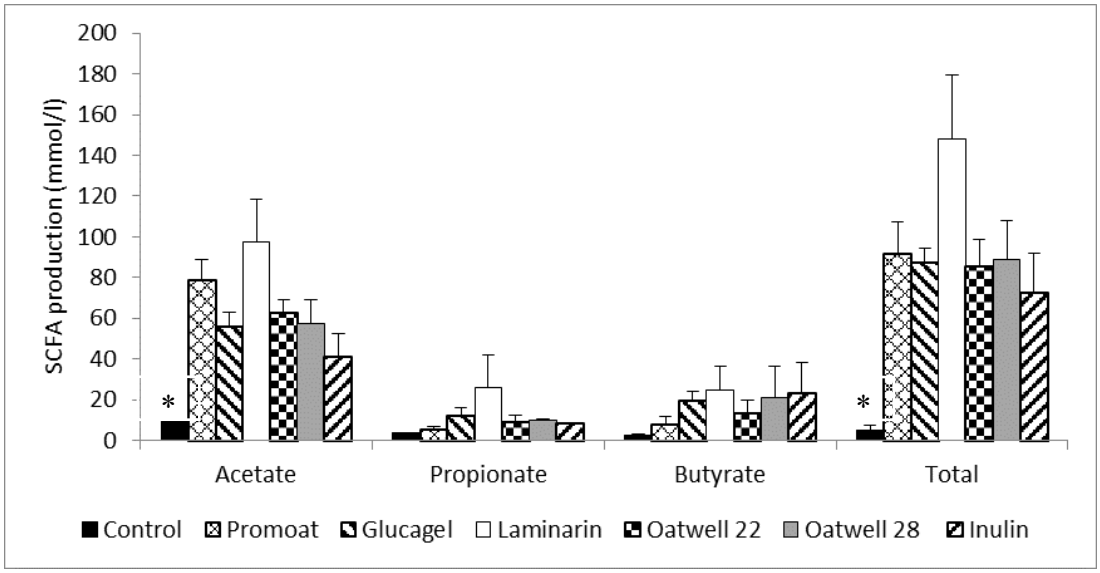

Figure 5-1: SCFA production (mmol/l) after 24 hours of fermentation.

Substrates fermented were the β-glucans: Promoat, Glucagel, Laminarin, Oatwell 22, and Oatwell 28. Presented are mean (+ SEM), n=3, * = $p<0.05$ vs all β-glucans

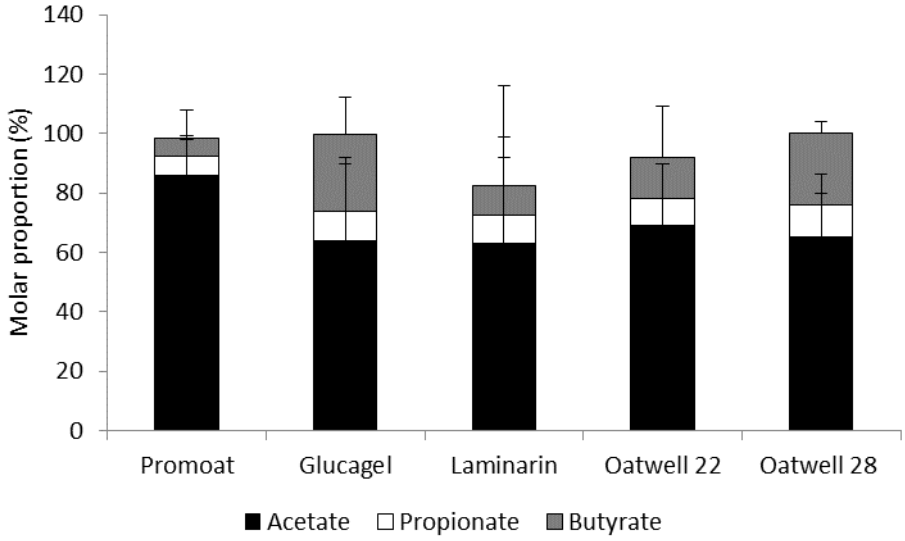

Figure 5-2: SCFA molar proportion (%) after 24 hours of fermentation.

Substrates fermented were the β-glucans: Promoat, Glucagel, Laminarin, Oatwell 22, and Oatwell 28. Median (+IQR), n=3

Laminarin fermentation led to non-significant increases in acetate, propionate and total SCFA production compared to the other substrates used. Although non-significant, total SCFA production with laminarin fermentation was 56.2 mmol/l higher than that of Promoat, the second highest β-glucan for total SCFA production (Figure 5-1).

There was also no significant effect (or trend) of β-glucan on the proportion of SCFA produced, however, Promoat resulted in the highest proportion of acetate (86.0 [13.4] %) and the lowest proportion of propionate (6.4 [5.5] %). Oatwell 28 and Glucagel generated the highest proportion of propionate (10.7 [10.4] % and 10.1 [5.6] %). Glucagel also yielded the highest proportion of butyrate with 25.7 (12.5) % of the total SCFA generating butyrate (Figure 5-2). This indicated that the increased fermentability of laminarin, increased all of the SCFA produced, and did not change the proportion.

The order of magnitude of SCFA production was ranked for each individual set of cultures (individual donors, Table 5-1). Acetate production by the different β-glucans varied by individual. On all occasions, Laminarin resulted in the highest propionate production, and that propionate production from Glucagel was consistently high. Ranking of butyrate production was more variable for the top producers, whereas it was clear that Promoat and the blank generated the lowest amount of butyrate for all donors (Table 5-1).

Table 5-1: Ranking of acetate, propionate and butyrate production from each stool donor after 24 hours of fermentation.

	Acetate			Propionate			Butyrate		
Rank	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**
1	Laminarin	Laminarin	Promoat	Laminarin	Laminarin	Laminarin	Glucagel	Oatwell 28	Laminarin
2	Promoat	Promoat	Oatwell 22	Glucagel	Glucagel	Oatwell 28	Laminarin	Glucagel	Glucagel
3	Oatwell 22	Oatwell 28	Glucagel	Oatwell 22	Oatwell 28	Glucagel	Oatwell 28	Laminarin	Oatwell 22
4	Oatwell 28	Glucagel	Oatwell 28	Oatwell 28	Control	Oatwell 22	Oatwell 22	Oatwell 22	Oatwell 28
5	Glucagel	Oatwell 22	Laminarin	Promoat	Oatwell 22	Promoat	Promoat	Promoat	Promoat
6	Control	Control	Control	Control	Promoat	Control	Control	Control	Control

NDC with a ranking of 1 is the top producers, 6 is the lowest produced.

5.5.2 Experiment 2: Legumes

Fermentations were conducted using the soluble fractions of peanut, kidney bean, and mung bean fibre. The pH for all legumes reduced compared to the control within 6 hours (Table 5-2). Oligofructose fermentation resulted in the lowest pH after 24 hours. The soluble fibre fraction of peanut had the smallest reduction in pH (0.9 units) after 24 hours compared to the other legumes tested. It was also observed that at 24 hours all substrates had significantly different pH from each other (Table 5-2).

Table 5-2: pH at 0, 6 and 24 hours of fermentation of the soluble fibre of legumes,

Time (hours)	Control (Blank)	Peanut	Kidney Bean	Mung Bean	Oligofructose	
0	7.09 (0.18)[a]	6.98 (0.2)	6.92 (0.21)[b]	6.90 (0.18)	7.0 (0.22)	$p<0.05$
6	7.04 (0.07)[a]	5.77 (0.28)[b]	5.13 (0.18)[c]	4.71 (0.66)[d]	4.36 (0.51)[d]	$p<0.01$
24	7.04 (0.08)[a]	6.0 (0.11)[b]	5.4 (0.48)[c]	4.32 (0.5)[d]	3.98 (0.35)[e]	$p<0.01$

Median (IQR), different letters within rows indicate significant differences.

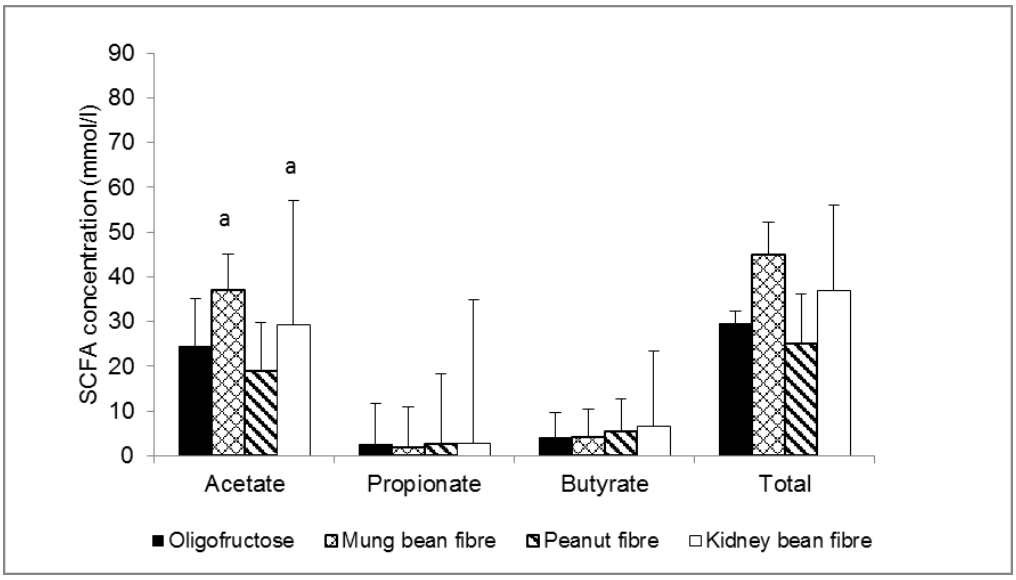

Figure 5-3: SCFA production with the soluble fibre from the legumes; mung bean, peanut and kidney bean (mmol/l) after 6 hours of fermentation.
Median + IQR, n=3. Significant differences from peanut are denoted as a: $p<0.01$

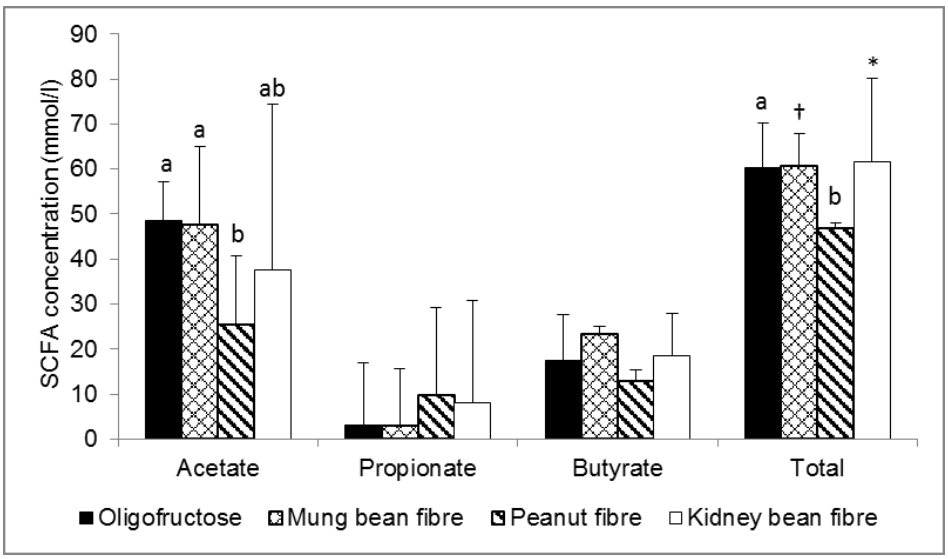

Figure 5-4: SCFA production with the soluble fibre of the legumes; mung bean, peanut and kidney bean (mmol/l) after 24 hours of fermentation.

Presented are median + IQR, n=3. Significant differences from peanut are denoted as a: $p<0.05$, and from oligofructose b: $p<0.05$, *; $p= 0.081$ vs Peanut, †; $p=0.062$,

Table 5-3: Ranking of the production of SCFA by the soluble fibre of legumes.

Rank	Acetate			Propionate			Butyrate		
	P1	**P2**	**P3**	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**
1	Mung bean	Oligofructose	Oligofructose	Peanut	Peanut	Oligofructose	Mung bean	Kidney bean	Mung bean
2	Oligofructose	Mung bean	Kidney bean	Kidney bean	Kidney bean	Mung bean	Oligofructose	Peanut	Oligofructose
3	Kidney bean	Kidney bean	Mung bean	Oligofructose	Mung bean	Kidney bean	Kidney bean	Oligofructose	Kidney bean
4	Peanut	Peanut	Peanut	Mung bean	Control	Peanut	Peanut	Mung bean	Peanut
5	Control	Control	Control	Control	Oligofructose	Control	Control	Control	Control

Ranking from highest (1) to lowest (5) Control = blank/ negative control. P1-P3 indicates stool donors 1, 2, and 3.

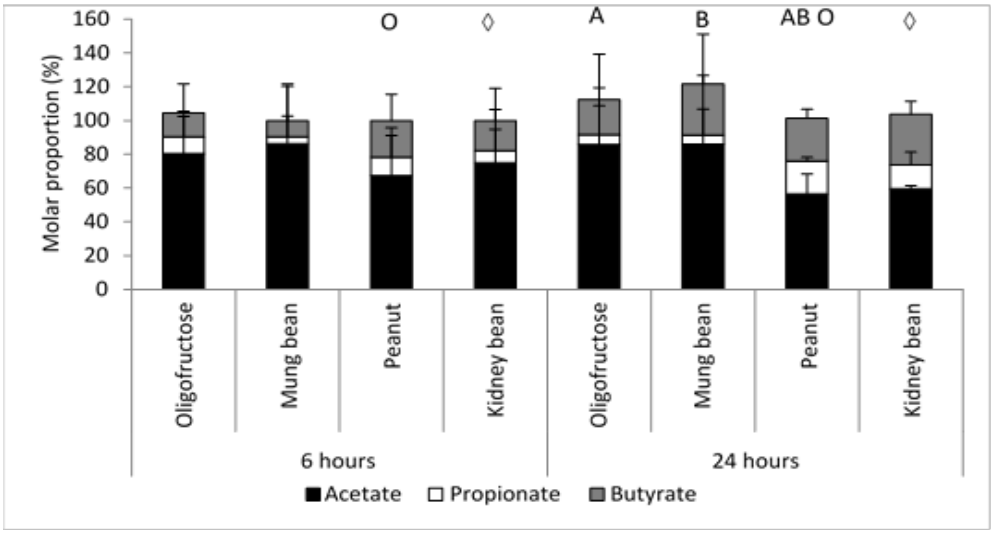

Figure 5-5: Ratios of SCFA production with the soluble fibre of legumes mung bean, peanut and kidney bean at 6 and 24 hours.

Substrates with same letter are significantly different from each other; A, p = 0.047 for acetate, B, p= 0.013 for propionate at 24 hours. Symbols indicate changes in proportion between 6 and 24 hours; O indicates changes in proportion of all SCFA $p<0.05$ with peanut, ◊, indicates a change in butyrate $p<0.001$ with kidney bean between 6 and 24 hours. Presented are median + IQR, n=3

Fermentation of all the soluble fraction legumes increased acetate production compared to the control at 6 hours (Figure 5-3). For the legumes, mung bean fibre fermentation generated the most acetate, producing 37.1 (9.2) mmol/l, and peanut fibre resulted in the lowest acetate (19.0 [5.5] mmol/l). The lowest producer of propionate and butyrate at 6 hours was the control (blank) which generated 1.4 (0.7) mmol/l and 0.9 (1.6) mmol/l of propionate and butyrate. Butyrate was produced in high quantities after fermentation with peanut and kidney bean soluble fibre fractions, which yielded 5.5 (7.3) mmol/l and 6.6 (10.9) mmol/l of butyrate (Figure 5-3).

Within 24 hours of fermentation all of the legume fibres had significantly more acetate production than the control ($p<0.001$), with oligofructose leading to the highest acetate production (48.5 [8.8] mmol/l). Peanut fibre produced the most propionate by 24 hours yielding 9.8 (1.7) mmol/l, however this only reached statistical significance compared to the control ($p<0.05$, Figure 5-4). This was also seen when the SCFA production was ranked per individual, with two of the three donors ranking peanut fibre as a top producer (Table 5-3). Butyrate production was the highest with kidney bean and mung bean fibres

resulting in 18.4 (1.2) mmol/l and 23.5 (19.6) mmol/l of butyrate respectively, this was significantly higher than the control (2.4 [0.43] mmol/l, $p<0.05$, Figure 5-4). As suggested by the pH data, total production was similar for oligofructose, mung bean, and kidney bean soluble fibre fractions, all of which produced approximately 61.0 (26.1) mmol/l and was significantly higher than the control ($p<0.001$). Total production indicated trends when compared with the soluble peanut fibre fraction vs oligofructose, $p= 0.032$, vs mung bean $p= 0.062$ and vs kidney $p= 0.081$ bean (Figure 5-4).

When the molar proportions were measured, acetate was predominant on all occasions. The proportion of SCFA forming acetate was significantly less than oligofructose after 24 hours for peanut (85.7 [33.6] % vs 56.5 [11.7] %, $p<0.05$). At 6 hours, propionate was produced in the highest proportion for the control (20.2 [2.7] %) and the lowest for mung bean (4.0 [12.4] %, $p<0.05$). Peanut fibre fermentation had the largest change in the proportion of propionate, significantly increasing by 8.3% between 6 and 24 to 19.6 (2.2) %, $p<0.01$. Peanut also had the highest proportion of propionate after the control, which was significantly higher than mung bean, which had the lowest proportion of propionate (19.6 [2.2] % vs 5.4 [15.4] %; $p<0.05$). The proportion of butyrate was highest at 6 hours for peanut (21.7 [15.5] %). At 24 hours, production of butyrate was significantly higher than at 6 hours for peanut, and kidney bean ($p<0.01$). At 24 hours, more than 25% of total SCFA was being produced as butyrate by mung bean, peanut and kidney bean fibre (Figure 5-5).

5.5.3 Experiment 3: Starch (and modified starch) fermentation

Of the starches tested, potato starch resulted in the lowest pH at 24 hours at 3.94 (0.26). At 6 hours, all of the starches had a significantly lower pH than the control, but were also significantly higher than oligofructose. After 24 hours of fermentation, the pH of the control had significantly less of a reduction in pH than that of all other substrates tested, $p<0.01$. Fibersol also had less of a reduction in pH than oligofructose and potato starch $p<0.05$, Table 5-4).

Table 5-4: Change in pH at 0, 6, and 24 hours of fibersol, potato starch, and hi-maize starch.

Time (hours)	Control	Oligofructose	Fibersol	Potato	Hi-Maize
0	6.74 (0.23)	6.63 (0.3)	6.66 (0.32)	6.75 (0.25)	6.65 (0.32)
6	6.8 (0.32)[a]	4.17(0.45)[b]	4.62 (0.44)[c]	4.76 (1.01)[c]	5.49 (1.95)[c]
24	6.84 (0.28)[a]	3.7 (0.72)[b]	4.34 (0.15)[c]	3.94 (0.26)[b]	4.02 (0.51)[b,c]

Median (IQR), n=3, Different letters within rows indicate significant differences p<0.05.

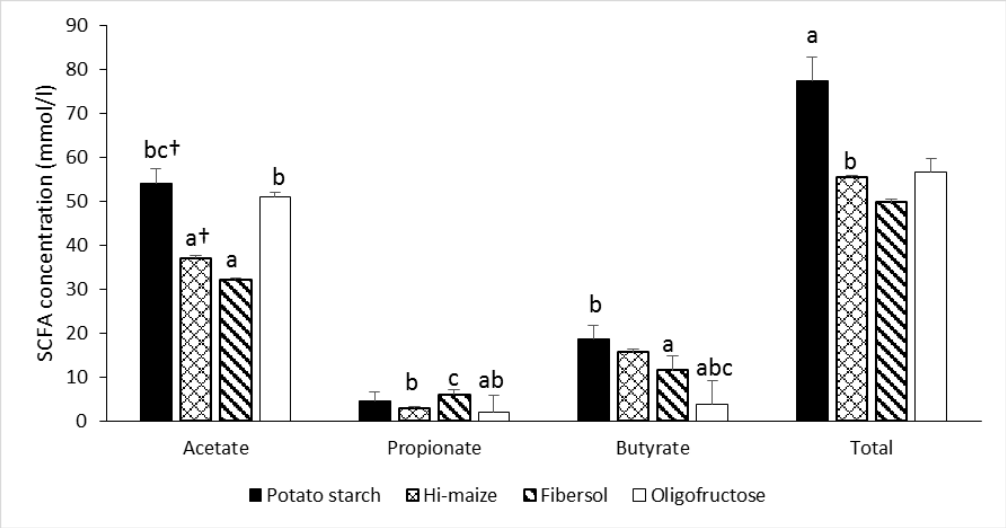

Figure 5-6: 24 hour SCFA production by potato starch, hi-maize and fibersol.
Presented are Mean (+ SEM), n=3. Significant differences denoted as a: vs potato starch, b: vs fibersol, c: vs high maize p<0.05. † indicated p= 0.05 vs Oligofructose

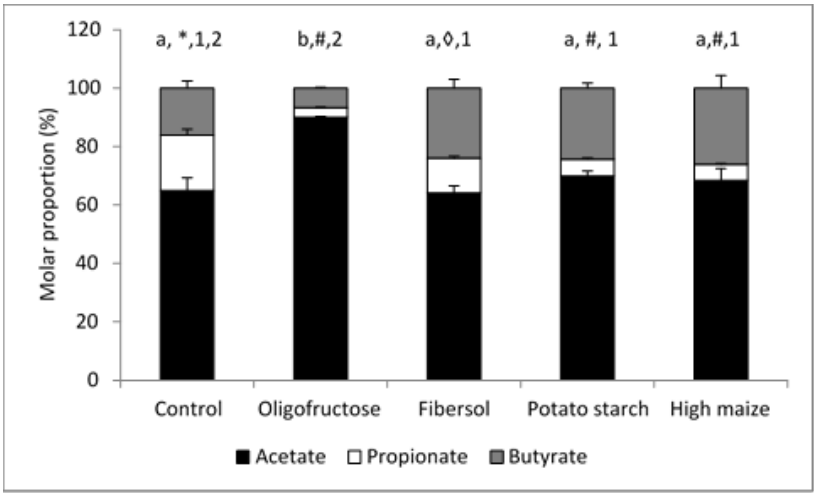

Figure 5-7: Molar ratios of 24 hour SCFA production of a selection of starches.

Mean +SEM, n=3. Different letters denote significant differences in acetate, different symbols denote significant differences in propionate, and different numbers denote differences in butyrate production

Potato starch produced significantly higher total SCFA compared to all other substrates tested (77.5 [3.8] mmol/l, $p<0.05$). Potato starch also yielded significantly more acetate than the other substrates (oligofructose, $p=0.05$), however this did not translate into a higher proportion of acetate production (69.9 [1.6] %). Oligofructose significantly increased the proportion of acetate compared to all other substrates (89.6 (0.27) %, $p<0.001$). Fibersol yielded the highest concentration of propionate (6.06 [0.65] mmol/l) which was significantly higher than oligofructose (1.91 [0.15] mmol/l $p<0.001$), and high maize (2.99 [0.33] mmol/l, $p<0.001$). Fibersol also generated a significantly higher proportion of propionate than all substrates except the control (19.0 [1.9] %, $p<0.001$, Figure 5-6, Figure 5-7). All starches increased butyrate production and molar proportions with potato starch, high maize, and, fibersol (Figure 5-6, Figure 5-7). This, along with the starch fraction indicates that the starches are butyrogenic.

5.5.4 Experiment 4 : Modified barley starches

Table 5-5 shows the change in pH of different fractions of the modified barley starches. The control and the whole grain fractions did not decrease in pH, but increased slightly (< 0.1 pH units) by 24 hours. For amylose only and wild type barley grains, at 6, and 24 hours, the pure starch and milled fraction gave rise to a significantly reduced pH compared to the other fractions. For example, at 24 hours, amylose only pure starch had a lower pH than the whole grain which was higher than the pH of the milled fraction (Table 5-5).

Table 5-5: pH after 0, 6, and 24 hours of fermentation of different fractions of amylose only and wild type barley.

		Amylose Only			**Wild type**			
Time (hours)	Control	Whole Grain	Milled	Pure Starch	Whole Grain	Milled	Pure Starch	
0	6.74 (0.23)	6.68 (0.64)	6.54 (0.62)	6.6 (0.35)	6.75 (0.53)	6.61 (0.85)	6.59 (0.45)	
6	6.8 (0.32)	6.7 (0.79)[b]	5.69 (0.91)[a]	4.86 (1.34)[a,b,c]	6.79 (0.54)[b]	4.76 (0.42)[a,c]	4.5 (0.71)[a,b,]	P<0.001
24	6.84 (0.28)	6.74 (0.66)[b]	4.67 (0.39)[a,c]	3.89 (0.27)[a,b,c]	6.81 (0.72)[b]	4.2 (0.4)[a,c]	3.57 (0.26)[a,b,c]	P<0.01

Median (IQR), n=3 (control and oligofructose n=6). a, different from whole grain of individual starch type, b different from milled fraction of individual starch, c is different from control

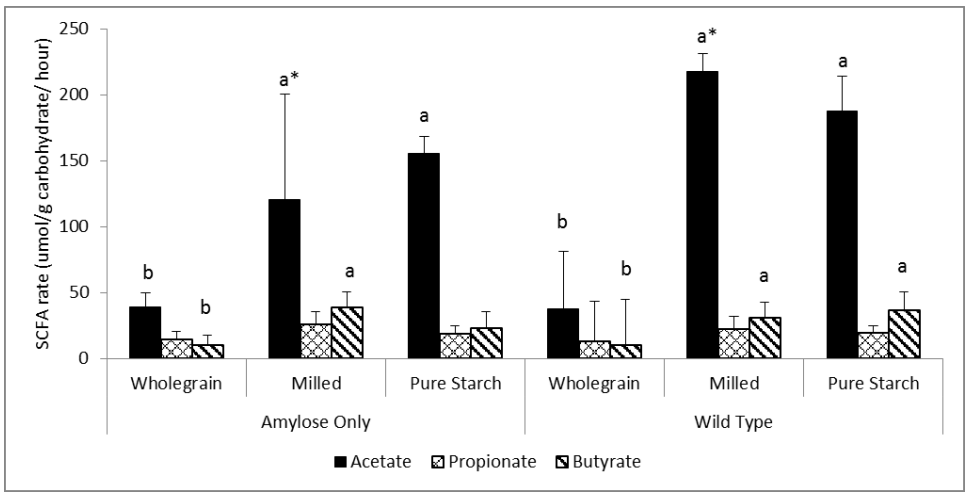

Figure 5-8: SCFA production after 6 hours of fermentation (µmol/ carbohydrate/ hour) Median (+ IQR), n=3 (control and oligofructose n=6). Data is presented as a rate to enable comparison of fermentations conducted using different fermenter sizes (see Chapter 2). Significant differences are denoted by: a = different from whole grain of each starch/grain

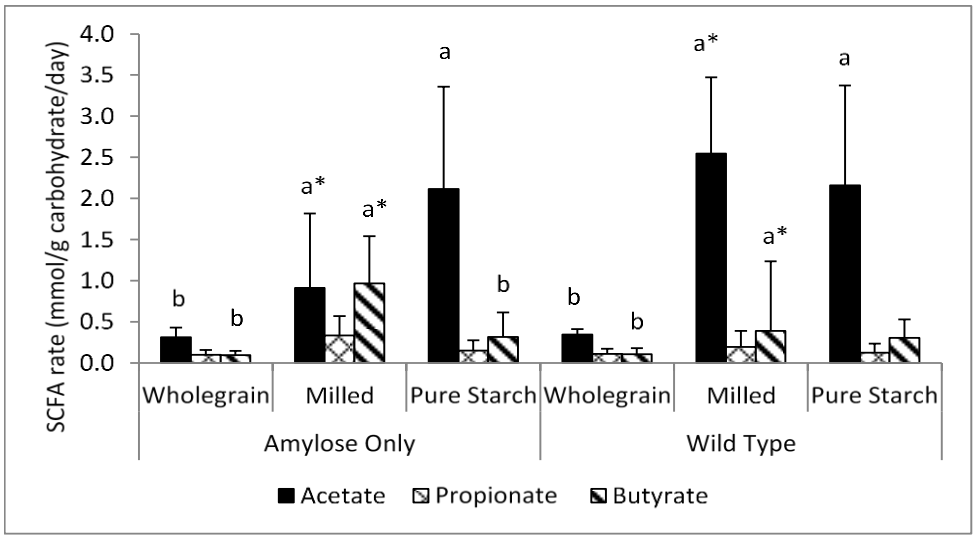

Figure 5-9: SCFA production rate per day at 24 hours of fermentation (mmol/g carbohydrate/day).

Median (+ IQR), n=3 (control and oligofructose n=6). Data is presented as a rate to enable comparison of fermentations conducted using different fermenter sizes (see Chapter 2). Significant differences are denoted by: a = different from whole grain of each barley, b = different from milled fraction of each starch/grain, * = difference between starch type for each SCFA.

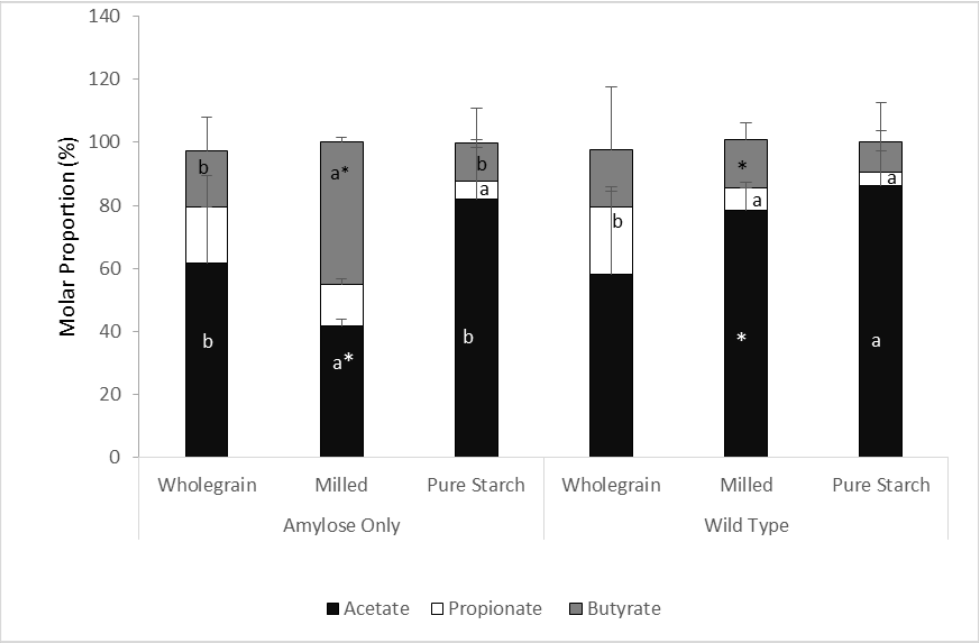

Figure 5-10: 24 hour SCFA molar proportion (%) of fractions of amylose only and wild type starch

Median (+ IQR), n=3 (control and oligofructose n=6). Significant differences are denoted by: a = different from whole grain of each starch/grain, b = different from milled fraction of each starch/grain, * = difference between the starch type, for each SCFA.

Information on the SCFA production by the starches was presented in the rate form, allowing comparison of all the starch fractions. For both barleys, the whole grain fraction did not result in higher SCFA than the control. This suggested that whole grain was poorly fermented (24 hour total: control vs AO- whole grain 0.5 [0.1] vs 0.5 [0.1] mmol/g CHO/ day, p= 1.0, control vs WT- whole grain 0.5 [0.1] vs 0.5 [0.1] mmol/g CHO/ day, p= 1.0, Figure 5-9).

After 6 hours of fermentation, the pure starch fraction led to the highest SCFA production of all the fractions tested. Pure starch for the wild type barley generated the highest rate of acetate production at 6 hours (218.8 [96.6] µmol/g CHO/hour), this however was less than that of the oligofructose (279.7 [113.9] µmol/g CHO /hour). At 24 hours the milled fraction of the wild type starch generated the highest rate of acetate production with 106.0 (38.6) µmol/g CHO/hour and 2.5 (0.9) mmol/g CHO/day. As well as for the pure starch, the milled fraction for both amylose only and wild type barley also increased rates of acetate production compared to the whole grains and the control at 6 and 24 hours, demonstrating that these fractions are fermentable (Figure 5-8, Figure 5-9). The

proportion of acetate was also highest with the starches, all of which had a SCFA proportion above 80% (Figure 5-10).

The milled and pure starch fraction for both starch types also led to increased propionate and butyrate production. The increase in propionate was not statistically significant at 6 hours, however, at 24 hours the milled fraction of amylose only starch increased propionate compared to the control (13.9 [9.8] μmol/g CHO/hour vs 3.7 [3.4] mmol/g CHO/hour). The rate of butyrate production of the milled fraction of amylose only barley did not differ between 6 and 24 hours (38.6 [34.5] vs 40.3 [23.9] μmol/g CHO/hour). The milled fraction of amylose only also generated increased butyrate compared to the other substrates at 24 hours. For example, the milled fraction of amylose only starch led to significantly higher rates and proportions of butyrate production (40.3 [23.9] μmol/g CHO/hour and 45.2 [19.8] %) compared to the wild type starch, which was at the cost of acetate (Figure 5-8 - Figure 5-10) suggesting that this is butyrogenic. Generally, the rate of production, per hour was also reduced at 24 hours, suggesting that for most of the substrates tested the majority of the SCFA production occurred at between 0 and 6 hours.

5.5.5 Comparison of all substrates screened

Table 5-6 shows the top 10 propionate producers from all substrates screened within this chapter (n=3 for all except oligofructose n=18). When ranked, laminarin led to the highest rate of propionate production. The legumes and the β–glucans generated increased propionate production after 24 hours with all (except mung bean, 12th) ranking in the top 10 of propionate producers.

Table 5-6: Ranking of the top 10 producers of propionate µmol/g CHO /hour at 24 hours

Rank		Acetate	Propionate	Butyrate	Total
1	Laminarin	198.7 (42.9)	53.1 (32.1)	50.2 (24.2)	302.0 (64.1)
2	Guar	64.9(10.0)	26.7 (9.3)	28.7 (8.5)	120.3 (20.9)
3	Glucagel	113.7 (14.8)	24.8 (8.1)	39.5 (9.6)	178.0 (15.0)
4	Oatwell 28	117.6 (13.5)	20.9 (40.6)	42.8 (3.1)	181.3 (12.4)
5	Pectin	136.7 (9.9)	20.5 (4.2)	32.4 (6.5)	185.1 (13.8)
6	Cellobiose	63.8 (13.1)	20.5 (10.5)	33.7 (7.9)	118.0 (27.9)
7	Peanut	54.8 (4.1)	19.7 (0.73)	25.8 (1.2)	98.2 (3.9)
8	Oatwell 22	128.4 (11.0)	18.9 (5.0)	27.2 (10.6)	174.4 (22.4)
9	Oligofructose	103.42 (4.5)	18.2 (7.7)	30.2 (6.0)	139.1 (17.55)
10	Kidney bean	78.3 (4.6)	17.4 (2.8)	37.9 (0.6)	131.0 (8.1)

Data presented are the mean (SEM) of the SCFA produced
For comparison, samples from fermentations were ranked and compared. If two sets of experiments used the same substrates, the lower ranking value was removed, this prevented the comparison of production by the same donor.

5.5.6 Comparisons of oligofructose

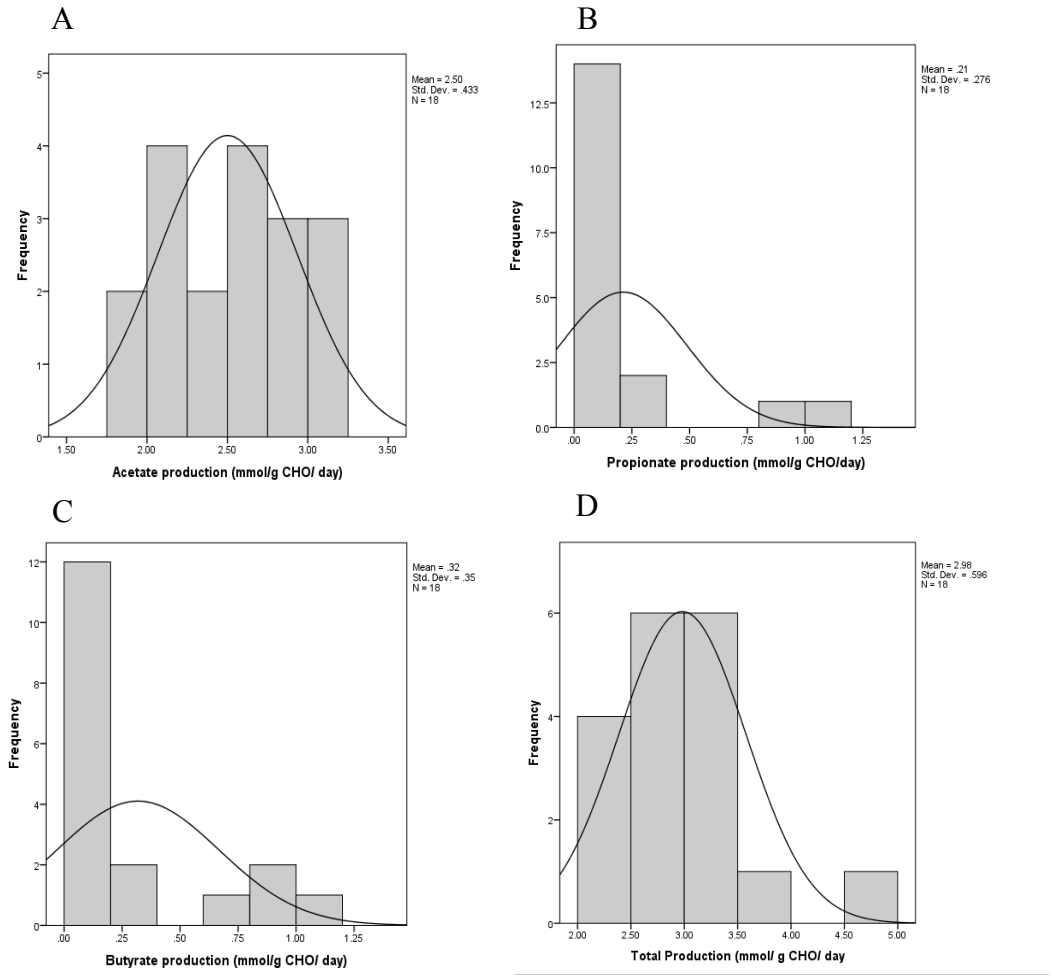

Figure 5-11: Histogram of SCFA after the fermentation of oligofructose.

A: Acetate production, B: Propionate production, C: Butyrate production, D: Total production. Unit: mmol/g CHO/ day, n=18

Oligofructose was often used as a control during fermentations, and as a result has been fermented many times, providing a high number of technical replicates (n=18). Fermentation of oligofructose was plotted for each individual (Figure 5-11). It became apparent that although the majority of individuals had similar propionate production (0.0-0.25 mmol/g CHO /day), two datasets had high rates of propionate production (Figure 5-11- B). These were from the same donor, but in other cases where they provided a stool sample, propionate production was not considered an outlier.

5.5.7 Experiment 5: Starch fermentation at two initial pHs (6.8 & 5.4)

A selection of substrates were fermented at two initial pHs (6.8 and 5.4), with the pH being controlled by buffer in the medium only (Section 2.2.1.2). Sometimes the pH of the negative control rose instead of fell, this was possibly due to the buffering capacity of the media used for the fermentations, particularly for the initial pH of 5.4. It could have also been associated with production of ammonia or hydrogen sulphide due to the fermentation of tryptone in the media with a lack of fermentable carbohydrate, which could have buffered against the decreasing pH (Walker et al., 2005, Magee et al., 2000). Increases in the pH of the 6.8 media could also be as a result of variability where there was an IQR of 0.5 after 24 hours of fermentation.

Starch fermentations were conducted with an initial pH of 6.8 and 5.4. Within the non-pH controlled fermentation systems, after 6 hours of fermentations the pH of all vessels did not differ regardless of initial pH, i.e. the initial pH of a substrate had no effect on the pH at 6 or 24 hours (Table 5-7). This was not the case for hi-maize where the pH was significantly higher ($p<0.001$) at 24 hours with the initial media with a pH of 5.4 (pH 4.66 [1.27]) compared to the 6.8 media (pH 4.02 [0.37]). Interestingly, once again the pH of the control resulted in an increased pH (Table 5-7). It was also observed that in all cases the pH was significantly lower than the control at both 6 and 24 hours.

Table 5-7: pH change after fermentation of a selection of starches and starch derivatives with different starting pHs.

Time	0 hours		6 hours		24 hours	
Initial pH	**6.8**	**5.4**	**6.8**	**5.4**	**6.8**	**5.4**
Control	6.70 (0.2)	5.55 (0.11)*	6.71 (0.18)^{a}	5.59 (0.14)^{a}	6.76 (0.09)^{a}	6.17 (0.07)^{a}
Oligofructose	6.65 (0.25)	5.49 (0.11)*	3.79 (0.3)^{b}	4.03 (0.51)^{b}	3.42 (0.34)^{d}	3.74 (0.2)^{c}
Potato	6.75 (0.18)	5.56 (0.12)*	4.76 (0.73)^{c}	4.57 (0.26)^{bc}	3.94 (0.17)^{de}	3.75 (0.04)^{c}
Hi- maize	6.65 (0.24)	5.53 (0.16)*	5.49 (1.51)^{c}	4.82 (0.67)^{bc}	4.02 (0.37)^{ce}	4.66 (1.27)^{b*}
Fibersol	6.66 (0.24)	5.52 (0.1)*	4.62 (0.31)^{d}	4.55 (0.15)^{bc}	4.34 (0.1)^{b}	4.53 (0.05)^{b}
Amylose only	6.6 (0.25)	5.55 (0.1)*	4.86 (0.94)^{c}	4.83 (0.68)^{c}	3.89 (0.19)^{e}	4.06 (0.15)^{c}
Wild Type	6.59 (0.34)	5.51 (0.08)*	4.5 (0.51)^{bc}	4.58 (0.56)^{bc}	3.57 (0.21)^{d}	3.79 (0.05)^{c}

Non pH-controlled (apart from buffer) batch fermentations were conducted with initial pHs of 6.8 and 5.4. Amylose only and wild type are the pure starch fractions, median (IQR), n=3. Different letters within columns indicate significant differences between substrates, * indicates significant differences between substrates at different initial pHs (e.g 5.4 vs 6.8).

Table 5-8, and Figure 5-12 show the proportions of SCFA production after 24 hours of fermentation. There were significant differences in the proportion of each SCFA as a result of the starting pH. For example, with an initial fermentation pH of 5.4, acetate was the predominant SCFA, and was increased compared to substrates with an initial pH of 6.8. Acetate proportion was in the range of 71.6 (24.7) % -98.2 (2.6) % with and initial pH of 5.4, compared to a range of 64.3 (12.6) - 89.9 (1.0) % or 6.8 (Table 5-8). This difference was significant for the control (83.09 [6.7] % vs 66.7 [21.2] %, $p<0.001$), potato starch (93.4 [5.4] % vs 69.6 [7.3] %, $p<0.001$), hi-maize starch (83.8 [18.9] % vs 64.2 [19.9] %, $p<0.026$), and fibersol (71.6 [24.7] % vs 64.3 [12.6] %, $p=0.006$). It was, however, observed that oligofructose generated the highest proportion of acetate, and lowest of propionate and butyrate regardless of initial pH.

An initial pH of 5.4 resulted in a significantly lower proportion of propionate for all substrates except high maize, and the control. After the control, fibersol resulted in the highest proportion of propionate with and initial pH of both 6.8 and 5.4 (12.1 [3.5] % and 3.6 [1.2] %). This increase was only significant with the fermentations of an initial pH of 6.8 ($p=0.013$) against the control ($p<0.001$ against all other substrates, Table 5-8).

Butyrate proportion was also altered by the different starting pHs, with the fermentations with the initial pH of 5.4 having significantly reduced butyrate compared to those with an initial pH of 6.8. Figure 5-12 demonstrates the differences in propionate proportion at all measured occasions. Here it was found that after 6 hours of fermentation for all starches tested there was a reduced proportion of propionate with the initial pH of 5.4 for fibersol (5.4 vs 6.8: 3.0 [3.2] % vs 10.2 [0.7], $p = 0.001$) and wild type (5.4 vs 6.8: 2.5 [3.5] % vs 7.3 [3.7] %, $p=0.004$). By 24 hours all starches tested (except high maize) resulted in the initial pH of 6.8 yielding increased proportions of propionate compared to the initial pH of 5.4 ($p<0.01$).

Table 5-8: Molar proportion of acetate, propionate and butyrate after 24 hours of fermentation

	Acetate			Propionate			Butyrate		
Initial pH	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**
Control	66.74 (21.2)^{b}	83.07 (6.7)^{a}	p<0.001	17.89 (9.4) ^{c}	7.93 (7.8)^{c}	p=0.07	15.37 (11.0)^{bc}	8.91 (0.5)^{bc}	NS
Oligofructose	89.90 (1.0)^{a}	98.15 (2.6)^{b}	NS	3.43 (0.4)^{bd}	0.88 (1.3)^{d}	p<0.001	6.81 (1.2)^{a}	0.89 (1.9)^{a}	p<0.001
Potato	69.6 (7.3)^{bc}	93.36 (5.4)^{b}	p<0.001	6.21 (1.9)^{b}	1.66 (0.6)^{ab}	p<0.001	24.09 (7.5)^{b}	4.97 (4.8)^{abc}	p<0.001
Hi- Maize	64.17 (19.9)^{b}	83.77 (18.9)^{a}	p=0.026	5.79 (1.8)^{b}	3.24 (1.0)^{a}	NS	30.86 (20.1)^{b}	12.71 (19.7)^{c}	NS
Fibersol	64.25 (12.6)^{b}	71.63 (24.7)^{a}	p=0.006	12.08 (3.5)^{a}	3.61 (1.2)^{abc}	p<0.001	21.98 (14.0)^{b}	24.39 (24.8)^{b}	NS
Amylose only	81.94 (7.0)^{c}	94.60 (1.8)^{b}	NS	5.75 (1.74) ^{b}	1.8 (3.3)^{ab}	p=0.006	12.17 (5.3)^{c}	2.79 (2.3)^{ab}	p=0.005
Wild Type	86.28 (6.6)^{a}	97.74 (2.3)^{b}	NS	4.40 (2.2)^{bd}	1.55 (1.6)^{b}	p<0.001	9.34 (5.8)^{ac}	1.07 (1.5)^{a}	p<0.001

Date presented are the median (IQR) of the molar proportions of SCFA after fermentations of a selection of starches. Different letters within columns indicate significant differences between substrates.

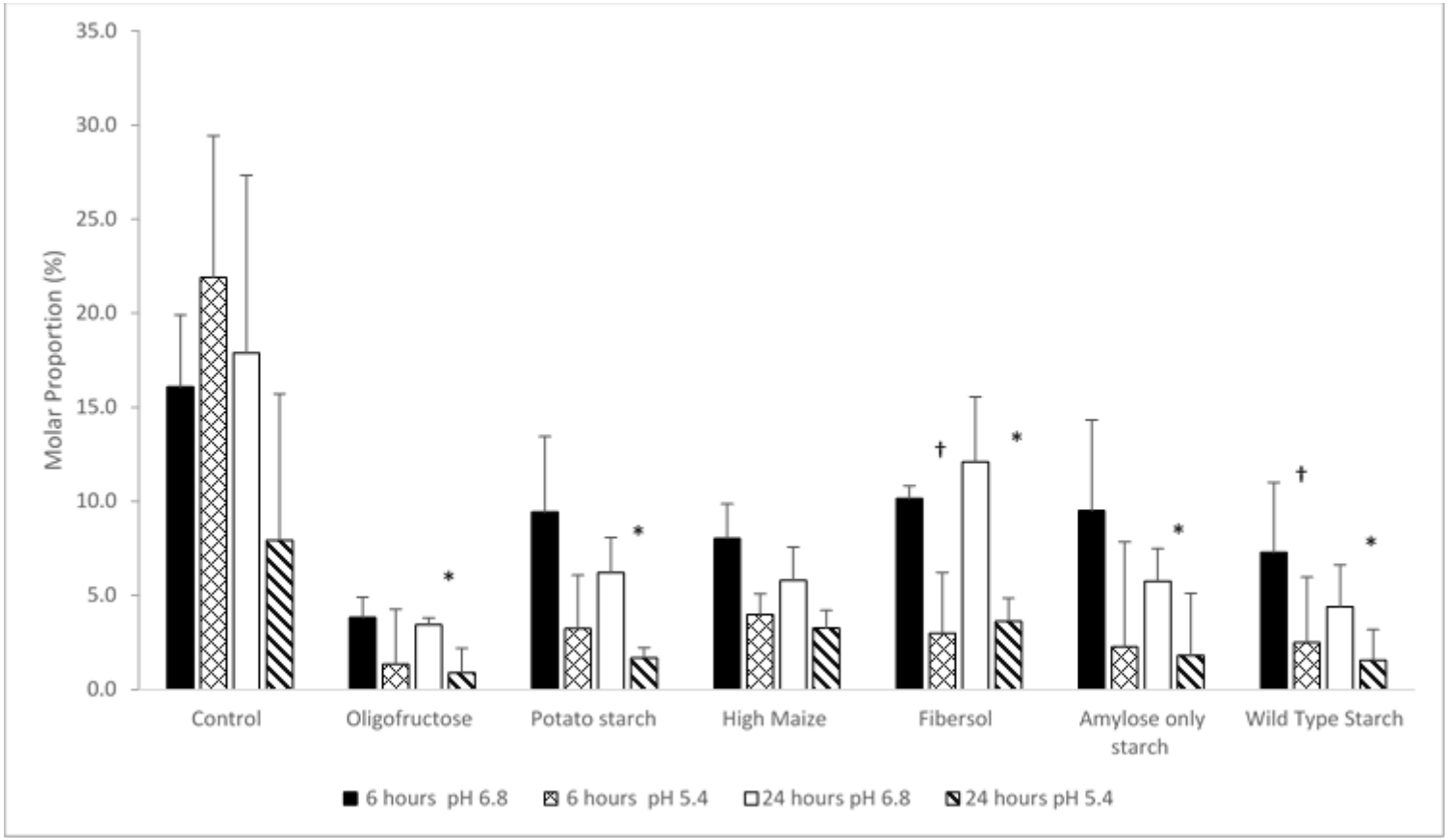

Figure 5-12: Molar proportion (%) of propionate production at both 6, and 24 hours with initial pHs of 6.8 and 5.4. Data presented are median + IQR. N=3, and the pH was not maintained during the fermentation. Symbols indicates different proportions of propionate between fermentations starting at pH 5.4 and 6.8 at 6 hours (†) and 24 hours (*).Wild type and amylose only are the pure starch fractions.

Table 5-9: Rate of acetate, propionate, butyrate, and total production after fermentation with an initial pH of 5.4 or 6.8 mmol/g CHO/ day

	Acetate			Propionate			Butyrate			Total		
Initial pH	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**
Control	0.33	1.78		0.10	0.19		0.09	0.19		0.52	2.16	
	(0.01)ac	(0.05)^{abc}	P=0.002	(0.02)b	(0.03)^{a}	NS	(0.02)b	(0.01)^{ab}	NS	(0.05)a	(0.04)^{ab}	p=0.003
Oligofructose	2.51	2.84		0.09	0.02		0.18	0.03		2.78	2.89	
	(0.16)bc	(0.09)^{b}	NS	(0.03)b	(0.0)^{b}	NS	(0.06)b	(0.02)^{b}	NS	(0.19)b	(0.09)^{a}	NS
Potato	2.66	2.40		0.22	0.04		0.92	0.10		3.80	2.54	
	(0.1)b	(0.16)^{bc}	NS	(0.02)c	(0.01)^{b}	p<0.001	(0.16)ac	(0.12)^{ab}	p<0.001	(0.26)c	(0.27)^{ab}	NS
Hi-Maize	1.82	1.60		0.15	0.06		0.76	0.34		2.73	2.0	
	(0.02)bc	(0.05)^{c}	NS	(0.02)bc	(0.02)^{b}	NS	(0.02)cd	(0.02)^{a}	p=0.002	(0.04)bc	(0.07)^{ab}	NS
Fibersol	1.58	1.13		0.3	0.05		0.56	0.29		2.44	1.47	
	(0.15)c	(0.09)^{ac}	NS	(0.03)a	(0.01)^{b}	p<0.001	(0.04)bcd	(0.08)^{ab}	NS	(0.15)b	(0.15)^{b}	NS
Wild type	2.49	2.42		0.15	0.03		0.31	0.03		2.94	2.48	
	(0.26)bc	(0.5)^{bc}	NS	(0.01)bc	(0.0)^{b}	p=0.006	(0.02)bd	(0.01)^{b}	NS	(0.29)bc	(0.5)^{ab}	NS
Amylose only	2.10	1.85		0.15	0.05		0.32	0.05		2.57	1.95	
	(2.57)bc	(0.24)^{abc}	NS	(0.03)bc	(0.01)^{b}	p=0.074	(0.05)bd	(0.01)^{b}	NS	(0.38)b	(0.24)^{ab}	NS

Mean (SEM) of the molar proportions of SCFA after fermentations of a selection of starches. Different letters within columns indicate significant differences between substrates. N=3. Wild type and amylose only are the pure starch fractions. Data presented as a rate to enable comparisons with the other rate changing data

Differences in acetate production at the two initial pHs were less apparent when presented as a rate, as opposed to a ratio. The negative control led to differences in acetate production, with the lower initial pH resulting in significantly more acetate (1.8 [0.1] vs 0.3 [0.0] mmol/g CHO/ day, p=0.002). Interestingly the lower initial pH favoured SCFA production for the control, but not for the substrates fermented, although this was not significant (Table 5-9).

Propionate production from fermentation of the starches tested was reduced with an initial pH of 5.4 compared to 6.8. This occurred to the greatest extent with fibersol and potato starch, in which the lower initial pH led to ~82% less production ($p<0.001$) for both substrates. It was also identified that like the ratio, fibersol fermentation generated the most propionate with an initial fermentation pH of 6.8 (0.3 [0.0] mmol/g CHO/day). This was not the case for the lower initial pH which resulted in high maize being the substrate generating the highest rate of propionate production (0.1 [0.0] mmol/g CHO/day). High maize also led to high rates of butyrate production with both initial pHs (pH 6.8: 0.8 [0.0] and pH 5.4: 0.3 [0.0] mmol/g CHO/ day). Potato starch generated the most butyrate with an initial pH of 6.8 (0.9 [0.2] mmol/g CHO/day). Both high maize and potato starch led to increased butyrate production when initiated at pH6.8 compared to pH5.4 (55%, $p =0.002$ and 89%, $p<0.001$). Oligofructose and wild type starch resulted in the lowest rate of production of butyrate compared to all other substrates tested (pH6.8; 0.2 [0.1] and 0.3 [0.0] mmol/g CHO/day, pH5.4; 0.03 [0.0] and 0.03 [0.0] mmol/g CHO/day, Table 5-9).

Initial pH did not affect the total rate of production with the fermentation of substrates, however, the lower initial pH increased total SCFA compared to the initial pH for the control (6.8 vs 5.4: 0.5 [0.1] vs 2.2 [0.0] mmol/g CHO/day, $p<0.001$). Fibersol was the substrate generating the lowest rate of total SCFA (pH6.8: 2.1 [0.1] and pH5.4: 1.5 [0.2] mmol/g CHO/day). Total production with an initial pH of 5.4 was similar for oligofructose, potato starch and wild type starch (2.9 [0.1], 2.5 [0.3], 2.5 [0.5] mmol/ g CHO/ day, Table 5-9).

5.5.8 Experiment 6: Guar, Trehalose dihydrate, Cellobiose and Glucagel fermentation at two initial pHs (6.8 & 5.4)

For all the substrates, except the control, there was no significant difference between the pH of both systems by 6 hours, and by 24 hours was also the case for the control. For all other substrates, except guar gum, where the pH was 5.0, the pH reduced to approximately pH 4.0 (Table 5-10).

When the molar proportions were considered at each pH the increase in acetate was more apparent with an initial pH of 5.4 compared to the 6.8 starting pH, although this was not significant. Molar percentages did not differ between the two initial pHs with the majority of the SCFA forming acetate. Cellobiose with an initial pH of 5.4 led to 92.1 (2.8) % of SCFA production yielding acetate whereas this was only 55.2 (7.2) % with an initial pH of 6.8 (Table 5-11). Acetate molar percentage was however the highest for oligofructose with both starting pHs, with 80.0 (10.7) % and 95.8 (2.2) % of SCFA producing acetate.

The proportion of propionate at pH 5.4 was lower than that at pH 6.8, although this was not significant. Propionate proportion at pH 5.4 varied between oligofructose (1.6 [0.6] %) and guar gum (7.7 [2.6] %). At an initial pH of 5.4; the control, trehalose dihydrate, and guar gum generated the highest proportions of propionate (7.1 [1.1] %, 7.6 [2.9] %, and 7.7 [2.6] %). Interestingly, the proportion of propionate yielded as a result of trehalose dihydrate did not significantly differ between the different pHs. Trehalose dihydrate did yield the lowest proportion of propionate with an initial pH of 6.8 (6.8 vs 5.4: 7.3 [3.5] % vs 7.6 [2.9] %). Like with the initial pH of 5.4, the initial pH of 6.8 resulted in guar gum and the control having the highest percentage yields of propionate (21.45 [3.0] % and 20.8 [4.2] %).

Generally, the proportion of butyrate was higher with an initial pH of 6.8, with trehalose dihydrate and glucagel resulting in the highest proportions (32.9 [16.2] % and 37.8 [3.2] %). Trehalose dihydrate also led to the highest percentage of butyrate production compared to the other substrates (28.4 [8.1] %), although non-significant. It was also observed that oligofructose resulted in the lowest proportion of butyrate at both initial pHs (9.2 [4.6] % and 3.2 [1.6] %).

Table 5-10: pH change in cultures with initial pHs of 6.8 and 5.4 at 0, 6 and 24 hours of fermentation.

	0 hours			6 hours			24 hours		
pH	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**
Control	6.65 (0.18)	5.38 (0.11)	p<0.001	6.83 (0.6) a	5.44 (0.07)	p = 0.011	6.93 (0.49)[a]	6.01 (0.19)	NS
Trehalose Dihydrate	6.64 (0.08)	5.38 (0.12)	p<0.001	5.34 (1.21) [b]	5.01 (0.36)	NS	4.46 (0.12)[b]	4.6 (0.62)	NS
Cellobiose	6.6 (0.06)	5.42 (0.11)	p<0.001	4.58 (0.56) [bc]	4.9 (0.52)	NS	4.11 (0.09)[b]	4.2 (0.29)	NS
Guar gum	6.54 (0.18)	5.54	p<0.001	4.86 (1.56) [bc]	5.04	NS	4.43 (0.51)[b]	5.04	NS
Glucagel	6.5 (0.2)	5.45 (0.11)	p<0.001	4.86 (0.23) [bc]	4.65 (0.17)	NS	4.57 (0.25)[b]	4.58 (0.12)	NS
Oligofructose	6.61 (0.1)	5.37 (0.11)	p<0.001	4.24 (0.06) [c]	4.59 (0.21)	NS	4.01 (0.36[b]	4.09 (0.36)	NS

Median (IQR). n=3 for all except guar gum where n=2 with an initial pH of 5.4. . Letters indicated differences between substrates with the same pH media. Data presented as a rate to enable comparisons with the other rate changing data. NS= non-significant

Table 5-11 Molar SCFA production after 24 hours of fementation

	Acetate %			Propionate %			Butyrate %		
pH	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**
Control	57.62 (1.8)	85.52 (0.4)	NS	21.46 (3.0)	7.12 (1.1)	NS	20.92 (2.6)	7.36 (1.4)	NS
Trehalose Dihydrate	59.81 (19.4)	63.94 (8.8)	NS	7.26 (3.5)	7.58 (2.9)	NS	32.94 (16.2)	28.48 (8.1)	NS
Cellobiose	55.16 (7.2)	92.07 (2.8)	NS	15.52 (4.4)	2.59 (0.6)	NS	29.32 (5.2)	5.34 (2.3)	NS
Guar gum	55.05 (5.3)	76.54 (3.8)	NS	20.84 (4.2)	7.68 (2.6)	NS	24.11 (5.8)	15.78 (6.4)	NS
Glucagel	44.88 (3.0)	81.14 (12.8)	NS	17.33 (5.3)	4.05 (1.5)	NS	37.79 (3.2)	14.8 (11.3)	NS
Oligofructose	80.09 (10.7)	95.76 (2.2)	NS	10.72 (6.9)	1.64 (0.6)	NS	9.19 (4.6)	3.20 (1.6)	NS

Presented are mean (SEM). N=3 except for guar, n=2. NS = non significant

Table 5-12 SCFA production as a result of fermentation of a variety of 'propiogenic substrates'-24 hours mmol/g CHO/day

	Acetate			Propionate			Butyrate			Total		
pH	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**	**6.8**	**5.4**	**6.8 vs 5.4**
Control	0.32 (0.1)a	1.86 (0.3)	p<0.001	0.14 (0.1)	0.11 (0.0)	NS	0.12 (0.0)	0.11 (0.1)	NS	0.59 (0.1)a	2.17 (0.3)	p<0.01
Trehalose Dihydrate	1.11 (0.6)b	1.08 (0.6)	NS	0.18 (0.18)	0.07 (0.2)	NS	1.07 (0.8)	0.75 (0.6)	NS	2.26 (0.4)b	1.90 (1.4)	NS
Cellobiose	1.68 (0.8)b	2.49 (0.6)	NS	0.26 (0.4)	0.07 (0.0)	NS	0.76 (0.3)	0.14 (0.1)	NS	2.41 (0.4)b	2.54 (1.4)	NS
Guar gum	1.41 (0.4)b	1.99 (0.1)	NS	0.59 (0.4)	0.19 (0.1)	NS	0.56 (0.3)	0.43 (0.2)	NS	3.09 (1.1)b	2.61 (0.2)	NS
Glucagel	1.40 (0.4)b	1.16 (0.7)	NS	0.29 (0.3)	0.07 (0.1)	NS	1.07 (0.2)	0.16 (0.4)	NS	3.00 (0.8)b	2.08 (0.8)	NS
Oligofructose	2.17 (0.6)b	2.62 (0.7)	NS	0.12 (0.4)	0.05 (0.1)	NS	0.16 (0.3)	0.11 (0.1)	NS	3.20 (0.5)b	2.72 (0.9)	NS

Median (IQR). N=3 except for guar, n=2 different letters within column = significant differences. Data presented as a rate to enable comparisons with the other pH change data. NS = non significant

The rate of acetate and total SCFA production was significantly lower for the control with an initial pH of 6.8 (Table 5-12). It was also found that acetate production was significantly lower than all other substrates measured with an initial pH of 6.8. The rate of acetate production did not differ with the different pHs, where oligofructose resulted in the highest rate of production (2.2 [0.6] and 2.6 [0.7] mmol/g CHO /day). No significant differences in propionate or butyrate production were observed, however an initial pH of 5.4 did lead to decreased production compared to the pH 6.8 counterpart, although this was not significant. An example of this was shown by cellobiose where with an initial pH of 6.8, 0.3 (0.4) and 0.8 (0.3) mmol/g CHO/day of propionate and butyrate were produced, which was higher than in 0.1 (0.0) and 0.1 (0.1) mmol/g CHO /day of propionate and butyrate production at an initial pH of 5.4. Total SCFA production also did not vary between the substrates with the initial pH of 5.4. For example, trehalose dihydrate had the lowest rate of production (1.9 [1.4] mmol/g CHO /day) and oligofructose had the highest rate (2.72 [0.9] mmol/g CHO/ day). Oligofructose also resulted in the highest rate of total production with an initial fermentation pH of 6.8 (3.2 [0.5] mmol/g CHO/ day, Table 5-12).

5.6 Discussion

5.6.1 Beta-glucans

A selection of commercial β-glucans containing different proportions of β-glucan, were fermented to determine their propionate producing capacity. Oatwell 22, Oatwell 28, Promoat, and Glucagel consisted of 22%, 28%, 35%, and 75% β-glucan respectively. All β-glucans tested resulted in a decreased pH and SCFA production. There was no significant differences in the production or proportion of SCFA between the β-glucans. Thus indicating that the percentage of β-glucan within a substrate has no effect on the production of SCFA *in vitro.*

This is supported by the investigation by Kim and White., (2011) who conducted *in vitro* batch fermentations of different molecular weight β-glucans and found no significant differences in the production of the individual SCFA (Kim and White, 2011b). No effect of different molecular weights β-glucan on feelings of satiety were observed in a feeding trial of 23 healthy males (Clegg and Thondre, 2014). Although there was no difference in SCFA production between the β-glucans, barley β –glucan has been previously shown to

alter individual SCFA production *in vitro*. Kaur et al., (2011) fermented a selection of substrates and identified that barley β-glucan resulted in significantly higher total acetate and butyrate production compared to the other substrates tested (e.g. inulin and psyllium). The barley β-glucan also produced significantly more propionate than inulin, FOS and resistant starch (Kaur et al., 2011). Although no significant increases in SCFA production by laminarin were observed, there was a trend for increased total and propionate production. This was supported by the *in vitro* study by Deville et al, (2007) where increased proportions of propionate were identified after 24 hours of fermentation (Deville, 2007). The role of laminarin in the production of propionate has also been demonstrated in a feeding trial in which consumption selectively increased in propionate production, at the cost of acetate (Lynch et al., 2010).

Increasing propionate to the detriment of acetate (by altering the acetate to propionate ratio) has been demonstrated to be beneficial for the regulation of cholesterol synthesis (Wolever et al., 1991). This indicates that β-glucan (and laminarin) consumption is beneficial but the type has little effect on SCFA production.

5.6.2 Legumes

Legume consumption is associated with a healthy diet, and has been shown to lead to positive roles in health that could be attributed to the colonic fermentation of these substrates (McCrory et al., 2010).

The legumes investigated here are formed of fat, protein, carbohydrates and dietary fibre. Based on the AOAC method 991.43 of assessment of the dietary fibre of freeze dried legumes, total fibre content of peanut, mung bean and kidney bean were 46.9%, 71.7%, and 60.4% and protein content was 22.1%, 14.6% and 28.3% (Mallillin et al., 2008). When the AOAC method 991.43 was used to assess fibre content of mung bean and kidney bean as consumed (e.g hydrated) total fibre content was 4.43% ,and 11.22% respectively (Aldwairji et al., 2014). Brummer et al., (2015) assessed the composition of the soluble fibre of a selection of legumes (not including those used within this thesis) and identified that the main sugars included galacturonic acid (also in pectin), arabinoxylose, galactobiose, and glucose (Brummer et al., 2015), all of which can lead to the production of SCFA (Mortensen et al., 1988).

This study showed that the soluble fibre of legumes is fermentable leading to a reduced pH and an increase in SCFA production. At 24 hours mung bean led to the lowest pH within the *in vitro* batch system. Peanut fibre increased propionate and butyrate proportion compared to controls (propionate: 19.59 [2.2] % vs 23.18 [11.4] %, butyrate: 25.30 [5.3] % vs 22.54 (4.7) %. Peanut fermentation reduced production of acetate, demonstrating that it may be an effective substrate in reducing the acetate: propionate ratio, which may be important for the regulation of cholesterol (Wolever et al., 1991). Consumption of 2.6g/day of peanut by overweight and obese women for 4 weeks was shown to reduce waist circumference and plasma TAG concentrations (Ha et al., 2015).

The *in vitro* SCFA producing capabilities of legumes have also been measured previously in batch fermenter systems, but with a sample from single subject, and also utilised total fibre (not the soluble fibre used in this thesis) (Mallillin et al., 2008). Mallillin et al., (2008) observed that propionate proportions by peanut, mung bean and kidney bean were 19.8%, 17.9% and 64.3%. This supports our findings with the proportion of propionate by peanut being 19.59 (2.2) %, but propionate proportions with mung bean and kidney bean were lower with 5.4 (15.4) % and 14.0 (7.7) %. This may be due to increased variability within this investigation due to using three different donors. For example, one donor yielded 18.6% propionate, whereas the two other donors yielded approximately 3.8% propionate with mung bean resulting in an overall IQR of 15.4%. Peanut production was less variable (IQR 2.2%), indicating that different individuals had different capabilities of utilising the legumes to produce SCFA.

Proportions of butyrate with mung bean (26.32%) were similar to our observation of 30.3 (29.5) %, whereas Mallillin et al., (2008) observed greater proportions of butyrate with of peanut fibre compared to this thesis (Mallillin et al., (2008) 54.0% vs this chapter 25.3 (5.3) %). Like propionate, butyrate production by peanut was similar for all individuals. The butyrate proportion for mung bean was approximately 1/3rd less than the other two individuals (~35 vs 9%), as Mallillin et al., (2008) only used one stool donor, this would not have been observed.

Although some differences were observed in the proportion of propionate and butyrate after 24 hours of fermentation, there was very little difference in the production of the SCFA between the legume fibres tested, whereas Mallillin et al., (2008) did find

differences. What was also observed was that the legumes yielded total SCFA concentrations that did not differ from oligofructose, the highly fermentable control (Rycroft et al., 2001a, Stewart et al., 2008). Consumption of oligofructose in rats has also been able to increased cellular FFAR2 densities as well as the satiety hormones PYY and GLP-1, this effect was attributed to the SCFA produced (Kaji et al., 2011). As these legumes yielded similar concentrations of SCFA to oligofructose, it could be proposed as one of the possible mechanisms for increased feelings of satiety identified after legume consumption (Spadafranca et al., 2013). Therefore, it would be interesting to assess the effects of commonly consumed legumes on propionate production, and to identify whether the feelings of satiety observed after consuming different legumes are related to the source of legume and type of fibre.

5.6.3 Starch

As expected, all of the starches were fermentable which was initially demonstrated by the observed reduction in pH. Potato starch resulted in the lowest pH of the individual starches tested, and generated the greatest increase in SCFA, particularly acetate. As expected, all of the individual starches tested led to increased butyrate production compared to oligofructose. Fibersol also significantly increased propionate production compared to the other starches tested. This observation is supported by *in vitro* trials by Laurentin and Edwards., (2004), who observed increased propionate after fibersol fermentation compared to native starch (Laurentin and Edwards, 2004). In contrast, consumption of up to 15 g/ day of fibersol by healthy individuals for 7 weeks did not alter faecal propionate production, and increased butyrate production (Fastinger et al., 2008).

After 24 hours of fermentation of both barley grains and purified starch fractions, the purified starch resulted in the lowest pH after 24 hours whereas whole grain had the lowest reduction in pH. This was also associated with increased total SCFA production for the pure and milled fractions compared to the whole grain. The whole grain fractions were not well fermented, yielding little SCFA production, independently of type of barley. This is likely due to the lack of processing on the barley grains, resulting in the starch being physically inaccessible to the colonic bacteria, preventing fermentation. It was also noted that the rate of SCFA production (μmol/g CHO/ hour) was greater at 6 hours compared to 24 hours, suggesting that the barley starches were rapidly fermented. Independently of

wild type barley or amylose only barley, total SCFA was similar for each level of processing. An exception to this was the milled fraction in which the acetate and butyrate production differed between amylose only and wild type. For example, the amylose only had a decreased acetate concentration, and an increased butyrate concentration compared to the wild type (Figure 5-9). Similar findings were also observed as the butyrate proportion was 45.2 (19.8) % (of total) after fermentation of the milled fraction of amylose only barley bran compared to 15.25 (16.2) % (of total) for the milled fraction of wild type barley ($p<0.05$). Unexpectedly butyrate production within the pure starch fraction did not differ between the amylose only and wild type barley.

Although the starches increased butyrate production, butyrate was higher in all commercial starch products compared with the modified barley. Increased butyrate production due to starch fermentation has been demonstrated in a variety of investigations (Sayar et al., 2007, Kaur et al., 2011). Surprisingly the amylose only starch was not more butyrogenic than the wild type starch as previously high-amylose starch has been associated with increased butyrate production when consumed (Bird et al., 2008) and *in vitro* (Christl et al., 1997).

5.6.4 Initial pH on SCFA production.

The initial pH of the fermentation vessel was successfully reduced to different initial pHs, which correspond to the proximal and distal colon. By 6 hours, there was no difference in the pH of the vessels; except for controls. Therefore, it was difficult to identify the role of pH on SCFA production. For example, for the potentially propiogenic substrates there was no significant difference in SCFA production or proportion by the different initial pHs. It was observed that for all substrates tested that acetate and total production was significantly higher than the control. The control also led to significantly lower acetate and total production with an initial pH of 6.8 compared to that of pH 5.4. This also occurred when a selection of different starches were fermented in the same conditions. Belenguer et al., (2007) assessed the impact of different pH's (5.2, 5.9, and 6.4) on the fermentation of lactate. Here it was also observed that after fermentation of a mix of carbohydrates with and without lactate, the lower pH favoured the production of acetate over propionate and butyrate. This was attributed to a reduction in *Eubacterium hallii* (a lactate utiliser) and the accumulation of lactate at this low pH altering the SCFA produced. Acetate can be formed independently of lactate in the Wood-ljundahl pathway, whereas propionate

requires lactate for production, and butyrate is formed by the interconversion of acetate, which likely explains the increased acetate production seen with the lower pH (Belenguer et al., 2007).

This was also identified when the pH was switched from 5.5 to 6.5 where the lower pH favoured acetate and butyrate production, and the higher pH favoured propionate production. These changes in SCFA production were also attributed to changes in the bacterial composition, with the lower pH increasing numbers of *F.prausnitzii* and *Roseburia* spp, both of which are considered butyrogenic bacteria (Walker et al., 2005). These increases in butyrate production were not observed within this thesis; this is likely due to the pH not being continuously controlled, and the pH of the two systems not differing after 6 hours.

Unlike for the 'propiogenic substrates', fermentations with different initial pHs led to differences in SCFA production and proportion after the fermentation of a variety of starches, even though the pH of the systems converged by 6 hours. An initial pH of 6.8 increased propionate production compared to the initial pH of 5.5 for potato starch, fibersol and wild type barley. Propionate production was increased proportionally for all substrates tested except hi-maize, which was not significantly higher at pH 6.8 versus the initial pH of 5.4. It was also observed that the starches led to increased butyrate production with an initial pH of 6.8 compared to an initial pH of 5.4 which was contradictory to that of (Walker et al., 2005). However, to further elucidate the impact of pH on SCFA production pH controlled systems would be required.

5.6.5 Conclusion

The SCFA production by different individuals was not always the same, with differences in production being observed. This was demonstrated in Figure 5-11, where production of propionate by the oligofructose was higher on some occasions. This was from a single donor, whom also provided stool samples not differing from the other donors. This variability has been observed previously, where two fold differences in SCFA production were observed between individuals (Carlson et al., 2016), and variation in SCFA production was also observed when the same donor provided two samples 30 days apart

(Mortensen et al., 1991). This further exemplifies the difficulties faced when comparing the SCFA production of different substrates by different donors.

Overall laminarin was identified as being the most propiogenic substrate in terms of rate of production yielding 53.1 (32.1) μmol/g CHO/ hour. The substrate which ranked second (guar) yielded approximately 50% less propionate than laminarin. After this, the ranking of the substrates did not differ, i.e. second vs third had approximately a 7% difference in propionate production and guar (2^{nd}) vs kidney bean (10^{th}) had a 35% difference in propionate production. This indicates that there were not large differences between top and bottom producers. This is also supported by a consistent lack of significant changes in propionate production observed throughout this chapter. Any differences may have been masked by inter-individual variability. The top propionate producers also had high total SCFA production, leading to increased propionate production. This indicates that in terms of actual propionate production, increasing total SCFA production is beneficial, not necessarily changing the proportion of propionate. As described in Chapter 3 the ratio can be misleading in terms of predicting propionate production. For example within this chapter after the controls (blank) the whole grain fractions of the wild type and amylose only starch led to 22.2 (1.3) % and 20.9 (2.4) % (of total SCFA) being propionate, and ranked highest for propionate proportion. The inaccuracy of the ratio in predicting propionate production is exemplified with these substrates ranking 30^{th} and 33^{rd} out of 37 for total propionate production.

This indicates that laminarin, which yielded high total production as well as propionate production was the best substrate tested.

Chapter 6 Investigation of the influence of glycosidic bond anomer configuration on production of propionate *in vitro*.

6.1 Introduction

As discussed in Chapter 1, the main determinants of propionate production remain unknown. The properties of NDC thought to be involved in determining the amount and pattern of SCFA production include solubility (and therefore chain length), sugar composition, and bond configuration. To-date there has been little systematic analysis of these factors and their impact on the production of propionate *in vitro*.

It is difficult to characterise the fermentation properties of NDC based on their MW/ solubility. This is because the solubility and MW do not take into account other physicochemical properties such as branching, bonding, or sugar composition, which can alter SCFA production (Salvador et al., 1993, Laurentin and Edwards, 2004). Another issue is that not all NDC are 100% soluble or insoluble, such as ispaghula consisting of 63% insoluble and 42% soluble fibre (Mortensen and Nordgaard-Andersen, 1993). Solubility is an indicator of fermentability, and is predictive of total SCFA production, not individual SCFA production. Associations between SCFA production and carbohydrate solubility were observed after analysis of eight different NDC sources; glucose, sorbitol free ispaghula (Vi-siblin), ispaghula (Lunelax), pectin, resistant starch, sterculia (Inolaxol), wheat bran (Fiberform) and cellulose. Each of the NDC tested had different solubilities and were fermented *in vitro*. It was identified that the greater the solubility of the NDC the more SCFA produced (Mortensen and Nordgaard-Andersen, 1993). Although increased solubility increases fermentability, insoluble fibre can also be fermentable, for example when equal amounts of the insoluble fibre of sugar beet and barley bran were fermented, SCFA were produced, but to different extents. The insoluble fibre of sugar beet produced more total SCFA than the insoluble fibre of barley bran. These differences in total SCFA production were attributed to differences in the sugar composition and bonding structure, not the solubility further demonstrating that solubility alone cannot determine SCFA production for all NDCs (Fardet et al., 1997).

It has recently been shown that the influence of carbohydrate solubility on fermentability and SCFA production is more complex. Stewart and Slavin., (2006) fermented guar gum at a range of different MW (15-1100 kDa) and observed that guar gum of 400 kDa MW had the fastest rate of SCFA production between 4 and 8 hours (9.5 μmol/ml/hr). In contrast, the rate of SCFA production for guar with MW of 15kDa (5.4 μmol/ml/hr) and 1100 kDa

(5.6 μmol/ml/hour) did not differ (Stewart and Slavin, 2006). Moreover, total SCFA production has not been previously seen to have a significant linear association with MW in a study of oat beta glucans (Kim and White, 2011b) (Figure 6-1, Figure 1-5). Between 0 and 12 hours oat β-glucan MW had little effect on SCFA production where there was no significant differences in the total SCFA production between all the different MW β-glucans. It was also seen that the lowest MW oat β-glucan (0.5 kDa), and the highest MW oat β-glucan (9 kDa) produced comparable total production which was less than the reminder of the MW of β-glucan tested. These different MW did not translate into differences in the production of propionate (Kim and White, 2011b).

This reduction of SCFA production with the different MW could be due to a number of factors. Such as the high MW altering the ability of different bacteria to adhere to the polysaccharides, thus preventing their utilisation. For example, for the utilisation of cellulose by *Ruminococcus flavefaciens* dockerin-cohesin pairs are required to allow the glucosidases to break down the cellulose (Flint et al., 2008). This could also occur as a result of steric hindrance due to differences in the outer structure preventing access to the carbohydrate (Valjamae et al., 1998). Differences in utilisation could also occur as a result of the requirement of different glycoside hydroxylases (GH) which are found in the colonic bacteria. This could result in a number of different bacteria utilising the substrate including generalist species such as *B.thetaiotaomicron* can express many different CAZymes and utilise a variety of substrates, although it is not efficient to activate them all concurrently. However, *B.thetaiotaomicron* did not grow on β-glucans or galactomannan as used in the investigation by Martens et al., (2014) indicating that a different bacterial populations such as *Bacteroides ovatus* which has the appropriate CAZymes utilise these substrates (Martens et al., 2014).

With the low MW, substrates reduced total SCFA production could have occurred; there may have been a lag period in the bacterial response. This is as the low MW were di and tetra saccharides not commonly encountered within the diet (due to the consumption of complex polysaccharides). Due to this different bacteria or the upregulation of different enzymes were likely to be required to catabolise these simple sugars, further highlighting the complexity of identifying drivers of SCFA production (Martens et al., 2014).

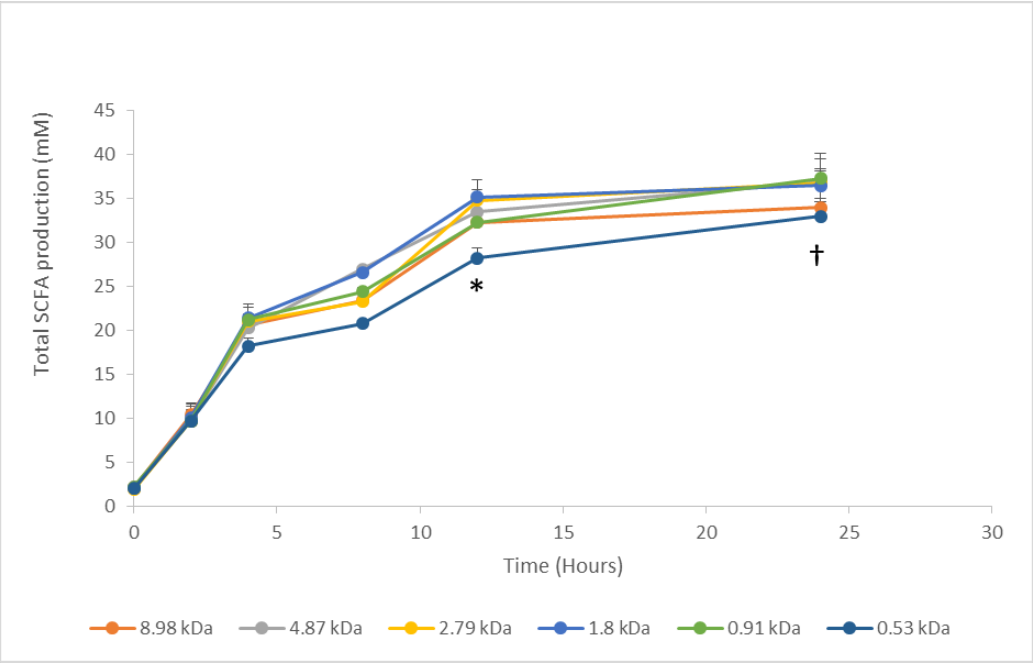

Figure 6-1: Total SCFA production over 24 hours after fermentation of different molecular weight oat β-glucans.

Time points plotted are 0, 2, 4, 6, 12, and 24 hours. Data plotted from that presented by (Kim and White, 2011b). Mean + SD, n=3. * indicates $p<0.05$ 0.53 KDa vs 2.79 kDa and † = $p<0.05$ 0.53kDa and 8.98 vs 4.87, 2.79, 1.8 and 0.91 kDa.

Although the systematic review conducted in Chapter 3 suggested that there was little difference in the ability of different NDC to produce propionate, few *in vitro* investigations have systematically considered the impact of the sugar composition of oligosaccharides on individual SCFA production - particularly propionate. The fermentability and the SCFA producing capability of the individual sugars within a substrate has been previously studied by Salvador et al., (1993) using the *in vitro* fermentation technique. Salvador et al., (1993) assessed the composition of wheat bran, sugar beet, cocoa, maize and pea hull fibre and these were fermented to assess how well the sugars were utilised by the colonic bacteria. It was observed that uronic acids were the most fermentable sugars in wheat bran, cocoa, maize and sugar beet. For pea and cocoa, glucose was the most poorly fermented sugar, whilst xylose for sugarbeet, and arabinose for wheat bran were also poorly fermented. Sugar beet, which consisted of high arabinose (17.8%) and uronic acid (19.4%) content which as a whole, as well as the individuals sugars were highly fermentable, and led to the highest concentration of propionate after 24 hours of fermentation (~35 mmol). In contrast, maize (10.7% arabinose) and pea fibre (14.7% uronic acid) did not lead to remarkable amounts of propionate production (~15 mmol) (Salvador et al., 1993). This suggests that within an NDC the fermentability of the different constituent sugars is not

uniform, and that the amount of SCFA produced is not only a sum of the SCFA production by each sugar within the NDC. This is likely as a selection of different CAZymes are required to utilise all of these sugars and bonds, which may or may not be produced by the same bacteria. For example the colonic bacteria are highly adaptable with sugars tested within this study can be utilised by the bacteria *B. thetaiotaomicron* and *B.ovatus.* This however does not take into account competition of the bacteria utilising these substrates or other properties of the substrates. For example, *B.ovatus* preferentially acts on less soluble carbohydrates whereas *B. thetaiotaomicron* acts on more soluble substrates (Martens et al., 2011). In contrast, Firmicutes bacteria preferentially act on insoluble fibre indicating that there may be competition as well as reduced access to the bonding resulting in altered SCFA production profiles (Martens et al., 2014, Ze et al., 2012).

Although not all monosaccharides and disaccharides reach the colon, the glucose disaccharides used are likely to be absorbed in the colon after breakdown by brush border hydrolases in the small intestine. However, *in vitro* fermentation studies of these sugars allow a more mechanistic approach to the identification of components determining propionate production. Monosaccharide analysis by *in vitro* batch fermentation investigations demonstrated that rhamnose, a sugar that can avoid digestion, selectively increased the generation of propionate (Gietl et al., 2012, Fernandes et al., 2000). Human feeding studies have also found varied effects of rhamnose on serum propionate concentration when approximately 25 g/day was consumed by healthy individuals. Vogt et al., (2004) observed that a drink containing rhamnose ingested for 4 weeks and in a single dose trial increased concentrations of serum propionate. In contrast, Darzi et al., (2015) found no effect on serum propionate after consumption of a rhamnose containing jelly for a week (Vogt et al., 2004b, Vogt et al., 2004a, Darzi et al., 2015).

Few studies have also investigated the role of disaccharides on propionate production. Sanz et al., (2005) conducted *in vitro* fermentation experiments with a selection of different disaccharides, including glucose disaccharides. This study appears to have used only one stool sample and sampled only at 12 hours of fermentation making extrapolation to the population difficult. Here, a few differences were observed, where beta-glycosidic bonding of glucoses generally increased propionate production compared to glycosidic bonding in the alpha anomeric form. Increased propionate with specific glycosidic bonds were identified with the fermentation of sophorose (β-1-2- glucose-glucose), β-β- trehalose

(β-1-1-β- glucose-glucose), laminaribiose (β-1-3- glucose-glucose), cellobiose (β-1-4-glucose-glucose), whereas isomaltose (α-1-2- glucose-glucose) and mannobiose (2α mannose – mannose) which also have alpha bonds also increased propionate production (Sanz et al., 2005a). In contrast when a small selection of disaccharides (isomaltose, maltose cellobiose and gentiobiose) underwent *in vitro* fermentation (n=3), after 24 hours there was no significant differences in the production of propionate (Gietl et al., 2012).

Changes as a result of alterations in bonding have also been observed when comparing pyrodextrinised starches which have increased solubility and water holding capacity compared to their native forms. Pyrodextrinisation does not alter the sugar composition, but pyrodextrinised forms have increased beta glycosidic bonding compared to native forms (Laurentin and Edwards, 2004, Campechano-Carrera et al., 2007). When pyrodextrinised forms of native starches were fermented *in vitro,* it was found that pyrodextrinised starch generated significantly more SCFA per kg of carbohydrate than native starch. Propionate proportions also increased with pyrodextrinised starches by an average of 50% and acetate proportions decreased by an average of 22.7% (Laurentin and Edwards, 2004), suggesting that the bond anomer may be a major player in the production of propionate.

Investigations of oligosaccharide structures with different types of bonds, structures, and sugars conducted *in vitro* also observed that bonding in the beta anomeric form was associated with increasing propionate production. For example, *in vitro* incubations and human feeding trials of dietary fibres consisting of bonding with the beta anomer such as laminarin (β[1-3] β[1-6] glucose bonding). After *in vitro* fermentation of laminarin, and when laminarin consisted of 2% of the diet in rats for 2 weeks resulted in approximately double the caecal concentration of propionate compared to a low MW form of alginate (7.49 [1.33] vs 4.51 [1.32] μmol/g content)(Deville, 2007, Kuda et al., 2005). Cereal β-glucans which are formed of linked glucoses (β[1-3] β[1-4] glucose bonding) have also been demonstrated *in vitro* and in feeding trials to increase production of propionate (Queenan et al., 2007). Feeding trials where 3% of the diet was barley β-glucan and was consumed for 2 weeks by rats produced significantly more propionate than the control diet and the low β-glucan diet (0.02% of the diet).

Guar gum, differing in sugar composition compared to cereal β-glucans and laminarin is also considered propiogenic. Guar gum is formed of a β-linked mannose back bone with α- linked galatobiose side chains where increased propionate has been demonstrated *in vitro* and after guar gum formed 8% of the diet in rats for 3 weeks. Guar gum consumption led to the production of double the amount of cecal propionate compared to resistant starch (50 [6.0] mM vs 27 [7.0] mM) (Khan and Edwards, 2005, Levrat et al., 1996). Pectin, also differing in structural composition (α[1-4] linked galactouronic acid) has been shown *in vitro* and in feeding trails where it formed 5% of the diet of rats diet for 3 weeks and caecal propionate production was higher than that of the control (88.1 vs 53.0 μmol/g) (Titgemeyer et al., 1991, Knapp et al., 2013).

As these all have different structures and bonding, the question of which factor in the most influential in determining propionate production remains. This demonstrates that although changes in propionate production have been observed as a result of different monosaccharides, disaccharides, and oligosaccharide structure, there have been no studies which have systematically considered the impact of individual glycosidic bonds on SCFA profiles. Using disaccharides of glucose covering the full range of glycosidic bond configuration available would allow analysis of the impact of the bond configuration, without the confounding effects of different monomer composition, MW or solubility.

6.2 Aim

To identify which glycosidic bond configurations selectively increase SCFA production during fermentation of all commercially available glucose-glucose disaccharides.

6.3 Methods

6.3.1 Substrates

All possible glucose-glucose disaccharides (diglucoses), except for β, β-trehalose (diglucose β[1-1]β) which was not a commercially viable option to study in a usable amount, were used to model the effect of glycosidic bonding on SCFA production (Figure 6-2).

Bond linkages (both anomers and all positional isomers) investigated were; α,α-D-Trehalose Dihydrate (diglucose α[1-1]), α,β,- Trehalose (diglucose β[1-1]), Kojibiose (diglucose α[1-2]), Sophorose (diglucose β[1-2]), Nigerose (diglucose α[1-3]), Laminaribiose (diglucose β[1-3]), D-Maltose Monohydrate (diglucose α[1-4]), D-cellobiose (diglucose β[1-4]), Isomaltose (diglucose α[1-6]), β-D-gentiobiose (diglucose β[1-6]). All disaccharide substrates were purchased from Carbosynth (Berkshire, UK). A blank (no-substrate) control was also used to adjust for background SCFA production.

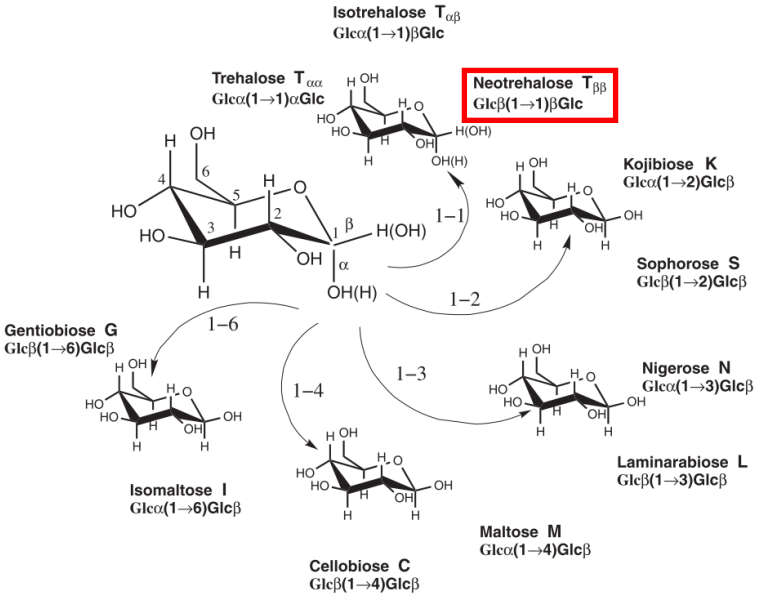

Figure 6-2: Bond linkages used in this investigation.

All linkes in the α and β anomer with all positional isomers were fermented. The β(1-1)β, which was not included due to cost is indicated by the red box. Adapted from (Peric-Hassler et al., 2010). Trehalose = trehalose dihydrate, Isotrehalse = Trehalose. Nb Iso and neotrehalose are annotated the wrong way around

6.3.2 *In vitro* batch Fermentations

Batch fermentations were performed as outlined in Section 2.2.1 and in Chapter 4. The fermentations were carried out in the validated 50mg 'small vessel size' due to the high cost of the substrates, therefore enabling a sufficient number of repeats. SCFA and pH were measured in samples from the fermentation vessels at 0, 8, and 24 hours. The SCFA were extracted and analysed by GC: FID as described in Section 2.3.

6.3.2.1 *Sample donors*

All sample donors were healthy Caucasians. No information on prior or habitual diet or weight were obtained. An initial power calculation using G.Power 3.1 ([Heine et al., 2016] University of Dusseldorf) was calculated using data generated during the miniaturisation (see Chapter 4). It indicated that differences in propionate production could be observed with approximately 12 fermentations would yield 80% statistical power. Due to a lack of substrate availability, 10 fermentations were conducted. Initially, stool samples were obtained from 10 individuals (4 males and 6 females) aged 20-52 (median age 26.5 years, mean age, 31 years). Due to a lack of substrate, diglucose β(1-1)α was n=9 (4 males and 5 females, aged 20-52, median age 28 years, mean age 31.9 years). A post hoc power calculation was also conducted and it was identified that for α(1-1) and β(1-4) an additional five fermentations should achieve sufficient statistical power. As it was feasible and affordable, five additional fermentations for these substrates were performed along with the blank. The additional five donors (three males and two females) were aged 23-52 (median age 30, mean age 35.4). This resulted in the final 15 stool samples being obtained from seven males and eight females, aged 20-52 (median age = 28 years, mean age 32.5 years).

6.3.2.2 *Statistics*

Statistical power was measured by post hoc power analysis using G.Power 3.1 ([Heine et al., 2016] University of Dusseldorf). The distribution of the data was assessed using the Shapiro Wilk test of normality. The effect of pH was tested by the Kruskal Wallis test with post-hoc pairwise comparisons with the Dunn-Bonferroni test. Analysis of the rate and ratio were conducted using one-way ANOVA with post hoc Bonferroni analysis on $\log_{(10)}$ transformed data. Statistical significance was considered at $p<0.05$.

6.4 Results

All substrates were easily dissolved enabling ease of sampling, with no substrates being viscous. The majority of gases were produced after 8 hours of fermentation. These gases were removed (but not measured) to allow for the sampling. Gas was also produced at 24 hours, indicating fermentation resumed and that the vessel remained anaerobic.

6.4.1 Statistical power

As indicated in Section 6.3.2.1 an initial power calculation using G.Power 3.1 identified that 12 fermentations would provide 80% power. Once the initial 10 fermentations were conducted, a post hoc power calculation was performed. Here it became apparent that an additional five fermentations could provide a statistical power that was >90%. Power at 8 and 24 hours differed with α(1-1), β(1-2), β(1-4), β(1-6) have >90% power for propionate production. All other disaccharides had >40% power after 8 hours of fermentation. Diglucoses α(1-1) and β(1-4) had power of; 98% and 93%, β(1-2) and β(1-6) had 70% power, the remainder of diglucoses had a power <20% after 24 hours of fermentation.

6.4.2 pH

All diglucoses were utilised by the bacteria within the slurry, as demonstrated by a reduction in pH (Table 6-1). Within 8 and 24 hours of fermentation, all of the diglucoses had a significantly reduced pH compared to the control and the initial pH. Diglucose α(1-4) had the lowest pH at both time points (pH 3.85 [0.15] and pH 3.57 [0.33], Table 6-1).

When comparing the effect of linkage on pH, diglucose α(1-1) had a significantly lower reduction in pH compared to all other linkages in the alpha anomer, after both 8 and 24 hours of fermentation (pH 5.07 [0.52] and pH 4.55 [0.13], $p< 0.05$). The pH did not differ between the disaccharide bonding positions with the beta anomer at 8 or 24 hours (Table 6-1).

When considering the effect of bond anomer on pH, few selective effects were seen across or between different types of glycosidic bond linkages. Differences in anomer were observed for the 1-4 position with the alpha anomer having a significantly lower pH than

the beta anomer at both 8 (pH =3.85 [0.15], vs 4.44 [0.32], p= 0.001) and 24 hours (pH= 3.57 [0.12] vs 3.99 (0.44), p< 0.002). Diglucoses with 1-1 bond positions had differences in pH with the alpha anomer having a significantly higher pH at both time points (8 hours 5.07 [0.52] vs 4.12 [0.40], p=0.002, 24 hours 4.55 [0.13] vs 3.80 [0.39], p = 0.003, Table 6-1).

Table 6-1: pH after fermentation of all possible diglucoses at 0, 8 and 24 hours

	0h		**8h**		**24h**	
	α	**β**	**α**	**β**	**α**	**β**
Diglucose	7.15	6.99	5.07	4.12	4.55	3.80
(1-1)	(0.62)	(1.11)	(0.52)a*	(0.40)	(0.13)a*	(0.39)
Diglucose	6.81	7.06	4.23	4.16	3.85	3.81
(1-2)	(0.98)	(0.93)	(0.49)b	(0.35)	(0.33)b	(0.19)
Diglucose	6.91	6.76	4.05	4.42	3.84	3.89
(1-3)	(0.83)	(0.84)	(0.34)b	(0.25)	(0.34)b	(0.39)
Diglucose	6.95	7.15	3.85	4.44	3.57	3.99
(1-4)	(0.89)	(0.63)	(0.15)b*	(0.32)	(0.12)b*	(0.44)
Diglucose	6.93	7.21	4.05	4.14	3.72	3.77
(1-6)	(0.83)	(0.37)	(0.18)b	(0.37)	(0.20)b	(0.40)+

Median (IQR) n= 15 for blank, α(1-1), and β(1-4), n= 9, β(1-1) all other diglucoses n=10. All diglucoses except α(1-1) had a pH significantly lower than the blank control at 8 and 24 hours ($p<0.0001$) . Different letters indicate significant differences between diglucoses with the same anomer, $p<0.05$, + indicated p=0.066 vs. β(1-1). * indicated significant differences as a result of bond anomer, $p<0.05$.

6.4.3 SCFA production after 8 hours of fermentation

SCFA production at 8 hours was not significantly affected by the disaccharide linkage. Trends were observed with the α(1-1) linkage, which generated the lowest amount of acetate, 107.38 (72.74) µmol/g carbohydrate/ hour where, p= 0.075 vs α(1-6) (261.08 [78.70] µmol/g carbohydrate/ hour) and p=0.056 vs β(1-6) (230.18 [148.87] µmol/ g carbohydrate/ hour, Table 6-2). Also observed at 8 hours was the lack of effect of anomeric orientation on acetate, propionate, or butyrate. Unlike for bonding position, no trends were observed due to the different anomers.

Table 6-2: SCFA production after 8 hours of diglucose fermentation µmol/g carbohydrate/ hour

	Acetate		Propionate		Butyrate		Total	
	α	**β**	**α**	**β**	**α**	**β**	**α**	**β**
Diglucose	107.38	187.17	19.00	12.58	28.10	9.92	182.07	247.22
(1-1)	(72.74)	(59.69)	(21.63)	(10.2)	(26.66)	(4.86)	(173.45)	(63.46)
Diglucose	174.03	225.28	16.68	18.44	10.34	34.01	250.85	265.62
(1-2)	(97.55)	(109.71)	(17.62)	(4.29)	(13.21)	(36.78)	(97.63)	(98.89)
Diglucose	224.36	119.85	14.21	15.49	8.66	14.14	278.73	189.71
(1-3)	(151.90)	(98.42)	(12.14)	(7.42)	(24.24)	(20.21)	(129.24)	(113.86)
Diglucose	224.91	130.73	13.63	21.55	7.57	31.66	260.02	223.37
(1-4)	(93.05)	(85.95)	(13.80)	(21.88)	(7.54)	(85.83)	(104.41)	(182.97)
Diglucose	261.08	230.18	16.46	13.99	10.08	32.30	304.04	290.66
(1-6)	(78.70)a	(148.87)b	(14.81)	(21.56)	(15.27)	(34.40)	(90.76)	(220.07)

Median (IQR) n = 15 for α(1-1), and β(1-4), n= 9 β(1-1) all other diglucoses n=10.
Letters indicate trends vs α(1-1) a; p = 0.075, b; p = 0.056

6.4.4 SCFA production after 24 hours of fermentation

6.4.4.1 *Influence of bond position on SCFA production*

After 24 hours of fermentation, there was no significant difference in the production of propionate or total SCFA as a result of bond anomer. In contrast, significant differences in acetate and butyrate production were observed (Table 6-3,Table 6-4, Figure 6-3). When considering bonding with an alpha anomer, diglucose α(1-1) led to the lowest rate of production and molar proportion of acetate and were significantly less than the values for diglucoses α(1-6) (57.05 [46.58] vs 125.51 [48.45] µmol/g carbohydrate/ hour, p= 0.001), and α(1-4) (57.05 [46.58] vs 108.97 [48.50] µmol/g carbohydrate/ hour, p= 0.006).

Acetate proportion for diglucose α(1-1) was significantly less than all other linkages in the alpha anomer yielding 61.47 (21.44) % compared all other linkages in the alpha anomeric form with 1-2= 90.8 (18.90) %, 1-3 = 92.2 (17.52) %, 1-4 = 93.94 (8.82) %, 1-6 = 92.16 (8.45) %, $p<0.01$.

Diglucose α(1-1) generated significantly more butyrate production (29.17 [36.74] µmol/g carbohydrate / hour) and molar proportion of butyrate (26.45 [32.40] %, $p<0.01$) than all other linkages with alpha anomer. Statistical p values for the rate were; α(1-1) vs α(1-2), p

= 0.057, α(1-1) vs α(1-3), p = 0.019 , α(1-1) vs α(1-4), p = 0.001, α(1-1) vs α(1-6), p= 0.006. This indicates that the bacteria were preferentially producing butyrate over acetate. The other diglucoses with alpha anomer did not differ in butyrate production from one and other (Table 6-3).

The diglucose with α(1-1) bonding position also had the highest rate and proportion of propionate production but this was not statistically significant (Table 6-3, Table 6-4). With propionate production from α(1-1) being 11.14 (15.51) μmol/g carbohydrate/ hour compared to α(1-4) yielding the least propionate with 4.3 (3.53) μmol/g carbohydrate (Table 6-3). Similar observations were also seen with the proportion of propionate with α(1-1) yielding 9.10 (9.38) % and the lowest proportion of propionate was from α(1-4) producing 3.89 (7.71) %Table 6-4).

When considering the beta anomer effects on the rate of acetate production were observed. Diglucose β(1-6) produced the highest rate of acetate which was significantly more than β(1-1); 191.47 (191.09) vs 95.62 (30.05) μmol/g carbohydrate/ hour, p = 0.004 and β(1-2); 191.47 (191.09) vs 125.32 (48.45) μmol/g carbohydrate/ hour, p = 0.036. For linkages in the beta anomer there was no difference in propionate, butyrate, or total production (Table 6-3, Figure 6-2, Figure 6-3). Proportionally, a trend for increased butyrate production with diglucose β(1-4) compared to β(1-1) was observed 19.2 (28.39) % vs 2.68 (8.27) %, p<0.051. The proportion of acetate production was significantly reduced for diglucose β(1-4) compared to diglucoses β(1-1); 68.56 (37.89) % vs 92.18 (12.74) %, p= 0.016 and β(1-6); 68.56 (37.89) % vs 85.27 (10.82) %, p = 0.023 (Table 6-4).

6.4.4.2 *Influence of bond anomer on SCFA production (alpha vs beta)*

Differences in butyrate production due to anomer were seen with the 1-1 diglucose linkage (α: 29.17 [36.74] vs β: 3.69 [2.54] μmol/g carbohydrate/ hour, p = 0.009), and 1-4 (α: 2.75 [2.60] vs β: 26.43 [35.80] μmol/g carbohydrate/ hour; p = 0.033, Table 6-3, Figure 6-3)

Contrasting proportions of acetate, propionate, and butyrate were seen in diglucoses with 1-1 and 1-4 linkages. Diglucoses with the 1-4 linkages in the alpha anomer observed a significantly higher proportion of acetate (α: 93.94 [8.82] % vs β: (68.56 [37.89] %, p=0.001) and a lower proportion of propionate (α: 3.89 [7.71] % vs β: 9.29 [7.22] %, p = 0.037) and butyrate (α: 2.86 [2.63] % vs β: 19.20 [28.39] %, p = 0.001) compared to the

beta anomer. A similar effect in the molar proportions of acetate and butyrate as a result of differing bond anomer were observed for diglucose 1-1 where the alpha anomer had a significantly lower proportion of acetate (61.47 [21.44] % vs 92.18 [12.74] %, p<0.001), and higher proportion of butyrate (26.45 [32.4] % vs 2.68 [8.27] %; p= 0.001, Table 6-4).

Table 6-3: SCFA production after 24 hours of diglucose fermentation µmol/g carbohydrate/ hour

	Acetate		**Propionate**		**Butyrate**		**Total**	
	α	**β**	**α**	**β**	**α**	**β**	**α**	**β**
DiGlucose (1-1)	57.05 (46.58)a	95.62 (30.05)b	11.14 (15.51)	4.59 (1.79)	29.17 (36.74)a*	3.69 (2.54)	126.21 (54.36)	103.74 (30.59)
DiGlucose (1-2)	101.16 (37.29)	125.32 (48.45)b	5.68 (8.25)	6.44 (1.22)	4.19 (9.61)‡	19.91 (32.56)	120.97 (64.45)	149.05 (78.12)
DiGlucose (1-3)	105.44 (67.52)	86.96 (44.63)b	5.39 (3.37)	7.25 (3.11)	3.51 (8.37)b	8.09 (20.72)	123.76 (59.59)	128.64 (60.94)
DiGlucose (1-4)	108.97 (48.50)b	110.03 (80.83)b	4.30 (3.53)	9.13 (21.09)	2.75 (2.60)b*	26.43 (35.80)	118.86 (51.86)	98.23 (78.75)
DiGlucose (1-6)	125.51 (43.50)b	191.47 (191.09)a	6.32 (3.75)	10.49 (24.41)	3.68 (4.91)b	15.18 (13.89)	134.54 (55.14)	165.64 (67.19)

Median (IQR) n = 15 for α(1-1), and β(1-4), n= 9 β(1-1) all other diglucoses n=10.
Different letters within colums show significant differences. ‡ shows α(1-1) p= 0.057. * indicates signifcant differences as a reault of anomer p= 0.035

Table 6-4: Ratio of SCFA after 24 hours of diglucose fermentation

	Acetate		Propionate		Butyrate	
	α	**β**	**α**	**β**	**α**	**β**
Diglucose	61.47	92.18	9.1	5.09	26.45	2.68
(1-1)	(21.44)b*	(12.74)b	(9.38)	(4.88)	(32.4)b*	(8.27)+
Diglucose	90.80	97.14	5.51	5.47	3.98	15.19
(1-2)	(18.90)a	(16.74)	(7.90)	(4.72)	(8.45)a	(17.13)
Diglucose	92.20	88.27	4.83	5.98	3.35	6.23
(1-3)	(17.52)a	(23.53)	(7.13)	(7.06)	(7.36)a	(18.79)
Diglucose	93.94	68.56	3.89	9.29	2.86	19.20
(1-4)	(8.82)a *	(37.89)a	(7.71)† *	(7.22)	(2.63)a*	(28.39)
Diglucose	92.16	85.27	4.68	4.47	3.51	7.60
(1-6)	(8.45)a	(10.82)b	(4.37)	(5.07)	(3.66)a	(9.20)

Median (IQR), α(1-1), β(1-4) n= 15, β(1-1) n=9 all others n=10. Different letters indicate significant differences between bond positions. $p<0.05$. † indicates $p = 0.086$ vs α(1-1) + indicates $p = 0.051$ vs. β(1-4). Significant differences as a result of bond anomer are shown as *.

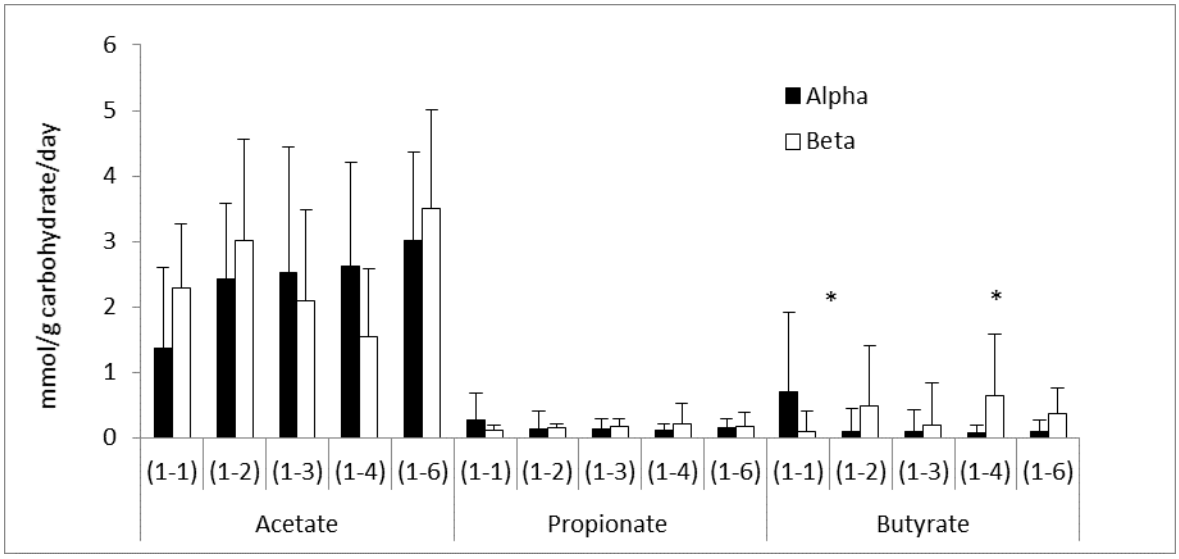

Figure 6-3: 24 hour SCFA production (mmol/g carbohydrate/day at 24 hours).

Data presented are Median + IQR. α(1-1), β(1-4) n= 15, β(1-1) n=9 all others n=10. * indicate differences between the α and β anomer $p< 0.05$.

6.4.5 Ranked propionate production

As there were no observed significant differences in the production of propionate, the production of propionate by the 10 individuals fermented were ranked (Figure 6-4). There was no substrate that ranked consistantly for propionate production. A sub-population was identified with preference for propionate with diglucoses with beta anomers. Subgroups of individuals are discussed below.

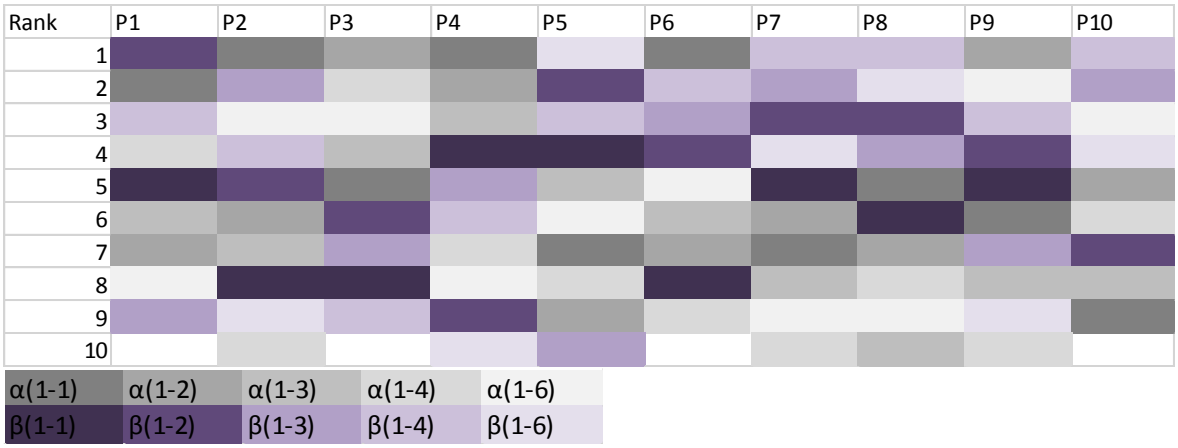

Figure 6-4: Heat map of ranked propionate production by the diglucoses tested by each individual.

Key: Grey- alpha anomer diglucoses, purple – beta anomer diglucose. Different shades indicate different bond positions. P1, P3, P6 β(1-6) not included. P10 β(1-1) not included

6.4.6 Individual variability in SCFA production

Figure 6-5 shows the variability for propionate, butyrate and total production between individuals with fermentation of α(1-1) and β(1-4). For propionate and butyrate production there was a subset of individuals appearing to have higher propionate (n = 3) and butyrate (n =4) producing capabilities. However, it was not same individual producing consistently higher propionate, butyrate, and total production suggesting that this may not have occurred due to of issues of the experimental set up. For example donor number 6 (green line) produced the most propionate with diglucose α(1-1), but butyrate and total production were unremarkable, indicating that the substrate added to the vial was not erroneous. It was also observed for donor number 6 that SCFA production with diglucose β(1-4) was also unremarkable indicating that there were no issues with the fermentation media/ experimental setup as all fermentations were performed concurrently. Similar effects were also observed with the top producers, with no individual having high

propionate, butyrate or total production on all occasions. Figure 6-5 shows that although some individuals produced high concentrations of propionate and butyrate, there were also others who produced low concentrations of propionate and butyrate. Interestingly, it was not always the same individuals yielding high or low propionate/ butyrate concentrations.

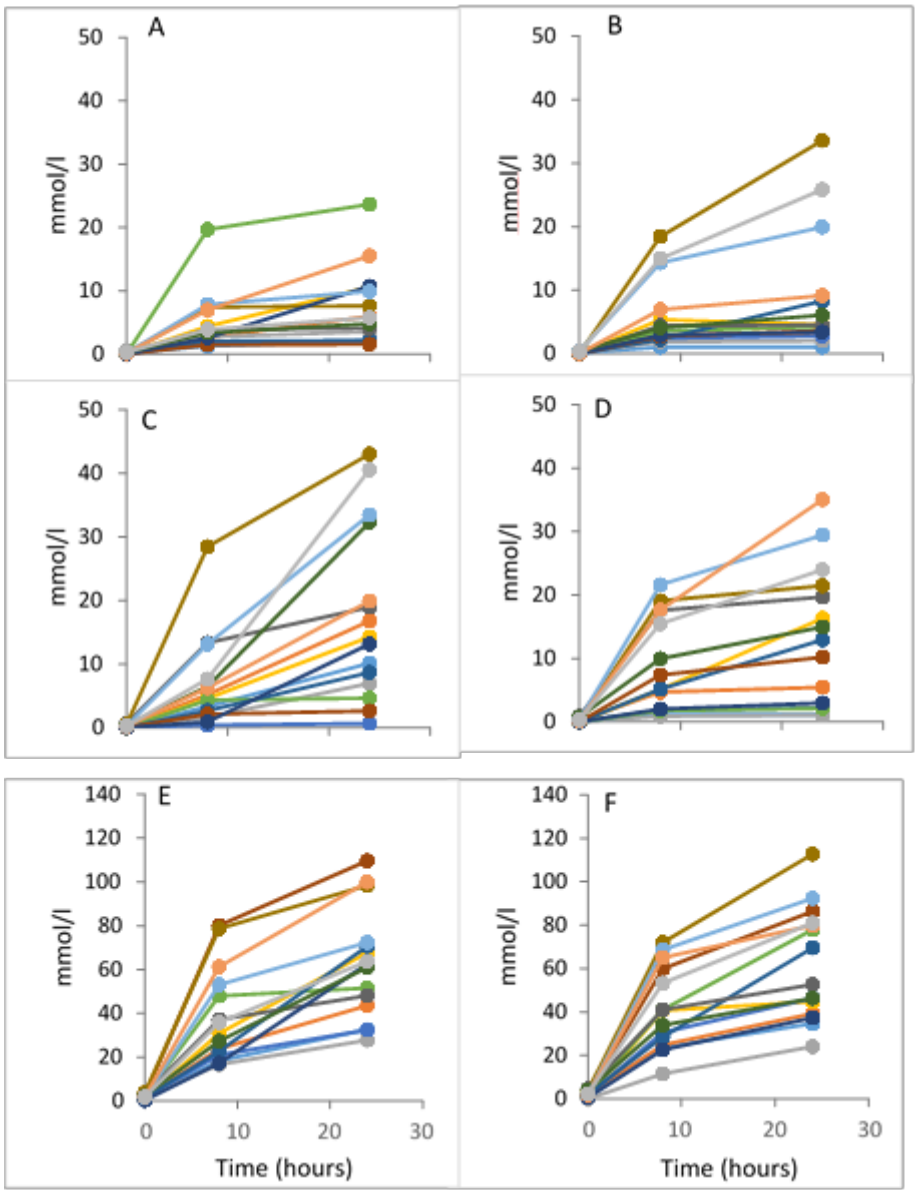

Figure 6-5: Individually plotted absolute SCFA production at 0, 8, and 24 hours.
Diglucose α(1-1): A, Propionate, C, Butyrate, E, Total SCFA. Diglucose β(1-4): B, Propionate, D, Butyrate, F, Total SCFA. Each coloured line is an individual

6.5 Discussion

Knowing which fractions of dietary fibres are responsible for driving the production of propionate would enable better selection for substrates to increase concentrations of propionate in the colon. As discussed in Chapter 1, selectively increasing the production of propionate could enable targeted activation of FFAR2 and FFAR3. This has been shown in principle to confer advantages to metabolic health, including the regulation of the appetite (Chambers et al., 2014, Byrne et al., 2016).

Different dietary fibres appear to be associated with increases in propionate production. NDC consisting of β-glucans, such as laminarin, and psyllium are associated with increased propionate production, whereas starch with α(1-4) and α(1-6) bonding is linked with increased butyrate production (Fässler et al., 2006, Deville, 2007, Kaur et al., 2011). Due to the lack of mechanistic studies it still remains unclear why these substrates increase the generation of these specific SCFA. Currently there are few investigations that have assessed the effect of mono/ disaccharide sugars and their linkages on the production of propionate (Mortensen et al., 1988, Gietl et al., 2012). The most comprehensive investigation consisted of only a single stool sample from one donor (Sanz et al., 2005a). This of limited use when there is such variation in different individual's abilities to produce SCFA (Carlson et al., 2016).

The disaccharide sugars tested within this study are unlikely to reach the colon *in vivo* due to digestion and/or absorption. However, this *in vitro* analysis has enabled the investigation of whether a specific bond linkage leads to the production of propionate. This was conducted by utilising all possible glucose-glucose disaccharides, except for β-β-trehalose β(1-1)β as the cost was too high to include within this experiment (Figure 6-2). With limited commercially viable availability of some substrates, and the requirement of a high number of replicates, the validated miniaturised method discussed in Chapter 4 was utilised with each fermentation vial requiring 50 mg of substrate. The use of these glucose disaccharides also enabled the analysis of the propionate producing capabilities of the bonding without confounding effects from the sugar composition, substrate solublity, or MW on SCFA production.

Although up to 15 different donors provided stool samples only α(1-1) and β(1-4) reached statistical power after 24 hours, these substrates along with β(1-2) and β(1-6) were sufficiently powered at 8 hours. This indicates that although generally underpowered, significant differences or trends would have been identified from the 10/ 15 fermentations. To gain sufficient power for some substrates would require more than 100 different fermentations, which due to substrate availability and time constraints was not feasible. Lack of power likely occurred as a result of the variability in an individual's ability to produce SCFA, which as shown in Figure 6-3 - Figure 6-5 is substantial. This variation in an individual's ability to produce SCFA may be as a result of their habitual diet prior to donating a sample, which was not controlled. Different diets are likely to have influenced the bacterial diversity in the stool as well as the bacterial enzymes expressed and as a result the SCFA produced (Bourriaud et al., 2005, Reichardt et al., 2014, David et al., 2014, O'Keefe et al., 2015).

Unexpectedly, there was little effect of bonding position or anomer on propionate production, and this was also apparent after ranking the substrates. All differences in SCFA production observed were associated with diglucoses α(1-1) and β(1-4). The final pH for glucose disaccharides with α(1-1) bonding was higher with the alpha anomer than all other diglucoses after 8 and 24 hours of fermentation. Although pH is an indicator of fermentation, this difference did not result in differences in SCFA production after 8 hours, or total SCFA production after 24 hours. No differences in total production of SCFA between all substrates were observed, but differences in acetate and butyrate were identified. After 24 hours of fermentation α(1-1) generated less acetate as a rate of production and as a proportion but had significantly increased butyrate production compared to the other diglucoses tested. This may have occurred as a result of interconversion of acetate to butyrate thus increasing butyrate concentrations and decreasing acetate concentrations (Morrison et al., 2006).

Differences linked with bond anomer were mostly isolated to (1-1) and (1-4) bond positions. The alpha anomer had a significantly reduced decrease in pH compared to the beta anomer with the 1-1 bonding position at 8 and 24 hours. Similar effects were also observed with the 1-4 bond position with the alpha anomer having a significantly lower pH at 8 and 24 hours compared to the beta anomer. This however did not alter the total SCFA production at 8 or 24 hours. This was also associated with changes in butyrate production

and propionate and butyrate proportion. Alpha (1-1) generated significantly more butyrate as a rate as well as molar proportion than the beta anomer, and the opposite was found with alpha (1-4) linkages. The alpha (1-4) also resulted in significantly less propionate proportion than the beta anomer. This could be associated with the requirement for different CAZymes for each different bonding positions and anomers. For example, α(1-4) glucose linkages (which are located in starch) can be utilised by enzymes encoded within starch utilisation systems such as the extracellular GH97 from *B.thetaoiotaomicron.* Bonding with β(1-4) glucose linkages can by catabolised by GH3 expressed by *B.ovatus* indicating that different bacterial species were likely required thus leading to different SCFA production (Martens et al., 2011, Martens et al., 2014).

An increase in propionate production as a result of the beta anomer within an NDC, has been previously reported (Laurentin and Edwards, 2004). Heat treatment of native starch (α[1-4], α[1-6]), resulting in pyrodextrisined starch and the conversion to β(1-4), β(1-6) bonds, led to increased in propionate production *in vitro*. The effect of this change on the bacterial profile was not assessed, however it is likely that the α(1-4) linkages are utilised with starch utilising systems such as those present in *B.thetaoiotaomicron* which use interconverstion to produce butyrate (although increased butyrate was not observed here) (Martens et al., 2011, Reichardt et al., 2014). In contrast β(1-4) are utilised by *B.ovatus* which forms propionate via the succinate pathway indicating that a shift from an alpha to a beta bond leads to different requirements for bacterial enzymes and as a result, alters the SCFA production profile (Martens et al., 2011, Reichardt et al., 2014). This may partially explain the increased proportion of propionate with β(1-4) bonding compared to the alpha anomer (Siljestrom et al., 1989, Laurentin and Edwards, 2004). Increased butyrate production from diglucoses with β(1-4) bonding is also supported by lactose which consist of this bonding and selectively generate butyrate *in vitro* (Mortensen et al., 1988, Hughes et al., 2008).

Studies assessing the *in vitro* fermentation capabilities of foods consisting of α(1-1) bonding, ie trehalose are scarce. Within foods these linkages are commonly found in mushrooms such as the ‘common’ mushroom (Wannet et al., 1998) and the shiitake mushroom (Chen et al., 2015) which also contain β-glucans. Shiitake mushrooms have been used in chinese medicine and have also been demonstrated to reduce body weight gain, and fat pad mass when rats were fed up to 6% wt/wt of shiitake mushroom powder

for 6 weeks (Handayani et al., 2014). Therefore it would be advantageous assess the propionate producing capabilities of these mushrooms. Wong et al., (2005) also carried out *in vitro* fermentations of mushroom sclerotia containing β-glucans containing β(1-3) and β(1-4) bonding and observed that they selectively increased propionate and butyrate proportions. However it was identified that two of the mushrooms tested were not well fermented, and that this did not lead to differences in the concentration of propionate. Within this investigation and few fermentations were conducted (n=1, with two technical repeats), this further makes it difficult extrapolate these findings to SCFA production in the wider population (Wong et al., 2005), also within this study the effect of β(1-6) linkages commonly identified in mushrooms was not present (Yu et al., 2009). A selection of mushrooms have also been demonstrated to have immunregulatory effects after response to an LPS challenge in mouse macrophage cells (RAW 264.7). Extracts of white button, shiitake, crimini and oyster mushrooms were able increase IL-1β, and TNFα expression, as well as decrease IL-10 expression which is favourable for immuno-regulation. It was also observed within this investigation that after feeding mice a diet which was 2% white button mushroom for 4 weeks, the expression of TNFα was increased after DSS mediated colitis (Yu et al., 2009). These immuno-regulatory affects have also been observed after the activation of FFAR2 by SCFA in mice (Maslowski et al., 2009b), suggesting that the benefical roles of fungal β-glucans may be occurring as a result of the colonic SCFA that are produced. Based on this, it would be interesting to assess the differences in propionate and butyrate production of different mushrooms, containing β-glucan bonding. It would also be interesting to assess the additional branching with trehalose and assess how that would alter propionate and butyrate production.

The results observed within this chapter are also in part supported by other investigations. The investigation by Sanz et al., (2005), assessing all of the possible diglucoses, with a stool sample from one donor for 12 hours also detected no trends in regards to linkage. It was however observed that the beta anomer did lead to selectively reduced acetate, and increased propionate and butyrate production. There were also no changes in the bacterial composition observed for all bond positions except for diglucose β(1-6) (gentiobiose) which generated decreased bifidobacteria numbers. As the investigation by Sanz et al., (2005) only used one donor, there was no inter-individual variability which may have made these differences more easily to observe. This investigation by Sanz et al., (2005) also did not continue the fermentations for 24 hours, so it is unknown whether these trends

are maintained after 24 hours. Within the small investigation by Gietl., 2012 comparing maltose (α[1-4]), cellobiose (β[1-4]), isomaltose (α[1-6]) and gentiobiose (β[1-6]) in *in vitro* fermentations with three stool donors for 24 hours no differences in propionate or butyrate were identified (Gietl et al., 2012).

This suggests that the specific bonding a glucose disaccharide is not the determining factor in propionate production. However this may be different for longer saccharides, which are broken down into smaller sugars, which is likely to be more beneficial to some bacteria than others (Martens et al., 2011). This may lead to differences in individual and total SCFA production observed as a result of different DP (Stewart et al., 2008).

In conclusion it is unlikely that the bonding alone determines the production of propionate and that other physicochemical determinants play role in driving this production.

Chapter 7 The propionate producing capacity of mycoprotein and extracted mycoprotein fibre.

7.1 Introduction

Mycoprotein is produced from cultures of the microfungus *Fusarium venenatum*. Mycoprotein is marketed in the UK as the meat replacement product Quorn®, and is widely consumed in the UK. Mycoprotein is high in protein and low in fat. It consists of 25% (dry weight, and 6% wet weight) dietary fibre. Chitin makes up 33% of the fibre fraction and the other 66% is β-glucan (Denny, 2008). Mycoprotein has been shown to be beneficial to health, with positive effects seen in human feeding trials including improved plasma cholesterol, and reduced energy intake (Table 7-1). A series of human feeding trials were conducted by Turnbull et al, in the early 1990s with mycoprotein consumption ranging from a single meal to 8 weeks of 80 g to 191 g/day. The 80 g dose corresponds to approximately 1.67 g chitin and 3.33 g β-glucan and the 191 g dose corresponds to approximately 3.98 g chitin and 7.96 g β-glucan which is about 1/3rd of current recommended intake of dietary fibre (SACN., 2015). Several advantages were associated with the consumption of mycoprotein in these studies, such as reduction in plasma cholesterol, glucose and insulin, energy intake, hunger, and desire to eat as well as increased fullness. These effects did not always occur and when measured did not lead to significant differences in body weight (Turnbull et al., 1990, Turnbull et al., 1991, Turnbull et al., 1993b, Turnbull and Ward, 1995)(Table 7-1).

The mechanisms for these effects of mycoprotein on health remain unclear. Mycoprotein is 11% (wet weight) protein, and the satiety inducing effects compared with other proteins have been assessed. Williamson et al., (2006) compared the effects on satiety of a preload of chicken, mycoprotein or tofu (soy protein). These meals were matched for protein but not fibre content. Tofu and mycoprotein resulted in significantly less food intake than chicken (Williamson et al., 2006). This is in agreement with other investigations comparing mycoprotein with chicken (protein content was matched) where subsequent energy intake after mycoprotein decreased (Turnbull et al., 1993a). Mycoprotein has also been shown to produce greater reductions in serum glucose and insulin than soy, although feelings of satiety were not measured (Turnbull and Ward, 1995). As the impact of the mycoprotein is seen when protein content is matched with other protein sources tested, studies have matched for overall protein content, but the amino acid content differed (Marlow foods LtD., 2016). Beef and soy protein have increased concentrations of many

amino acids compared to mycoprotein, but in a feeding study of beef and chicken where the amino acid composition was similar, no effect on satiety was observed (Uhe et al., 1992). Soy and beef protein leucine content is approximately double that of mycoprotein (Marlow foods LtD., 2016) and leucine in rat studies has been shown to reduce food intake (Morrison et al., 2007). Therefore, it is unlikely that the mycoprotein-induced effects on satiety occurred due to the protein content.

However, many of the effects observed with mycoprotein are often associated with increased fibre intake and thus the impact of mycoprotein may be due to its fibre, especially β-glucan.

Chitosan, which is the soluble fraction of chitin, formed by the deacetylation of chitin, and can occur with large scale bioprocessing. Chitin can be consumed as it is contained within the shells of crustaceans and fungi (Ravi Kumar, 2000). Chitosan has also been shown to reduce body weight (Mhurchu et al., 2004) and cholesterol (Bokura and Kobayashi, 2003) after consumption by overweight women. Beta glucan, which has a health claim for reductions in cholesterol, and glycaemic response ((EFSA Panel on Dietetic Products, 2011)) and is fermented, forming SCFA promotes the production of propionate (Queenan et al., 2007). These effects have also been seen when an inulin-propionate ester was consumed for 24 weeks by overweight individuals and resulted in reduced energy intake as well as reduced cholesterol (Chambers et al., 2014).

In Chapter 5, laminarin from seaweed (β[1-3], β[1-6], bonded glucose polymers, [Deville et al., 2007]) was the best candidate for propionate production *in vitro*. Mushrooms have been shown to contain similar bonded β glucans with additional β(1-4) bonds (Wong et al., 2005). Thus if mycoprotein fibre has similar bonding, its effects on satiety and plasma lipids could be mediated through propionate production and stimulation of FFAR receptor mediated gut hormone response.

Mycoprotein is approximately 25% dietary fibre (dry mass) and of that over 65% of the fibre is β-glucan with β(1-3), β(1-6) bonding. The remainder is chitin consisting of N-acetyl glucosamine monomers bound by β(1-4) linkages (Turnbull et al., 1991, Denny, 2008). Beta glucans from sources such as oats (Hughes et al., 2008), laminarin (Deville, 2007), and mushroom (Wong et al., 2005) also contain β(1-3), β(1-6), and β(1-4) linked

glucoses and have been associated with increased propionate and butyrate production, not only within published literature, but also within previous chapters (Chapter 3, Chapter 5). This indicates that these health benefits of mycoprotein may in part be due to increased production of SCFA, and more specifically propionate and butyrate.

Therefore in this chapter, the fermentation and propiogenic properties of mycoprotein and its extracted fibres was investigated.

Table 7-1: Effects of mycoprotein consumption assessed by human feeding trials

Study	Dose (daily)[1]	Form	Control	Length of feeding	[2]N	Health status	Outcome (empirical)	Outcome (subjective)
Single meals								
(Turnbull et al., 1991)	~140 g	Meal	Chicken	single meal	13	Healthy	Decreased energy intake (18%, vs chicken)	Reduced desire to eat (7%), and hunger (6%, vs chicken)
(Burley et al., 1993)	~ 180 g	meal and a cake	Chicken, standard cake	single meal	19	Healthy	Reduced subsequent energy intake (18%), NE on intake after 36 hours	Decreased hunger at 4 hours (NE over time), NE desire to eat, prospective fullness. Reduced taste (12%) and pleasantness (14%)*
(Turnbull et al., 1993b)	130 g	meal	Chicken	single meal	13	Healthy	Decreased energy intake on test day (24%) and the next day (16.5%)	Reduced desire to eat (25%), hunger (16%), and increased fullness (11%)
(Turnbull and Ward, 1995)	~80 g (20g/dw)	Milkshake	Soy protein	single meal	19	Healthy	Decreased serum glucose (13%) and insulin (36%)	
(Williamson et al., 2006)	44 g	Pasta meal	Chicken and tofu	single meal	42	Overweight	**vs chicken:** decreased food (12%) and energy intake (12%) **vs tofu:** NE on food or energy intake	NE on hunger or fullness
Long term studies								
(Turnbull et al., 1990)	19 1 g	Pie	Equicaloric meal	3 weeks	9	Raised cholesterol (5.2-6.2 mM)	NE body weight, dietary intake, glycaemic control, TAG or insulin. Decreased total (13%) and LDL cholesterol (9%), increase in HDL cholesterol (12%)	
(Udall et al., 1984)	~80 g (20g/dw)	Cookie	Mycoprotein free cookies	30 days	100	Healthy	Decreased cholesterol (7%)	
(Turnbull et al., 1992)	130 g	Cookie	Soy protein	8 weeks	11	Raised cholesterol >5.2 mM	NE body weight, energy intake, TAG, or HDL cholesterol. Total (16%) and LDL (18%) cholesterol decreased	

(Ruxton and McMillan, 2010)	88 g (21g/dw)	Meal	Standard diet	6 weeks	21	50% healthy, 50% high cholesterol	**Overall:** NE on BMI, waist circumference, blood pressure, or cholesterol (total, HDL, LDL). **High cholesterol:** Reduced total cholesterol (35%) **Compliance**: Reduced waist circumference (2.6%) and cholesterol (21%), NE on BMI or blood pressure

1: wet weight, 2: number of individuals consuming mycoprotein. DW= dry weight, NE= no effect, TAG= triacylglycerol, LDL= low-density lipoprotein, HDL= high-density lipoprotein. Dietary fibre content is 6% wet weight, and 25% dw. Percentage in brackets indicate percentage change. * reported that differences in taste and pleasantness were within the neutral range

7.2 Aim

To compare the propionate producing capacity of mycoprotein (and its extracted fibre) with a selection of 'propiogenic' substrates when fermented by gut bacteria *in vitro*.

7.3 Methods

7.3.1 Experimental Controls

All fermentation runs included a positive control of oligofructose (OF; Beneo P95, Mannheim, Germany) and a negative control (no fibre faecal blank).

7.3.2 Experiment 1: Fermentability and production of propionic acid from mycoprotein

7.3.2.1 *Substrates fermented were:*

- Mycoprotein, Quorn® (Marlow foods ltd, Yorkshire, UK)
- Fibersol-2, resistant maltodextrin (Matsutani Chemical Industry Ltd. Co, Hyogo, Japan).
- Oligofructose (OF/FOS) (Beneo p95, Mannheim, Germany)

Fermentation model used: 100 mg (medium) vessel, pH 6.8 media
Stool donors: 2 males and 1 female aged 23-25 (mean age 24 years), n = 3 (duplicate vials)
Time points: 0, 6, and 24 hours

7.3.3 **Experiment 2: Comparison of mycoprotein and mycoprotein fibre with other propiogenic substrates.**

7.3.3.1 *Substrates fermented were:*

- Mycoprotein, (Quorn®- Marlow Foods (Stokesley, UK))

- Mycoprotein fibre, - isolated from whole mycoprotein by a proprietary process in collaboration with Marlow Foods (Stokesley, UK) and Premier Analytical Services (High Wycombe, UK). The substrate was supplied as a dry white powder fibre extract containing > 75% fibre.
- Rhamnose (Sigma, Poole, UK)
- Laminarin (Sigma, Poole, UK)
- Oligofructose (OF/FOS) (Beneo p95)
- Inulin-propionate ester (DE = 0.8, IPE0.8) – provided by Dr Douglas Morrison

Fermentation model used: 100 mg (medium) vessel, pH 6.8 media
Stool donors: 2 males and 1 female aged 24-25 (mean age 24.7 years), n = 3 (duplicate vials)
Time points: 0, 6, and 24 hours

7.3.4 pH measurements

At each sampling time point, the pH of the fermentation fluid was measured using a pH meter (HANNA) (Chapter 2- Section 2.1.3).

7.3.5 SCFA analysis

SCFA production was analysed by GC: FID of ether extractions. This was conducted as described in Chapter 2 section 2.3

7.3.6 Statistical Analysis

Statistical analysis was conducted on IBM SPSS version 22. Tests of normality was analysed by the Shapiro Wilks test. One-way ANOVA with post hoc Bonferroni, or Students t-test were used when normally distributed and Kruskall Wallis, with post hoc Dunn Bonferroni or Mann Whitney U test when normal distribution was not present as required.

7.4 Results

7.4.1 Experiment 1: Fermentability and production of propionic acid from mycoprotein

7.4.1.1 *pH*

Using pH as a marker of fermentation, mycoprotein was less well fermented than fibersol and oligofructose, and was similar to the control (Table 7-2).

Table 7-2: pH changes after fermentation of mycoprotein in comparison to fibersol and oligofructose.

	0h	6h	24h
Control	6.70 (0.2)	6.71 (0.18) †	6.76 (0.09) †
Mycoprotein	6.57 (0.23)	6.44 (0.34) †	6.26 (0.18) †
Fibersol	6.66 (0.24)	4.62 (0.31)	4.34 (0.1)
Oligofructose	6.65 (0.25)	3.79 (0.3)	3.42 (0.34)

Median (IQR), n=3.
Between substrate, analysis used Kruskal Wallis with pairwise comparison and differences vs Oligofructose indicated by †.

7.4.1.2 *Pattern and amount of SCFA production*

Acetate

At 6 hours, mycoprotein fermentation resulted in much lower acetate production compared with oligofructose and fibersol, producing only 26% of that produced by oligofructose ($p<0.001$; Table 7-3, Figure 7-1, Figure 7-2). After 24 hours of fermentation, acetate production from mycoprotein was 73.5% less than from oligofructose fermentation ($p<0.001$, Table 7-3).

When considered as molar proportions, mycoprotein generated a significantly lower percentage of acetate compared with oligofructose (62.9 [6.9] % vs. 88.3 [2.7] %, $p<0.01$) and fibersol (62.9 [6.9] % vs. 73.1 [9.5] %, $p<0.05$) at 6 hours. At 24h hours 50.1 [5.5] % of SCFA produced by mycoprotein fermentation formed acetate, this was lowest of the all substrates tested and was significantly less than acetate proportion with oligofructose fermentation (89.9 [1.0] %, $p<0.001$).

Table 7-3: 6 and 24 hour acetate concentration and proportion

	Concentration (mmol/l)		Molar proportion (%)	
Time	**6 hours**	**24 hours**	**6 hours**	**24 hours**
Control	5.28 (1.87)†	6.77 (0.76)†	67.6 (3.8)†	66.7 (19.3)
Mycoprotein	7.85 (2.80)†	13.36 (1.55)†	62.9 (6.9)†	50.1 (5.5)†
Fibersol	25.77 (4.0)†*	33.35 (11.04)	73.1 (9.5)	64.3 (11.5)
Oligofructose	35.05 (1.54)*	50.05 (11.23)*	88.3 (2.7)*	89.9 (1.0)*

Median (IQR), n=3. * vs mycoprotein, † vs oligofructose. Concentration = ANOVA and post hoc Bonferroni, Molar Proportions = Kruskal Wallis and post hoc Dunn Bonferroni

Propionate

After 6 hours of fermentation, propionate production from mycoprotein and fibersol was similar and much higher than from oligofructose, which produced 43% less propionate than from mycoprotein (Table 7-4, Figure 7-1, Figure 7-2).

After 24 hours, mycoprotein and fibersol produced the highest amount of propionate (and highest molar proportion) of all the substrates tested (7.22 [1.2] mmol/l and 6.41 [2.53] mmol/l respectively), which was significantly greater than the control and oligofructose ($p<0.001$).

Proportionally, after 6 hours of fermentation propionate production by the control and mycoprotein were similar. This did not occur after 24 hours with propionate proportion from mycoprotein (27.2 [3.2] %) was greater than that of the control – albeit not significantly so ($p = 1.0$). Propionate molar proportions with oligofructose did not vary over time, leading to ~3.5% propionate; this was significantly less than the propionate proportion from mycoprotein ($p<0.001$, Table 7-4).

Table 7-4: 6 and 24 hour propionate concentration and proportion

	Concentration (mmol/l)		Molar proportion (%)	
Time	**6 hours**	**24 hours**	**6 hours**	**24 hours**
Control	1.09 (0.45)	1.85 (1.54)*	16.1 (3.2)†	17.9 (9.1)†
Mycoprotein	2.68 (0.76)	7.22 (1.20)†	16.2 (7.6)†	27.2 (4.0)†
Fibersol	3.49 (0.47)†	6.41 (2.53)†	10.1 (0.6)	12.1 (3.2)
Oligofructose	1.53 (0.19)	1.93 (0.46)*	3.8 (0.3)*	3.4 (0.3)*

Median (IQR) n=3. * vs mycoprotein, † vs oligofructose. Concentration = ANOVA and post hoc Bonferroni Molar Proportions = Kruskal Wallis and post hoc Dunn Bonferroni

Butyrate

The 6 hour butyrate production from mycoprotein was significantly less than production by fibersol (2.7 [0.2] mmol/l vs 6.3 [1.6] mmol/l, $p<0.022$; Table 7-5 , Figure 7-1, Figure 7-2). Fibersol also led to greater production of butyrate compared with oligofructose increasing by 47% after 6 hours ($p= 0.041$) and 91% after 24 hours ($p=0.013$, Table 7-5). Although not significant, mycoprotein fermentation led to the formation of 90% more butyrate than oligofructose after 24 hours of fermentation.

Proportionally, oligofructose led to the lowest molar proportion of butyrate, which was significantly less than all other substrates after 6 hours of fermentation (Table 7-5). For example, mycoprotein formed 20.4 (0.9) % butyrate versus 8.1 (1.0) % produced by oligofructose ($p<0.05$). After 24 hours mycoprotein and fibersol led to similar proportions of butyrate (~23%), which was significantly greater than the butyrate molar proportion after oligofructose fermentation (6.8 [1.0] %, $p<0.05$).

Table 7-5: 6 and 24 hour butyrate concentration and proportion

	Concentration (mmol/l)		Molar proportion (%)	
Time	**6 hours**	**24 hours**	**6 hours**	**24 hours**
Control	1.44 (0.86)	3.91 (0.28)	18.3 (2.9)†	15.4 (10.5)
Mycoprotein	2.72 (0.23)	6.44 (1.32)	20.4 (0.9)†	23.5 (4.8)†
Fibersol	6.26 (1.59) †*	7.04 (7.43) †	16.6 (10.8)†	22.0 (13.0)†
Oligofructose	3.32 (0.73)	0.62 (0.66)	8.1 (1.0)*	6.8 (1.0)*

Median (IQR). * vs mycoprotein, † vs oligofructose. Concentration = ANOVA and post hoc Bonferroni, Molar Proportions = Kruskal Wallis and post hoc Dunn Bonferroni test.

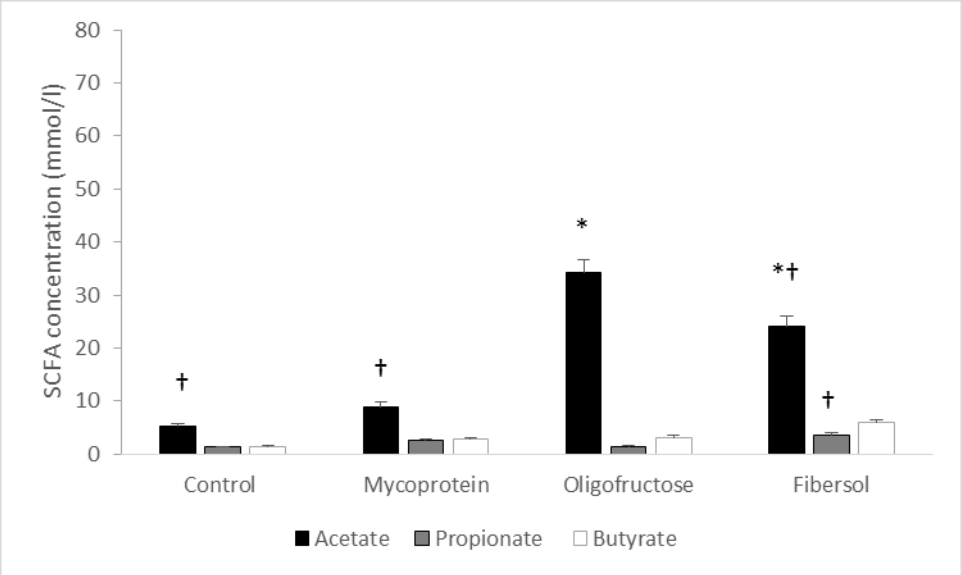

Figure 7-1: SCFA production after 6 hours of fermentation.
Mean + SEM. * indicates significant differences against mycoprotein and † indicates significant differences against oligofructose $p<0.05$.

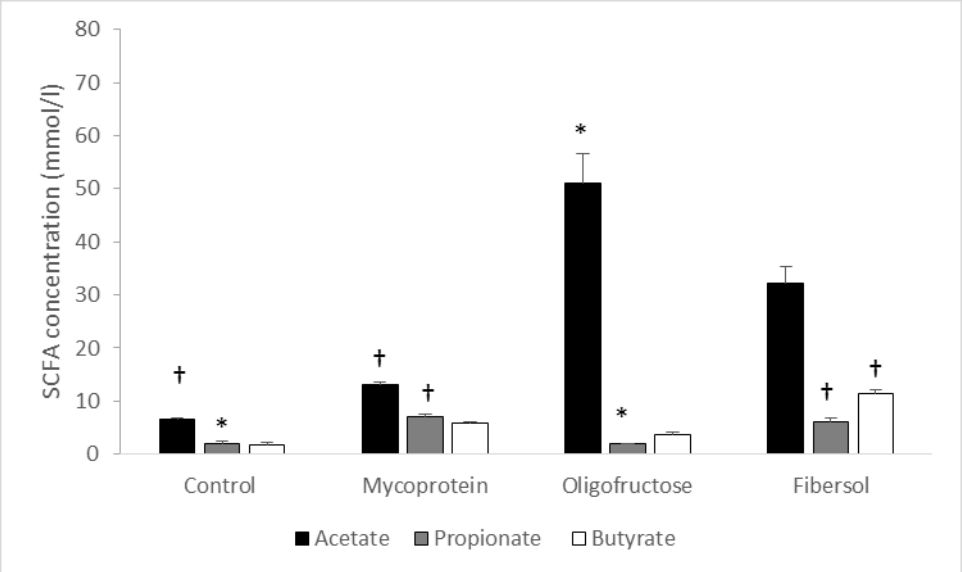

Figure 7-2: SCFA production after 24 hours of fermentation.

Mean + SEM. * indicates significant differences against mycoprotein and † indicates significant differences against oligofructose, $p<0.05$.

7.4.2 Experiment 2: Mycoprotein compared with mycoprotein fibre.

To explore the propiogenic properties of the fibre component of mycoprotein, SCFA production from mycoprotein and mycoprotein fibre was compared with that from the 'propiogenic' substrates; rhamnose and laminarin, as well as oligofructose and the synthetic inulin-propionate ester 0.8 (IPE0.8).

7.4.2.1 *pH*

After 6 hours of fermentation mycoprotein and mycoprotein fibre did not greatly reduce in pH, with both decreasing by less than 0.5 pH units, and did not differ from the control. In contrast, the pH for oligofructose reduced by 2.82 pH units and was significantly lower than mycoprotein and mycoprotein fibre ($p<0.01$ for both).

After 24 hours fermentation the pH of the control, mycoprotein and IPE 0.8 did not differ. Unlike after 6 hours of fermentation the mycoprotein fibre (4.94 [0.63]) had a significantly reduced pH from the control (6.54 [0.24]) after 24 hours of fermentation ($p=0.022$) suggesting that it was slowly, but well fermented (Figure 7-3).

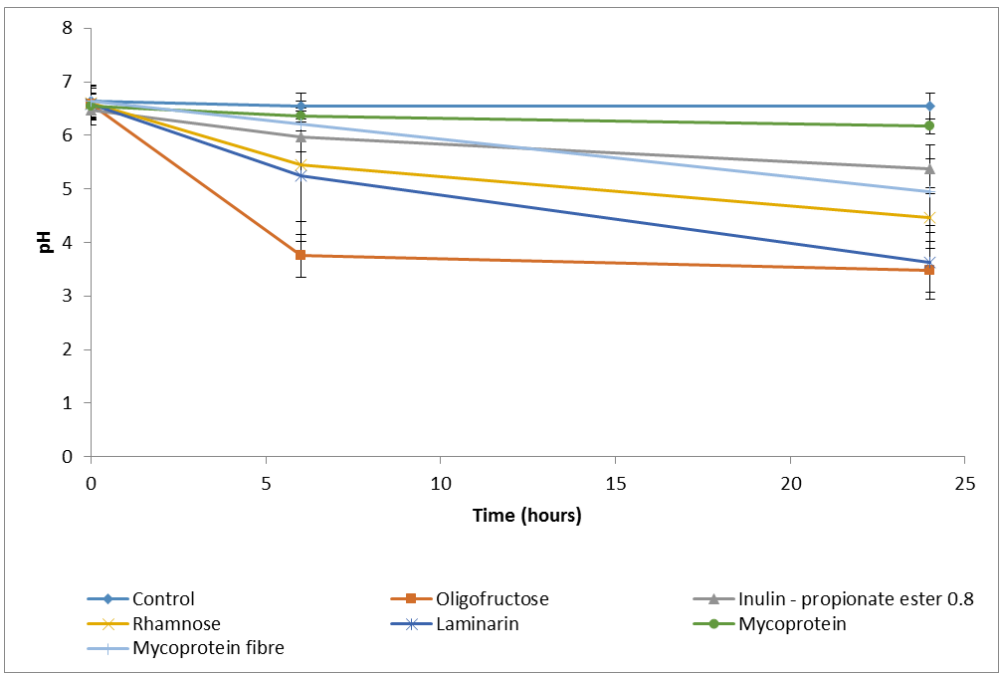

Figure 7-3: Change in pH over 24 hours of fermentation
Mean +/- Standard deviation, N=3. Time points measured are 0, 6, and 24 hours. Significant differences are discussed within text.

7.4.2.2 *SCFA production*

Acetate

At 6 hours mycoprotein fibre yielded the second highest concentration of acetate (15.1 [3.0] mmol/l), although this did not significantly differ from the other substrates tested (Figure 7-4, Figure 7-5). By 24 hours, mycoprotein fibre generated significantly more acetate (37.6 [10.3] mmol/l) than the control (6.5 [1.2] mmol/l, $p< 0.01$), IPE 0.8 (11.3 [2.4] mmol/l, $p=0.035$), and mycoprotein (6.4 [0.8] mmol/l, $p=0.045$). Oligofructose remained the highest producer of acetate generating 46.5 (4.9) mmol/l.

Propionate

As expected, IPE0.8 yielded the highest rate of propionate concentration at both 6 and 24 hours yielding 36.7 (2.2) and 37.4 (3.1) mmol/l which was significantly higher than all other substrates tested ($p<0.01$). Propionate production by mycoprotein and its extracted fibre was similar after 6 hours of fermentation, yielding ~2.5 mmol/l. After 24 hours, mycoprotein fibre generated approximately double the amount of propionate compared to the mycoprotein (12.3 [3.0] vs 6.4 [0.5], Figure 7-4, Figure 7-5). Rhamnose also generated high concentrations of propionate yielding 8.0 (3.0) mmol/l at 6 hours and 23.2 (1.3) mmol/l at 24 hours was significantly larger than all substrates except after 24 hours $p<0.05$.

This was not a significant increase in propionate ($p=0.848$), which may be due to variation as 'participant 2' did not rank high for SCFA production with mycoprotein fibre (see below). In opposition to findings within Chapter 4, laminarin yielded lower concentrations of propionate when tested at 6, and 24 hours, and production was only greater than that of the control and oligofructose. For example, after 6 hours, laminarin generated (2.0 (0.3) mmol/l) of propionate which was only greater than the control (1.3 [0.0] mmol/l) and oligofructose 1.2 [0.2] mmol/l), although this was not significant. After 24 hours laminarin produced 4.7 (1.64) mmol/l of propionate only surpassing the control (2.2 [0.1] mmol/l) and oligofructose (1.3 [0.1] mmol/l), although this was not significant (Figure 7-4, Figure 7-5).

Butyrate

In contrast, laminarin generated the highest rate of butyrate after 6 (6.5 [2.7] mmol/l) and 24 hours (18.4 [5.5] mmol/l). After 24 hours butyrate production significantly greater than all substrates tested ($p<0.05$) except for mycoprotein (6.3 [0.3] mmol/l, $p=0.072$) and mycoprotein fibre (11.3 [3.0] mmol/l, $p=1.0$). Butyrate production was the lowest for oligofructose (2.29 [0.6] mmol/l, Figure 7-4, Figure 7-5).

Total

All substrates tested were fermented, and all substrates (except for mycoprotein) resulted in significant increases in total SCFA production, $p<0.05$. Laminarin and mycoprotein generated the highest concentrations of total SCFA which was significantly higher than mycoprotein (laminarin vs mycoprotein: 64.1 [9.0] mmol/l vs 24.9 [1.7] mmol/l, $p=0.044$, mycoprotein fibre vs mycoprotein: 61.2 [15.7] mmol/l vs 24.9 [1.7] mmol/l, $p=0.078$).

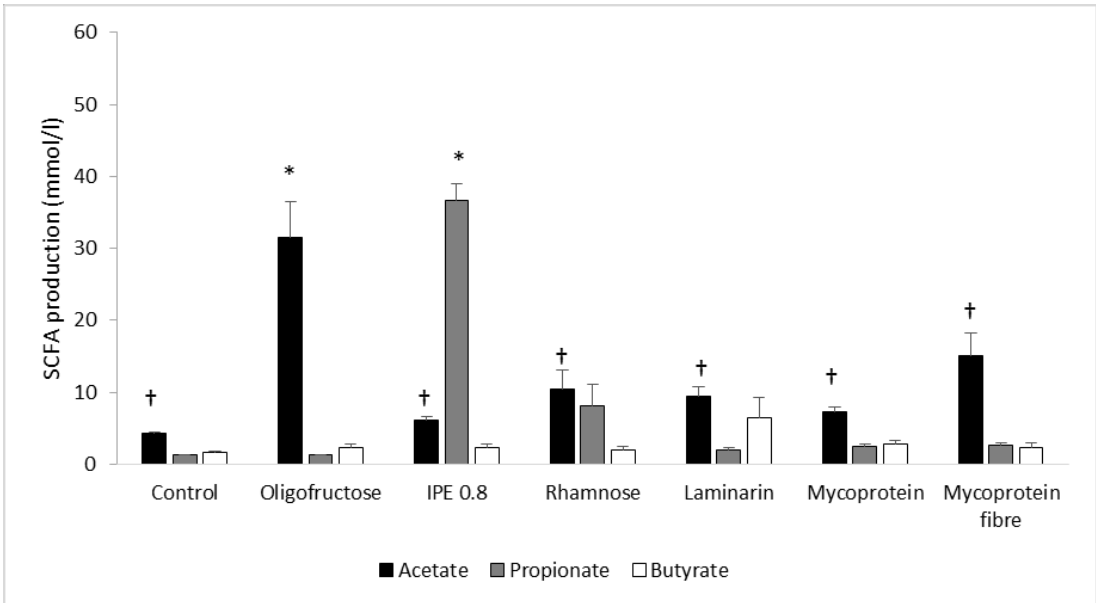

Figure 7-4: Acetate, propionate and butyrate production after 6 hours of fermentation (mmol/l)

Data presented is mean + standard deviation, n= 3. Statistical significant differences are shown by * vs mycoprotein fibre and † vs FOS $p<0.05$. IPE0.8 vs all for propionate $p<0.001$. IPE0.8 = inulin propionate ester 0.8.

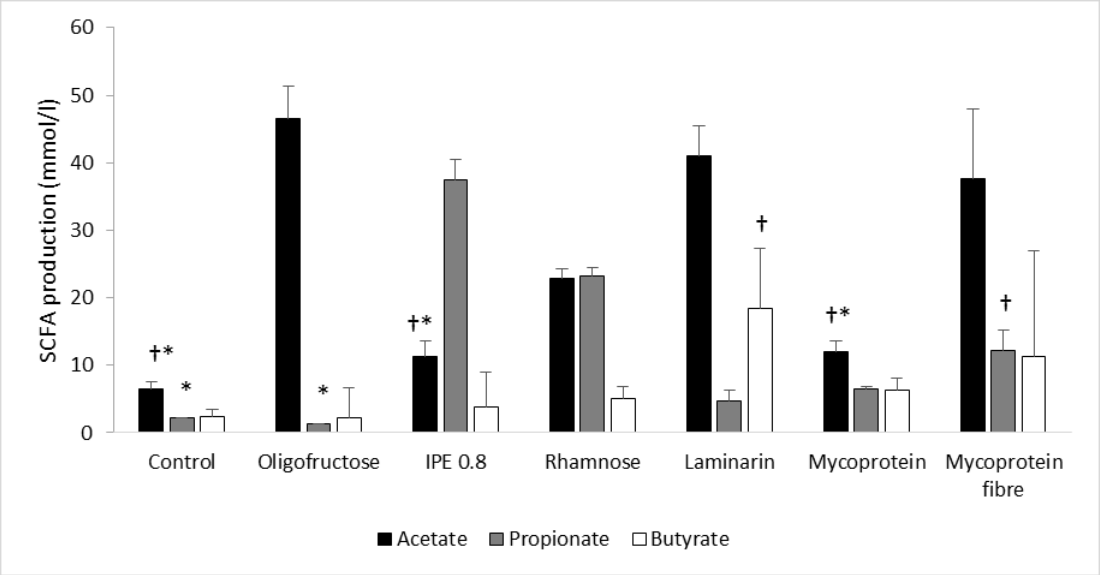

Figure 7-5: Acetate, propionate and butyrate production after 24 hours of fermentation (mmol/l)
Mean + standard deviation, n= 3. Statistically significant differences are shown by * vs mycoprotein fibre and † vs FOS $p<0.05$. IPE0.8 and rhamnose vs all for propionate $p<0.001$. IPE0.8 = inulin propionate ester 0.8.

7.4.2.3 *Molar proportions*

The proportions of propionate and butyrate produced with mycoprotein and mycoprotein fibre were similar, with mycoprotein producing acetate: propionate: butyrate in the approximate ratio 50:25:25 and mycoprotein 61:20:18 (Table 7-6). The molar proportion of acetate at 24h was highest for oligofructose with 92.6 (2.8) % of total SCFA. Mycoprotein fibre and laminarin both generated similar proportions of acetate yielding approximately 63% acetate, whereas both mycoprotein and rhamnose yielded approximately 46% of acetate. Rhamnose had the highest molar proportion of propionate (45.33 [1.95] %, except for the inulin ester IPE 0.8; 71.4 [2.1] %, $p<0.001$). Unexpectedly laminarin led to a significantly lower proportion of propionate than all other substrates except FOS ($p<0.01$). Laminarin generated the highest proportion of butyrate (28.0 [10.0] %), which was similar to that of the control and mycoprotein.
$p<0.01$.

7.4.2.4 *Ranked production*

The ability of the individual stool donors to produce acetate, propionate and butyrate from each substrate after 24 hours of fermentation was ranked (Table 7-7). The rank for acetate production varied between stool donors with each participant having different 'top' acetate substrates. The bottom three substrates of acetate for all participants were mycoprotein, IPE0.8 and the control. Ranked production of propionate was very similar for all individuals with IPE0.8, rhamnose and mycoprotein fibre ranking 1st, 2nd and 3rd for production. Mycoprotein, ranked 4th for participant 1 (p1) and participant 2 (p2) and 5th for stool donor 3 (p3). At all time points, oligofructose and the control ranked as the lowest producers for both propionate and butyrate. Laminarin ranked highest for butyrate with all participants. Mycoprotein fibre ranked 2nd for p1 and p3, and ranked 4th for p2.and was higher than mycoprotein except for p2.

Table 7-6: Molar proportions (%) of acetate, propionate and butyrate after 24 hours of fermentation

	Control	FOS	IPE0.8	Rhamnose	Laminarin	Mycoprotein	Mycoprotein Fibre
Acetate	56.91 (6.33)^{c}	92.57 (2.82)^{b}	20.94 (4.65)^{a}	44.77 (3.13)^{c}	65.04 (11.51)^{c}	48.31 (6.53)^{c}	61.36 (4.17)^{c}
Propionate	20.12 (2.57)^{a}	2.57 (0.34)^{b}	71.44 (2.07)^{d}	45.33 (1.95)^{c}	6.95 (2.48)^{b}	25.99 (3.51)^{a}	20.25 (1.28)^{a}
Butyrate	22.97 (3.88)^{c}	4.86 (2.72)^{b}	7.63 (2.58)^{b}	9.90 (2.70)^{bc}	28.02 (10.04)^{ac}	25.70 (3.11)^{c}	18.39 (5.18)^{abc}

Mean (standard deviation). FOS = oligofructose, IPE0.8 = inulin propionate ester 0.8.
Difference letters within rows indicate significant differences.

Table 7-7: Ranked production of acetate, propionate, and butyrate by each stool donor

	Acetate			Propionate			Butyrate		
Rank	**P1**	**P3**	**P3**	**P1**	**P3**	**P3**	**P1**	**P2**	**P3**
1	FOS	Laminarin	Mycoprotein fibre	IPE 0.8	IPE 0.8	IPE 0.8	Laminarin	Laminarin	Laminarin
2	Laminarin	FOS	FOS	Rhamnose	Rhamnose	Rhamnose	Mycoprotein fibre	Mycoprotein	Mycoprotein fibre
3	Mycoprotein fibre	Mycoprotein fibre	Laminarin	Mycoprotein fibre	Mycoprotein fibre	Mycoprotein fibre	Mycoprotein	Rhamnose	Mycoprotein
4	Rhamnose	Rhamnose	Rhamnose	Mycoprotein	Mycoprotein	Laminarin	Rhamnose	Mycoprotein fibre	Rhamnose
5	Mycoprotein	Mycoprotein	IPE 0.8	Laminarin	Laminarin	Mycoprotein	IPE 0.8	IPE 0.8	IPE 0.8
6	IPE 0.8	IPE 0.8	Mycoprotein	Control	Control	Control	FOS	FOS	Control
7	Control	Control	Control	FOS	FOS	FOS	Control	Control	FOS

P1 = stool donor 1, P2 = stool donor 2, P3= stool donor 3. Rank number 1 = top producer, Rank number 7 = bottom producer

7.5 Discussion

It has been demonstrated that mycoprotein, the commercial meat replacement product, known commercially as Quorn® and its extracted fibre are both well fermented and produce SCFA *in vitro*. Initially this was unexpected as the pH for mycoprotein barely dropped over the 24-hour duration of the fermentation. Although mycoprotein did not generate high yields of SCFA compared to the other substrates tested, the SCFA appeared to lead preferentially to the production of propionate. This was demonstrated by ~27% of total SCFA produced by mycoprotein forming propionate. Mycoprotein fibre on the other hand was highly fermentable and produced similar concentrations of SCFA to laminarin (Deville, 2007), and oligofructose (De Preter et al., 2010), which are both considered as highly fermentable dietary fibres.

However, it needs to be noted that the mycoprotein did not undergo a pre-digestion step, or a freeze-drying step. As a result, the fat and protein, which would not reach the colon after consumption were also available for fermentation in the batch culture systems. As only approximately 6% of the mycoprotein (as fibre) would reach the colon for fermentation indicating that protein fermentation may have occurred.

Propionate production from mycoprotein fibre was 61.5 μmoles/75.1 mg fibre (production in 5 ml, and based on the extracted fibre having >75% fibre; personal communication with Premier Analytical Services). Based on this it was likely that only 4.9 μmoles propionate was produced from the 6 mg (6% of 100 mg) of fibre in the mycoprotein. However when considering the propionate production from (whole) mycoprotein, 31 μmoles/ 100 mg propionate were produced within fermentation the fermentation vial (5 ml). This is over 6x more than that would have been expected from the fibre alone.
As mycoprotein also consists of 11% protein and 9% carbohydrate, it is likely that these are also contributing to the propionate produced. Mortensen et al., (1990) fermented a selection of amino acids and identified that they lead to the production of SCFA, although this was to a lesser extent than BCFA. During the fermentation of the protein within the mycoprotein, it is likely that low concentrations of ammonia and hydrogen sulphide, which occur as a result of bacterial fermentation of proteins were produced (Walker et al., 2005, Magee et al., 2000). As both of these are alkali, this could have led to buffering the pH in the system, preventing a reduction in pH.

High proportions of propionate (and butyrate) as a result of mycoprotein fibre are likely due the β(1-3), β(1-4) and β(1-6) glucose linkages within the mycoprotein fibre. These bonds types have previously been associated with the selective increase in the production of propionate and butyrate *in vitro* (Queenan et al., 2007, Hughes et al., 2008, Deville, 2007). Although few studies have looked at the fermentability of chitin, SCFA have been observed from the fermentation of mushroom sclerotia, this along with the presence of β-bonding suggests that chitin is also likely to selectively increase propionate and butyrate.

Feeding of chitosan (derived from chitin), and using the ruminal fluid and faeces of sheep generated significantly increased proportions of propionate and an increased propionate-acetate ratio in the rumen (0.17 vs 0.22, p=0.007) and faeces (0.13 vs 0.15, p=0.045). Chitosan consumption had no effect of total ruminal SCFA but reduced faecal SCFA (19.4 to 13.4 mmol/l, p=0.01). *In vitro* fermentation of starch ruminal fluid from both groups also demonstrated similar effects with no effect on total production and increased propionate proportion and ratio. Fermentation with cellulose resulted in the control yielding more propionate than the chitosan after the fermentation of cellulose but total production was significantly reduced (48.98 vs 35.30 mmol/l, p=0.001) (Goiri et al., 2009).

Further indications that these effects discussed in Table 7-1 of mycoprotein may occur via SCFA production have been found in other fungi. For example in *in vitro* batch fermentations of *Polyporous rhinoceros* and *Wolfiporia cocos,* both consisting of β(1-3) linked glucoses have been performed. Fermentation of these fungi resulted in 27% propionate production (of total SCFA), This was particularly apparent for *W. cocos* which yielded the most SCFA of the mushrooms tested, as well as the most propionate production (Wong et al., 2005). Similar effects were also observed when a selection of β-glucans, including mushroom sclerotia (*Pleurotus rhinoceros*) consisting of β(1-3), β(1-4) and β(1-6) bonding were fermented *in vitro* after the addition of different strains of bifidobacteria. Propionate production of the mushroom sclerotia was similar to other β-glucans tested, some of which are associated with the increased production of propionate (barley and seaweed) and was significantly higher than inulin, when fermented *in vitro* along with *Bifidobacterium infantis* (Zhao and Cheung, 2011).

Other substrates were fermented along with the mycoprotein and mycoprotein fibre, and in many cases 'propiogenic' substrates were selected. This enabled the comparison of multiple propionate producing fibres. In the initial fermentations with the mycoprotein alone, rate proportions of propionate were similar to that of fibersol, which been demonstrated to increase propionate production *in vitro* (Laurentin and Edwards, 2004). Mycoprotein and its extracted fibre were also compared with rhamnose, a monosaccharide sugar, and laminarin, a derivative of seaweed both of which lead to the increased production of propionate *in vitro* (Deville, 2007, Gietl et al., 2012). Rhamnose production from propionate exceeded that of mycoprotein, and the mycoprotein fibre. In contrast, laminarin production favoured butyrate and the mycoprotein produced more propionate. The propionate production by these fibres was dwarfed by that of the synthetic substrate where propionate is conjugated to inulin (IPE 0.8). This fibre directly delivers propionate to the colon leading to downstream propionate associated effects, including a reduction in weight gain (Chambers et al., 2014). Although the IPE 0.8 produces high concentrations of propionate, the mycoprotein fibre showed potential in terms of propionate production. For example, the mycoprotein fibre consistently ranked 3rd for propionate production (1st = IPE 0.8, 2nd rhamnose). Proportionally, mycoprotein and its extracted fibre generated increased amounts of propionate, (mycoprotein; 26.0 [3.5] % and the extracted fibre (20.3 [1.3] %) which was only exceeded by IPE 0.8 (71.4 [2.1] %) and rhamnose (45.3 [2.0] %). It was also observed that for mycoprotein and mycoprotein fibre the increased propionate was at the cost of acetate, a SCFA profile which has been shown to be beneficial for cholesterol regulation (Wolever et al., 1991).

Other advantages of mycoprotein include the fact that it is currently mass-produced within the UK, and is easily accessible to the general public. This therefore may be an effective avenue to increase the production of propionate within a standard diet as well as being beneficial for regulation of cholesterol and energy intake. The increased propionate observed with mycoprotein, and in particular, the extracted fibre may be involved in the increased feelings of satiety and energy intake that were also observed in feeding studies (Table 7-1) (Turnbull et al., 1990, Turnbull et al., 1993b, Turnbull et al., 1994, Turnbull and Ward, 1995).

This likely occurred as 25% of the dry weight of mycoprotein is fibre, and an 80g dose provides 5 g of fibre. The extracted fibre ranked high for propionate production,

proportion, and total fibre concentrations were not dissimilar from other highly fermentable substrates tested. This, and the observation that consumption of IPE 0.8 reduced energy intake and weight gain (Chambers et al., 2014) indicates that the effects on satiety and energy intake observed with mycoprotein are possibly due to colonic propionate production by fermentation of the mycoprotein fibre. The production of propionate by the ester and the mycoprotein could be compared. This is as 10 g IPE, produced 2.65 g propionate, which is the equivalent of 36.23 mmoles. Within this assessment, 100 mg of mycoprotein fibre yielded 61.25 μmoles of propionate, which is 612.5 μmoles per gram of fibre. Based on this, to achieve the same amount of propionate as the IPE, approximately 60 g of mycoprotein fibre would be required. Mycoprotein fibre yielded approximately 10x the amount of propionate as oligofructose, and based on the study by (Pedersen et al., 2013), <10 g of mycoprotein fibre would likely achieve appetite regulation. Indicating that although mycoprotein is beneficial to selectively increase propionate production compared to oligofructose, it remains to be seen if it can induce a similar effect on appetite regulation as seen IPE, within similar dose ranges.

Although protein was within the mycoprotein it is unlikely that the effects occurred as a result of the protein fermentation as when protein content was matched; differences in energy intake were observed indicating the effects are likely due to the fibre content (Turnbull et al., 1993b). Based on this it would be interesting to observe the effects of consumption of mycoprotein and/or its extracted fibre on the production of propionate. In conclusion, it has been demonstrated that mycoprotein and its fibre are propiogenic. This capacity to produce propionate as well as butyrate may go to some way to explain the ability of mycoprotein to reduce cholesterol. Although further investigations into the ability of mycoprotein to increase propionate and butyrate after consumption is required, mycoprotein, marketed as Quorn®, is easily available within the UK could be used tool to potentially increase propionate within the diet.

Chapter 8 General Discussion

What is becoming more and more apparent is the importance of the gut microbiota, and their metabolites in human health. Previously, the role of the colon was thought to be solely for the absorption of water and salts. The advent of improved anaerobic and molecular techniques has shown that the colon is a diverse microbial ecosystem consisting of over 150 different species and has a symbiotic relationship with the human host providing selective advantage (Qin et al., 2010). It has long been known that gut microbial fermentation is important for the generation of energy by ruminants (Sunvold et al., 1995), but the role of the NDC fermentation in humans and the consequences on SCFA production of is much less well understood.

As discussed in the general introduction (Chapter 1), the main SCFA are acetate, propionate, and butyrate. These have a selection of roles in health including in the mediation of cancer, gluconeogenesis, cholesterol synthesis and the satiety response. This satiety response, at least in part, likely occurs via the release of the satiety hormones PYY and GLP-1 mediated by the receptors FFAR2 and FFAR3. These receptors also have other roles including the production of hormones not only for satiety, but also leptin for the regulation of adipogenesis which has also been postulated to have regulatory effects in inflammation. Leptin also alters signals in the brain as it can cross the blood - brain barrier. Propionate, in particular may be an important signalling molecule responsible for many of these effects, it is the most potent activator of both FFAR2 and FFAR3 (den Besten et al., 2013b, Byrne et al., 2015).

The ratio of acetate to propionate is also of importance. In the recent study by Perry., et al (2016) assessing colonic acetate production when feeding rats a high fat diet, identified that increasing acetate, led to increased body weight, insulin secretion, and fatty acid production (Perry et al., 2016). In contrast propionate has the opposite effect to acetate, where supplementation has been shown to reduce weight gain, reduced glycaemic response and reduced adipose tissue (Chambers et al., 2014). The importance of the role of the acetate to propionate ratio was also identified in earlier studies where propionate was shown to compete with acetate by preventing acetate mediated FA synthesis in a dose dependent manor *in vitro* (Wright et al., 1990). Infusion studies have also demonstrated the inhibition of HMG-CoA reductase by propionate, thus preventing cholesterol synthesis (Wolever et al., 1991). Therefore, although acetate is consistently the most abundant

SCFA produced, reducing the acetate to propionate ratio, by increasing colonic concentrations and proportions of propionate is potentially beneficial.

8.1 Functional foods targeting selective SCFA production

Prebiotics are substrates which are fermented in the colon to selectively stimulate beneficial bacteria (Gibson and Roberfroid, 1995). The prebiotic capability of many different substrates have been assessed, but the most commonly assessed are inulin and oligofructose (although there are others) (Roberfroid et al., 2010). *In vitro* inulin has been associated with increased *Bifidobacterium* spp such as *B.longum, B.infantis, B.adolscentis* but inhibited *E.coli* and *C.perfringens* (Wang and Gibson, 1993). These species along with a number of lactobacilli species (along with many others) are also considered to be probiotics. Consumption of these substrates is also associated with a number of positive effects. Bouhnik et al., (1999), identified increased faecal bifidobacteria after consumption of oligofructose. What is also of interest is that oligofructose has other health benefits. For example, high doses of oligofructose increase feelings of satiety, and concentrations of the appetite hormones PYY and GLP-1 (Pedersen et al., 2013). The SCFA, and in particular propionate, are associated with the release of these hormones (Lin et al., 2012), which is one of the beneficial roles of these SCFA. Other roles of propionate also include gluconeogenesis, regulation of fatty acid and cholesterol synthesis (Chapter 1) (den Besten et al., 2013b). These SCFA are produced when NDC reach the colon and are utilised by the colonic bacteria. The colon is an ecosystem which has the capacity to metabolise a vast array of substrates, providing sustenance for the colonic bacteria, and generating SCFA as terminal reduced products. Different species are able to use the substrates to produce different SCFA and SCFA production profile can alter depending on the dietary source available. The ability of the colonic bacteria to be modified by diet has been demonstrated in a number of studies (David et al., 2014, O'Keefe et al., 2015). O'Keefe et al., (2015), fed African Americans a native African diet (high fibre, low fat), and native Africans an American diet (low fibre, high fat). The switch to a high fibre diet increased faecal SCFA production, as well as increased the bacterial diversity, compared to the high fat diet. Similar effects were also seen in the study by David et al., (2014) where diet was changed from a standard diet to a plant or animal based diet, changes in the bacteria such as increases in *Roseburia, E.rectale*, and *R.bromii* were associated with increases in SCFA production. This ability to adapt can also be used to manipulate the colonic bacteria. A

prime example of this is resistant starch, which is fermented by many species such as *E.rectale,* and *R.bromii* resulting in the production of butyrate.

It is important to consider that fibres are not eaten in isolation but more as a mixture, this may influence their impact on the microbiota, and SCFA produced. For example, consuming porridge and an apple for breakfast exposes the colonic bacteria to oat β-glucan with β(1-3), β(1-4) linked glucoses from the porridge and pectin with from the apple consisting of α(1-4) linked galacturonic acids. Therefore, for the colonic bacteria to survive they need to adapt so that they can utilise these energy sources. This can be demonstrated from *in vitro* studies where β-glucans are associated with increased populations within the clostridia cluster IX group, *Bacteroides-prevotella* group*,* and the *Clostridium histolyticum* subgroup, all of which are associated with the production of propionate (Hughes et al., 2008). Pectin on the other hand has also been associated with increased populations of *B.thetaiotaomicron* (Dongowski et al., 2000) which, along with previous studies suggests that *B.thetaiotaomicron* can use many different substrates demonstrating the adaptability of the bacteria within the colon (Martens et al., 2011). Thus, eating pectin and oat β-glucan may result in different populations of bacteria than each eaten on its own.

This is a feature that can be exploited for functional foods. If the diet could be used to optimise the microbiome so that the ecosystem selectively increased the production of propionate would be advantageous. This could occur by manipulating which bacteria are present and their functions, or ensuring that the bacteria present have the appropriate enzymes to utilise the substrates to produce propionate directly, or by interconversion from a different substrate.

8.2 Increasing colonic propionate

Human feeding trials where propionate is added directly to food have been problematic, particularly in the study of appetite regulation. Propionate is absorbed in the small intestine and does not reach the colon. Independently of this, addition of propionate to directly foods leads to feelings of satiety and effects on glucose tolerance are often attributed to the poor organoleptic properties of the food product (Darzi et al., 2012). To date- the one way to selectively deliver propionate to the colon is via the recently developed inulin-

propionate ester, in which propionate is delivered to the colon through conjugation to inulin. This inulin-propionate ester has been shown to suppress appetite, reduce weight gain, and reduce intra-abdominal adipose tissue in overweight individuals with elevated hepatic fat (Chambers et al., 2014). Although this is beneficial in overweight individuals, positive effects in healthy individuals are less clear and may revolve around the prevention of obesity-related metabolic phenotypes. The identification of a natural (non-synthetic) food source as a functional food, which might also selectively promote propionate production in the colon, would also be advantageous for carrying out long-term feeding trials, which could also have very high consumer acceptance.

Identifying 'propiogenic' foods or food ingredients has been hindered by the paucity of techniques to assess colonic production of SCFA within the literature. Selecting NDCs for feeding trials and mechanistic analysis is difficult due to a lack of systematic assessment of the properties which selectively increase the production of propionate. In order to assess the drivers of propionate production a multipronged approach was adopted. The overall aim of this thesis to be a systematic and step-wise approach to identify which factors are identifiable that lead to the preferential production of propionate.

During this PhD, the aim was to try to tease out the ideal composition of NDC to selectively elevate the production of propionate *in vitro*. This was carried out in a step-wise fashion:

1. A systematic review of the literature to identify any previously identified propiogenic substrates
2. Screening of selection of potentially propiogenic substrates
3. Assessing the effect of specific bonding on propionate production

8.3 Assessment of the literature

The aim of the systematic review of *in vitro* batch fermentations was to identify NDC which could be targeted for further analysis to elucidate why it was propiogenic. Unfortunately, many issues became apparent when carrying out this analysis. The lack of uniformity of methodologies and units provided made comparisons difficult. Due to this limitation, a rate term (mmol/g CHO/day) was developed to enable the comparison of these investigations. This somewhat enabled the SCFA producing capabilities of the

substrates tested to be evaluated and compared side-by-side. The rate term was beneficial as the ratio is often used for comparisons of different studies, a unit that was identified to be misleading, as it does not take into account the amount of propionate produced, just a proportion, i.e. 50% of four is the same as 25% of eight.

Within the non-pooled studies, no NDC stood out as being propiogenic (based on rate), for example, the top three producers were a monosaccharide or disaccharide sugar. Differences were found with the pooled data where β-glucans were identified to be the highest propionate producers. In contrast, pectin and guar gum were seen to produce high proportions of propionate, based on ratio. Differences in the pooled and non-pooled data could have occurred for many reasons, for example rhamnose which is deemed as highly propiogenic (Vogt et al., 2004b), did not have enough data for analysis within the pooled data and was not included. Pooling may have reduced inter-individual variation which could have down-stream effects on propionate production. Although differences did occur as a result of pooling and non-pooling data, each group had over 50 studies for comparison, providing a good overview of the literature. These studies where compared separately due to potential effects of combining bacteria. As discussed in Chapter 3, when stool samples are pooled the bacteria within the samples are likely to interact with each other and a dominant ecosystem is likely to prevail. This has not only been shown in faecal transplantation (Fuentes et al., 2014), but also *in vitro* where the bacteria of individual stool samples and the combined pool were compared (Aguirre et al., 2014). Aguirre et al., (2014) identified that the bacterial composition of the individuals was different compared to the pool, and that these differences were not uniform for all individuals. This was exemplified by the change of *Roseburia* populations in which one individual has a 63.5 fold change, and another had a 2 fold change compared with the pool. It was reported that this did not translate to differences in the SCFA production, however this was using the TIM-2 models and not batch fermentations. This is also in contrast to what was observed when the variability between pooled and non-pooled was assessed within this thesis. In Chapter 3, where pooled and non-pooled studies were compared the pooled samples had more overall variability, indicating that pooling may mask population variation in SCFA production.

8.4 In vitro analysis

A selection of NDC that are generally considered 'propiogenic' as well as others which seemed to produce propionate but did not fulfil the inclusion criteria for the systematic review were screened. As this was a screening of a selection of different substrates, batch *in vitro* investigations were carried out. This fermentation system lent itself to high-throughput screening allowing direct and simultaneous comparison of a range of NDC within each individual participant. We were unable to study all substrates in one huge fermentation batch and so this would have meant using different faecal samples for different substrates. We therefore grouped the substrates into related batches with similar properties which reduced the variability of faecal samples used for each comparison.

This fermentation system was also effectively 'miniaturised' which enabled fermentations to be carried out when there was reduced substrate availability, however there was a limit to this particularly with the 50 mg system. For example, it became apparent during the validation of the miniaturisation that the smallest fermentation system requiring 50 mg of substrate did not lend itself to some soluble fibres which increased supernatant viscosity (e.g pectin) As a result this system was only utilised for the disaccharide substrates which were very expensive and completely and freely soluble (discussed below).

An issue with the screening was that due to the relatively small sample size and the variability in SCFA production by different stool donors, led to difficulties in identifying statistical significances in production (Further discussed below). In contrast, if a greater number of biological replicate fermentations for each substrate were conducted, fewer substrates would have been compared due to time restraints, and on occasion (e.g glucose disaccharides) thus reducing the ability to screen a variety of different substrates.

Substrates which produced propionate were ranked after screening to examine the nature of the top 25% of propionate producing substrates. The top 10 producers of propionate included laminarin, oat and barley β-glucan, guar gum and legumes. Also within the top 10 propionate producers was oligofructose, although oligofructose is not generally considered as a 'propionate producer' (although it does consists of β[2-1] linkages [Khan and Edwards., 2005]). Oligofructose ranked in the top 25% as it produced high total SCFA production, which lead to increased propionate production as a consequence. A

common theme of the top 10 producers was the presence of linkages with beta bonding. For example laminarin has β(1-3), β(1-6) bonding (Deville, 2007), oat and barley β-glucan has β(1-3), β(1-4) bonding (Hughes et al., 2008), guar gum has a β(1-4) backbone (Stewart and Slavin, 2006). Legume fibre (although not in the substrates test here) consists of a variety of sugars. The soluble fibre fraction having high proportions of galacturonic acid (found in pectin), but also xyloglucan which also consists of β- bonding (Brummer et al., 2015), both of which have been associated with increased propionate production *in vitro* (Gulfi et al., 2005, Hughes et al., 2008). Therefore, it would have been interesting to assess the sugar structure of the legume fibres tested, however this was not in the scope of this thesis.

This also led to the assessment of the commercial mycoprotein product, known as Quorn. The polysaccharide within mycoprotein (mycoprotein fibre) also contains β(1-4), β(1-3), and β(1-6) bonding. After fermentation of the mycoprotein, and its extracted fibre it became apparent that the SCFA produced preferentially led to the production of propionate. This further supports other observations in this thesis that β-bonding plays an important role in the selective production of propionate.

The assessment of the effect that bond linkage had on the production of propionate was within the scope of this thesis, although only assessment of glucose-glucose disaccharides was possible. Due to the high cost of the substrates used for this experiment, the fermentation system was successfully miniaturised, allowing 10 fermentations in duplicate to be conducted initially. Although the majority of *in vitro* investigations carry out fermentations with stool donors from approximately 3 to 6 donors (Stewart and Slavin, 2006, Bourquin et al., 1992, Wang and Gibson, 1993), a post hoc power calculation indicated that the study was underpowered to assess the production of propionate with 10 different sample donors. It did indicate that for α(1-1) and β(1-4) bonding an additional five fermentations would provide power for robust statistical assessment. For the remainder of the substrates an unfeasible number of replicates would have been required to reach statistical power.

These disaccharide fermentations showed that β(1-4) bonding had an increased proportion of propionate compared to the α(1-4) linkage. However, linkage and bond orientation had

no effect on the production of propionate, and minimal effects were seen in differences in butyrate production.

These findings were not supported by a similar study by Sanz et al., (2005) who observed increased propionate (and butyrate) production with β-bonding when compared to the same linkage in the alpha orientation. However, this investigation differed from the one presented within this thesis in a number of different ways. For example, only 7 mg of substrate was used by Sanz et al, (2005) and within their study they validated the reduced size fermentation system based on bacterial populations and not SCFA production. Differing from this thesis, the system that they validated against was pH controlled, and their miniaturised system did not appear to have any form of pH control. In contrast, within this thesis the validation occurred with miniaturisations with identical methodologies. However, total SCFA production was consistently lower for the 50 mg system; this difference would likely have been more prevalent if the amount of substrate was further reduced. However, the system was valid for cross-comparison of substrate fermentation within and between subjects.

Another difference when compared to Sanz et al., (2005) was that fermentation was only carried out for 12 hours, whereas within this thesis 8 and 24 hour time points were used. By 12 hours, the substrate would likely have been fully utilised, but this does not take into account the interconversion of the SCFA which may have occurred between 12 and 24 hours of fermentation (although this often occurs within 12 hours). Interestingly, differences in the SCFA production were not associated with differences in the bacterial composition, which were not affected by the different bond linkages in the study by Sanz et al., (2005). This either indicates that the functionality of the bacteria did not differ or they were able to express different enzymes to utilise the different linkages. As assessment of the bacterial populations or the functionality of the bacteria was not accomplishable within this thesis it is difficult to compare the findings of Sanz et al., (2005) with the findings within this thesis.

The main difference and possibly the most important was the use of a single stool donor in the study by Sanz et al., (2005), negating any effect of inter-individual variability on SCFA production. Variability in the colonic microbiota has been observed on many different occasions, with a variety of different methods used to assess the colonic diversity. For

example, the type of food consumed has been previously shown to rapidly alter the composition of the colonic bacteria, which would then lead to down-stream effects on SCFA production (David et al., 2014). Similar effects were also observed by Walker et al., (2011) where overweight individuals consumed a maintenance diet, a non-starch polysaccharide diet, a resistant starch diet, and a weight loss diet, each for three weeks. Here it was seen that people had differences in their abilities to digest the individual diets but that these differing diets also altered the bacterial profiles. Within this thesis, the diets were not assessed, and no dietary control was instigated prior to stool collection. This may have played a role in the variability of SCFA production that was observed throughout this thesis and in particular during the disaccharide analysis. This variation could possibly have been reduced with a pre-study diet which may have 'normalised' the initial colonic bacteria.

Variation in SCFA production capabilities has also been discussed in other *in vitro* studies (Bourriaud et al., 2005, Rosendale et al., 2012, Carlson et al., 2016). Together this indicates the complexities of the diet - bacteria interactions within the colon of the donor, as well as the substrate being tested by fermentation. This indicates that SCFA production is multifactorial and complex, thus predicting SCFA formation by different NDCs is challenging. The disaccharide model used consisted only of glucose disaccharides, and although NDC such as β-glucans consist of β-linked glucoses, not all NDC do. This is exemplified by guar gum which is considered propiogenic and is formed of a β(1-4) mannose sugar backbone and α(l-6) linked galactose side chain (Stewart and Slavin, 2006). Therefore, saying that substrates such as guar gum is propiogenic is simplistic and does not address the fact that NDC are complex and there utilisation and the production of propionate is determined by not only the bacteria that are present but also the enzymes which they produce. However, what was identified throughout this thesis was that a propiogenic substrate for one individual may not be for another. This could have been for varieties of reasons, such as habitual diet, meal consumed prior to food intake and environment which may have, alter the SCFA producing capabilities of the donors. Based on this it would be interesting to assess the bacterial composition of sample donors who vastly differed in SCFA production compared to the other donors.

8.5 Overarching conclusions

Within this thesis, it has become increasingly apparent that determining which factors drive propionate production is highly complex. Very few studies have assessed the propionate producing capabilities of monosaccharides or disaccharides; allowing the effects of a particular sugar and/or linkage to be addressed. This thesis has estimated the propionate producing capabilities of all possible glucose-glucose linkages; however, in terms of all of the food that is consumed it is more complex than a linear chain of glucose bonding. This linear bonding has been demonstrated within oat and barley β-glucans which consist of β(1-3), β(1-4) bonding (Hughes et al., 2008). Due to a lack of side chains these β-glucans likely have a more open structure, thus making the bonding connecting the sugars more easily accessible. However NDC are often more complex that this, they often have multiple types of bonding with various branches. An example of this is pectin which consists of α(l-4) linked galactuoronic acids but also consists of branches containing a selection of sugars such as rhamnose, arabinan, galactan and arabinogalactan, which utilise different linkages (Gulfi et al., 2005). For SCFA production a selection of different bacterial enzymes are required, and access may only be possible after a different linkage has been catabolised, so if the initial bond is catabolised, even if the correct enzymes for the secondary bond are present, breaking the bond to utilise the sugar is not possible.

Another level of complexity is that often these fibres are also encapsulated as part of a food. The fibre of mycoprotein is an example of this. Approximately 25% (dry weight) of mycoprotein is dietary fibre, and the remainder consists of protein, fat, and a selection of different micronutrients (Marlow foods Ltd., 2016). How this is digested when consumed may affect what reaches the colon. If a substrate has not fully digested once reaching the colon the bacteria may utilise the non-digested material which may alter the SCFA that are produced but also the bacterial composition. This could also make it difficult for the bacterial enzymes to catabolise the different bonds and as a result release the sugars for bacterial utilisation. This indicates that increasing the overall fermentability of a substrate is key to increasing propionate production.

A main observation within the investigations carried out here are that β(1-3), β(1-4), and β(1-6) bonding increased propionate production, or at least ranked high for propionate production. Diglucoses with β(1-4) bonding were the only sugars that selectively

increased the proportion of propionate when tested. This bond is present in oat and barley β-glucans which have been shown to propiogenic as well as butyrogenic. What was also seen was that many of the more unusual substrates that lend themselves to production of propionate seem to have β(1-6) bonding which seem to occur in foods that are less common in the British diet. For example, brown seaweed (laminarin) (Deville, 2007), pyrodextrinised starch (Laurentin and Edwards, 2004), mycoprotein fibre (Quorn, 2016), and some fungi (Wong et al., 2005) all contain β(1-6) linkages which are all associated with increased propionate production. This was not observed when assessing the propionate producing capabilities of β(1-6) linked glucose disaccharides. This may be as the β(1-6) linkages are part of a chain in which the other linkages have influenced the bacteria that are present. The accessibility of these bonds may be improved when it is a branch point, also the number of branch points may also alter the accessibility of the bacterial enzymes. The ability of fungi to produce propionate and butyrate is also interesting as the α(1-1)α bonding (trehalose) has been seen to selectively increase butyrate production occurs in a variety of different fungi as well as other sources. Trehalose is present in shiitake mushroom (Chen et al., 2015), and the 'common' mushroom (*Agaricus Bisporus*) (Wannet et al., 1998) where it is used as a storage polysaccharide alongside beta glucans. It is found in other sources too such as the Arabidopsis plant (Müller et al., 2001). This suggests that trehalose could be exploited for the development of functional foods to increase butyrate production and that less common types of food, as well as those that are yet to have their SCFA producing capabilities measured would be interesting pursuits when further investigating the production of propionate.

Within this thesis and the overview of the literature, it has become increasingly clear that determining the production of a single SCFA is not simple, and is likely reliant on increased overall fermentability of the substrate. Production of a single SCFA relies on a series of different events to come together at the same time. It is likely that it is reliant on the sugars that are in the dietary fibre, the bonds that they have, the branching and the degree of branching present. All these effects likely alter the bacterial enzymes expressed by the colonic bacteria that are present and the pathways that occur and as a result the SCFA produced. Therefore, a next logical step would have been to assess which pathways were used to assess the production of propionate by different dietary fibres. This could be done using stable isotopes to assess which pathways were being utilised. Within the PhD, this was initiated in fermentation using $^{13}C_3$ lactate, and $^{2}H_5$-propionate with a number of

the propiogenic substrates *in vitro*. This, along with possible changes in the bacterial composition could have provided more mechanistic information on how propiogenic substrates, lead to the production of propionate. However, due to instrumental issues and time constraints these aspects were unable to be completed.

Within this thesis, it was identified that targeting a single substrate solely for increasing colonic propionate is unlikely to be achievable by manipulating the diet (without the use of IPE). Many different fibre sources were tested, and a systematic review of the current literature performed, and not one substrate was found to consistently increase propionate production. It was also observed within the systematic review that there were not large differences in the ability of the substrates to produce each individual SCFA.

It was found that increasing overall SCFA production, also led to increased propionate production. If fermentation of a substrate produced a high proportion of propionate, this did not always translate into high concentrations of propionate. It is important to consider not only what is produced but how the SCFA when they enter human tissues interact. Thus, the different proportions of each SCFA may affect the overall impact on metabolism. Previous infusion studies have showed that in terms of fatty acid and cholesterol production propionate can inhibit the effects of acetate (Wolever et al., 1991, Wolever et al., 1995). Recently opposing effects on energy intake of acetate and propionate have also been shown. Perry., et al (2016) found acetate increased energy intake, whereas propionate has opposing effects and reduces energy intake, however the mechanisms for this are unclear (Chambers et al., 2014). These results also conflict with the effects of acetate observed by Frost et al., (2014) suggesting that further work is needed at the whole animal level to understand the role of SCFA.

Extrapolating from the published studies on fibre, it would appear that to have a significant effect on satiety high intakes of fibre are needed. A major mechanism for the effects of fibre on satiety is now thought to be via the actions of propionate. The studies in this thesis suggest that rather than eating specific foods or fibres the best means to increase colonic propionate production within the diet is by increasing overall consumption of highly fermentable, non-digestible carbohydrates.

Appendices

Appendix 1- Participant information sheet

Participant Information Sheet

Title of Study
Faecal samples for the study of *In vitro* fermentation by human faecal microbiota

Invitation to take part
Thank you for reading this.
You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is unclear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?
The role of the bacteria in the gut in promoting health but also in increasing risk of some diseases is being investigated. The composition and metabolism of the bacteria in the large intestine is currently being linked to the risk of obesity, allergy, gut disease and other conditions. It is very important that we understand how the metabolism of the bacteria change on different diets and with different possible compounds to metabolise and then how this is related to disease risk. We can carry out much research using simple 'test tube' models of the colon where we grow bacteria from faeces in bottles mimicking conditions in the gut. We use these models to screen the effects of a wide range of possible dietary compounds and thus devise possible diets to promote health and reduce disease risk. To carry out these studies we need fresh human faecal samples.

Why have I been chosen?
You are:
i) A healthy individual, Caucasian, aged 18-65, in good general health, not taking any medication effecting bowl health or antibiotics within the past 6 months
ii) You do not suffer from any allergy or condition affecting bowel health

Do I have to take part?
No, it is your decision. If you do decide to take part you will be given this information sheet to keep and will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving reason.

What do I have to do?
You will have the study explained by one of the research team. You will be able to stay on your normal diet and will be asked to provide a faecal sample. You will be given full instructions on how to collect the sample and we will provide a special collection device that can be placed on the toilet seat to make collection as convenient as possible. We will ask you to place the sample in a special container and to add tap water to a sachet to provide an atmosphere to preserve the activity of the bacteria, you will then seal the pot and either bring the sample to our unit or we can collect it from you. We need to process the sample within 2 hours of passage. You will also be given a telephone number, to be able to call a member of the Research Team with any queries.

What are the possible disadvantages and risks of taking part?
There are no risks or advantages associated with this study other than time loss

What are the possible benefits of taking part?
There are no direct benefits to the volunteers associates with taking part. This study will provide us with a better understanding of the metabolism of the colonic bacteria and

their possible role in a variety of diseases as well as possible dietary changes that may reduce risk.

Will my taking part in this study be kept confidential?
Very little information will be required from you but all information will be kept strictly confidential. Any information about you that leaves the hospital/university will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?
Results will be presented at meetings of learned societies and published in scientific journals. Results will also be included in student project reports, when applicable. We will arrange a meeting to discuss the results with participant volunteers if they would like that.

Who is organising and funding the research?
This project is being organised by the Human Nutrition Group, at the University of Glasgow. The funding comes from a variety of sources including scholarships and some industry sources. Details of each funding source involved in the study your samples are used for can be provided if you require. Your samples will be used for research funded by the BBSRC.

Who has reviewed the study?
This project has been reviewed by the University of Glasgow, College of MVLS, ethics committee.

Contact for further information
If you require further information please contact Prof Christine Edwards Christine.Edwards@glasgow.ac.uk or Hannah Harris at h.harris.1@research.gla.ac.uk.

Thank you for reading this information sheet.

Study number
Version no. 4 Date 14.12.12

Appendix 2- Participant consent form

University of Glasgow

Centre number:

Study number:

Subject identification number for this trial:

CONSENT FORM

Title of Project: Faecal samples for the study of *In vitro* fermentation by human faecal microbiota

Name of Researcher: Hannah Harris

Please initial box

1. I confirm that I have read and understand the information sheet dated 14/12/12 (version 4) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected. ☐
3. I agree to take part in the above study. ☐

Name of participant	Date	Signature
Name of person taking consent (if different from the researcher)	Date	Signature
Researcher	Date	Signature

1 for subject; 1 for researcher

Faculty of Medicine
Ethics Committee

P.I.S (June 2003)

Human Nutrition Department
University of Glasgow
Yorkhill Hospital
Glasgow
G3 8SJ

List of References

(EFSA PANEL ON DIETETIC PRODUCTS, N. A. A. 2011. Scientific Opinion on the substantiation of health claims related to beta-glucans from oats and barley and maintenance of normal blood LDL-cholesterol concentrations (ID 1236, 1299), increase in satiety leading to a reduction in energy intake. *EFSA Journal,* 9.

(SACN), S. A. C. O. N. 2015. Carbohydrates and Health. *In:* ENGLAND, P. H. (ed.). London: TSO.

ADAM, T. C. & WESTERTERP-PLANTENGA, M. S. 2005. Glucagon-like peptide-1 release and satiety after a nutrient challenge in normal-weight and obese subjects. *Br J Nutr,* 93**,** 845-51.

ADIOTOMRE, J., EASTWOOD, M. A., EDWARDS, C. A. & BRYDON, W. G. 1990. Dietary fiber: in vitro methods that anticipate nutrition and metabolic activity in humans. *Am J Clin Nutr,* 52**,** 128-34.

AGUIRRE, M., RAMIRO-GARCIA, J., KOENEN, M. E. & VENEMA, K. 2014. To pool or not to pool? Impact of the use of individual and pooled fecal samples for in vitro fermentation studies. *J Microbiol Methods,* 107**,** 1-7.

AL-LAHHAM, S., ROELOFSEN, H., REZAEE, F., WEENING, D., HOEK, A., VONK, R. & VENEMA, K. 2012. Propionic acid affects immune status and metabolism in adipose tissue from overweight subjects. *Eur J Clin Invest,* 42**,** 357-64.

AL-TAMIMI, M. A., PALFRAMAN, R. J., COOPER, J. M., GIBSON, G. R. & RASTALL, R. A. 2006. In vitro fermentation of sugar beet arabinan and arabino-oligosaccharides by the human gut microflora. *J Appl Microbiol,* 100**,** 407-14.

ALDWAIRJI, M. A., CHU, J., BURLEY, V. J. & ORFILA, C. 2014. Analysis of dietary fibre of boiled and canned legumes commonly consumed in the United Kingdom. *Journal of Food Composition and Analysis,* 36**,** 111-116.

AMREIN, T. M., GRÄNICHER, P., ARRIGONI, E. & AMADÒ, R. 2003. In vitro digestibility and colonic fermentability of aleurone isolated from wheat bran. *LWT - Food Science and Technology,* 36**,** 451-460.

AOYAMA, M., KOTANI, J. & USAMI, M. 2010. Butyrate and propionate induced activated or non-activated neutrophil apoptosis via HDAC inhibitor activity but without activating GPR-41/GPR-43 pathways. *Nutrition,* 26**,** 653-61.

ARORA, T., LOO, R. L., ANASTASOVSKA, J., GIBSON, G. R., TUOHY, K. M., SHARMA, R. K., SWANN, J. R., DEAVILLE, E. R., SLEETH, M. L., THOMAS, E. L., HOLMES, E., BELL, J. D. & FROST, G. 2012. Differential Effects of Two Fermentable Carbohydrates on Central Appetite Regulation and Body Composition. *PLoS One,* 7.

ARPAIA, N., CAMPBELL, C., FAN, X., DIKIY, S., VAN DER VEEKEN, J., DEROOS, P., LIU, H., CROSS, J. R., PFEFFER, K., COFFER, P. J. & RUDENSKY, A. Y. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature,* 504**,** 451-5.

ARRIGONI, E., BROUNS, F. & AMADO, R. 2005. Human gut microbiota does not ferment erythritol. *Br J Nutr,* 94**,** 643-6.

ARRIGONI, E., JÖRGER, F., KOLLÖFFEL, B., ROULET, I., HERENSPERGER, M., MEILE, L. & AMADÒ, R. 2002. In vitro fermentability of a commercial wheat germ preparation and its impact on the growth of bifidobacteria. *Food Research International,* 35**,** 475-481.

ARUMUGAM, M., RAES, J., PELLETIER, E., LE PASLIER, D., YAMADA, T., MENDE, D. R., FERNANDES, G. R., TAP, J., BRULS, T., BATTO, J. M., BERTALAN, M., BORRUEL, N., CASELLAS, F., FERNANDEZ, L., GAUTIER, L., HANSEN, T., HATTORI, M., HAYASHI, T., KLEEREBEZEM, M., KUROKAWA, K., LECLERC, M., LEVENEZ, F., MANICHANH, C., NIELSEN, H. B., NIELSEN, T., PONS, N., POULAIN, J., QIN, J., SICHERITZ-PONTEN, T., TIMS, S., TORRENTS, D., UGARTE, E., ZOETENDAL, E. G., WANG, J., GUARNER, F., PEDERSEN, O., DE VOS, W. M., BRUNAK, S., DORE, J., ANTOLIN, M., ARTIGUENAVE, F., BLOTTIERE, H. M., ALMEIDA, M., BRECHOT, C., CARA, C., CHERVAUX, C., CULTRONE, A., DELORME, C., DENARIAZ, G., DERVYN, R., FOERSTNER, K. U., FRISS, C., VAN DE GUCHTE, M., GUEDON, E., HAIMET, F., HUBER, W., VAN HYLCKAMA-VLIEG, J., JAMET, A., JUSTE, C., KACI, G., KNOL, J., LAKHDARI, O., LAYEC, S., LE ROUX, K., MAGUIN, E., MERIEUX, A., MELO MINARDI, R., M'RINI, C., MULLER, J., OOZEER, R., PARKHILL, J., RENAULT, P., RESCIGNO, M., SANCHEZ, N., SUNAGAWA, S., TORREJON, A., TURNER, K., VANDEMEULEBROUCK, G., VARELA, E., WINOGRADSKY, Y., ZELLER, G., WEISSENBACH, J., EHRLICH, S. D. & BORK, P. 2011. Enterotypes of the human gut microbiome. *Nature,* 473**,** 174-80.

ASANO, I., UMEMURA, M., FUJII, S., HOSHINO, H. & IINO, H. 2004. Effects of mannooligosaccharides from coffee mannan on fecal microflora and defecation in healthy volunteers. *Food Science and Technology Research,* 10**,** 93-97.

ASTBURY, N. M., TAYLOR, M. A. & MACDONALD, I. A. 2013. Polydextrose results in a dose-dependent reduction in ad libitum energy intake at a subsequent test meal. *Br J Nutr,* 110**,** 934-42.

AURA, A.-M., KARPPINEN, S., VIRTANEN, H., FORSSELL, P., HEINONEN, S.-M., NURMI, T., ADLERCREUTZ, H. & POUTANEN, K. 2005. Processing of rye bran influences both the fermentation of dietary fibre and the bioconversion of lignans by human faecal flora in vitro. *Journal of the Science of Food and Agriculture,* 85**,** 2085-2093.

AURA, A. M., OIKARINEN, S., MUTANEN, M., HEINONEN, S. M., ADLERCREUTZ, H. C., VIRTANEN, H. & POUTANEN, K. S. 2006. Suitability of a batch in vitro fermentation model using human faecal microbiota for prediction of conversion of flaxseed lignans to enterolactone with reference to an in vivo rat model. *Eur J Nutr,* 45**,** 45-51.

BARRETT, M. L. & UDANI, J. K. 2011. A proprietary alpha-amylase inhibitor from white bean (Phaseolus vulgaris): a review of clinical studies on weight loss and glycemic control. *Nutr J,* 10**,** 24.

BARRY, J. L., HOEBLER, C., MACFARLANE, G. T., MACFARLANE, S., MATHERS, J. C., REED, K. A., MORTENSEN, P. B., NORDGAARD, I., ROWLAND, I. R. & RUMNEY, C. J. 1995. Estimation of the fermentability of dietary fibre in vitro: a European interlaboratory study. *Br J Nutr,* 74**,** 303-22.

BATES, B., LENNOX, A., PRENTICE, A., BATES, C., PAGE, P., NICHOLSON, S. & SWAN, G. 2014. *National Diet and Nutrition Survey: results from Years 1 to 4 (combined) of the rolling programme for 2008 and 2009 to 2011 and 2012* [Online]. Available: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/310997/NDNS_Y1_to_4_UK_report_Executive_summary.pdf [Accessed].

BATTERHAM, R. L., COHEN, M. A., ELLIS, S. M., LE ROUX, C. W., WITHERS, D. J., FROST, G. S., GHATEI, M. A. & BLOOM, S. R. 2003a. Inhibition of food intake in obese subjects by peptide YY3-36. *N Engl J Med,* 349**,** 941-8.

BATTERHAM, R. L., LE ROUX, C. W., COHEN, M. A., PARK, A. J., ELLIS, S. M., PATTERSON, M., FROST, G. S., GHATEI, M. A. & BLOOM, S. R. 2003b. Pancreatic polypeptide reduces appetite and food intake in humans. *J Clin Endocrinol Metab,* 88**,** 3989-92.

BAUMGARTNER, M. R., HORSTER, F., DIONISI-VICI, C., HALILOGLU, G., KARALL, D., CHAPMAN, K. A., HUEMER, M., HOCHULI, M., ASSOUN, M., BALLHAUSEN, D., BURLINA, A., FOWLER, B., GRUNERT, S. C., GRUNEWALD, S., HONZIK, T., MERINERO, B., PEREZ-CERDA, C., SCHOLL-BURGI, S., SKOVBY, F., WIJBURG, F., MACDONALD, A., MARTINELLI, D., SASS, J. O., VALAYANNOPOULOS, V. & CHAKRAPANI, A. 2014. Proposed guidelines for the diagnosis and management of methylmalonic and propionic acidemia. *Orphanet J Rare Dis,* 9**,** 130.

BEARDS, E., TUOHY, K. & GIBSON, G. 2010a. Bacterial, SCFA and gas profiles of a range of food ingredients following in vitro fermentation by human colonic microbiota. *Anaerobe,* 16**,** 420-5.

BEARDS, E., TUOHY, K. & GIBSON, G. 2010b. A human volunteer study to assess the impact of confectionery sweeteners on the gut microbiota composition. *Br J Nutr,* 104**,** 701-8.

BELCHEVA, A., IRRAZABAL, T., ROBERTSON, S. J., STREUTKER, C., MAUGHAN, H., RUBINO, S., MORIYAMA, E. H., COPELAND, J. K., KUMAR, S., GREEN, B., GEDDES, K., PEZO, R. C., NAVARRE, W. W., MILOSEVIC, M., WILSON, B. C., GIRARDIN, S. E., WOLEVER, T. M., EDELMANN, W., GUTTMAN, D. S., PHILPOTT, D. J. & MARTIN, A. 2014. Gut microbial metabolism drives transformation of MSH2-deficient colon epithelial cells. *Cell,* 158**,** 288-99.

BELENGUER, A., DUNCAN, S. H., HOLTROP, G., ANDERSON, S. E., LOBLEY, G. E. & FLINT, H. J. 2007. Impact of pH on Lactate Formation and Utilization by Human Fecal Microbial Communities▽. *Appl Environ Microbiol,* 73**,** 6526-33.

BELENGUER, A., HOLTROP, G., DUNCAN, S. H., ANDERSON, S. E., CALDER, A. G., FLINT, H. J. & LOBLEY, G. E. 2011. Rates of production and utilization of lactate by microbial communities from the human colon. *FEMS Microbiol Ecol,* 77**,** 107-19.

BERGGREN, A. M., NYMAN, E. M., LUNDQUIST, I. & BJORCK, I. M. 1996. Influence of orally and rectally administered propionate on cholesterol and glucose metabolism in obese rats. *Br J Nutr,* 76**,** 287-94.

BEYER-SEHLMEYER, G., GLEI, M., HARTMANN, E., HUGHES, R., PERSIN, C., BOHM, V., ROWLAND, I., SCHUBERT, R., JAHREIS, G. & POOL-ZOBEL, B. L. 2003. Butyrate is only one of several growth inhibitors produced during gut flora-mediated fermentation of dietary fibre sources. *Br J Nutr,* 90**,** 1057-70.

BHASKARAN, K., DOUGLAS, I., FORBES, H., DOS-SANTOS-SILVA, I., LEON, D. A. & SMEETH, L. 2014. Body-mass index and risk of 22 specific cancers: a population-based cohort study of 5.24 million UK adults. *Lancet,* 384**,** 755-65.

BINDELS, L. B., PORPORATO, P., DEWULF, E. M., VERRAX, J., NEYRINCK, A. M., MARTIN, J. C., SCOTT, K. P., BUC CALDERON, P., FERON, O., MUCCIOLI, G. G., SONVEAUX, P., CANI, P. D. & DELZENNE, N. M. 2012. Gut microbiota-derived propionate reduces cancer cell proliferation in the liver. *Br J Cancer,* 107**,** 1337-44.

BIRD, A. R., VUARAN, M. S., KING, R. A., NOAKES, M., KEOGH, J., MORELL, M. K. & TOPPING, D. L. 2008. Wholegrain foods made from a novel high-amylose barley variety (Himalaya 292) improve indices of bowel health in human subjects. *Br J Nutr,* 99**,** 1032-40.

BLISS, D. Z., WEIMER, P. J., JUNG, H.-J. G. & SAVIK, K. 2013. In vitro degradation and fermentation of three dietary fiber sources by human colonic bacteria. *Journal of agricultural and food chemistry,* 61.

BLOEMEN, J. G., VENEMA, K., VAN DE POLL, M. C., OLDE DAMINK, S. W., BUURMAN, W. A. & DEJONG, C. H. 2009. Short chain fatty acids exchange across the gut and liver in humans measured at surgery. *Clin Nutr,* 28**,** 657-61.

BOBIN-DUBIGEON, C., LAHAYE, M. & BARRY, J.-L. 1997. Human Colonic Bacterial Degradability of Dietary Fibres from Sea-Lettuce (Ulva sp). *Journal of the Science of Food and Agriculture,* 73**,** 149-159.

BODINHAM, C. L., AL-MANA, N. M., SMITH, L. & ROBERTSON, M. D. 2013. Endogenous plasma glucagon-like peptide-1 following acute dietary fibre consumption. *Br J Nutr***,** 1-5.

BOETS, E., DEROOVER, L., HOUBEN, E., VERMEULEN, K., GOMAND, S. V., DELCOUR, J. A. & VERBEKE, K. 2015. Quantification of in Vivo Colonic Short Chain Fatty Acid Production from Inulin. *Nutrients,* 7**,** 8916-8929.

BOKURA, H. & KOBAYASHI, S. 2003. Chitosan decreases total cholesterol in women: a randomized, double-blind, placebo-controlled trial. *Eur J Clin Nutr,* 57**,** 721-5.

BOUHNIK, Y., VAHEDI, K., ACHOUR, L., ATTAR, A., SALFATI, J., POCHART, P., MARTEAU, P., FLOURIE, B., BORNET, F. & RAMBAUD, J. C. 1999. Short-chain fructo-oligosaccharide administration dose-dependently increases fecal bifidobacteria in healthy humans. *J Nutr,* 129**,** 113-6.

BOURQUIN, L. D., TITGEMEYER, E. C. & FAHEY, G. C. 1996. Fermentation of various dietary fiber sources by human fecal bacteria. *Nutrition Research,* 16**,** 1119-1131.

BOURQUIN, L. D., TITGEMEYER, E. C., FAHEY, G. C., JR. & GARLEB, K. A. 1993. Fermentation of dietary fibre by human colonic bacteria: disappearance of, short-chain fatty acid production from, and potential water-holding capacity of, various substrates. *Scand J Gastroenterol,* 28**,** 249-55.

BOURQUIN, L. D., TITGEMEYER, E. C., GARLEB, K. A. & FAHEY, G. C., JR. 1992. Short-chain fatty acid production and fiber degradation by human colonic bacteria: effects of substrate and cell wall fractionation procedures. *J Nutr,* 122**,** 1508-20.

BOURRIAUD, C., ROBINS, R. J., MARTIN, L., KOZLOWSKI, F., TENAILLEAU, E., CHERBUT, C. & MICHEL, C. 2005. Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. *J Appl Microbiol,* 99**,** 201-12.

BROWN, A. J., GOLDSWORTHY, S. M., BARNES, A. A., EILERT, M. M., TCHEANG, L., DANIELS, D., MUIR, A. I., WIGGLESWORTH, M. J., KINGHORN, I., FRASER, N. J., PIKE, N. B., STRUM, J. C., STEPLEWSKI, K. M., MURDOCK, P. R., HOLDER, J. C., MARSHALL, F. H., SZEKERES, P. G., WILSON, S., IGNAR, D. M., FOORD, S. M., WISE, A. & DOWELL, S. J. 2003. The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *J Biol Chem,* 278**,** 11312-9.

BRUMMER, Y., KAVIANI, M. & TOSH, S. M. 2015. Structural and functional characteristics of dietary fibre in beans, lentils, peas and chickpeas. *Food Research International,* 67**,** 117-125.

BURLEY, V. J., PAUL, A. W. & BLUNDELL, J. E. 1993. Influence of a high-fibre food (myco-protein) on appetite: effects on satiation (within meals) and satiety (following meals). *Eur J Clin Nutr,* 47**,** 409-18.

BYRNE, C. S., CHAMBERS, E. S., ALHABEEB, H., CHHINA, N., MORRISON, D. J., PRESTON, T., TEDFORD, C., FIZPATRICK, J., IRANI, C., BUSZA, A., GARCIA-PEREZ, I., FOUNTANA,

S., HOLMES, E., GOLDSTONE, A. P. & FROST, G. S. 2016. Increased colonic propionate reduces anticipatory reward responses in the human striatum to high-energy foods. *Am J Clin Nutr*.

BYRNE, C. S., CHAMBERS, E. S., MORRISON, D. J. & FROST, G. 2015. The role of short chain fatty acids in appetite regulation and energy homeostasis. *Int J Obes (Lond),* 39**,** 1331-8.

CAMPECHANO-CARRERA, E., CORONA-CRUZ, A., CHEL-GUERRERO, L. & BETANCUR-ANCONA, D. 2007. Effect of pyrodextrinization on available starch content of Lima bean (Phaseolus lunatus) and Cowpea (Vigna unguiculata) starches. *Food Hydrocolloids,* 21**,** 472-479.

CARCIOFI, M., BLENNOW, A., JENSEN, S. L., SHAIK, S. S., HENRIKSEN, A., BULEON, A., HOLM, P. B. & HEBELSTRUP, K. H. 2012. Concerted suppression of all starch branching enzyme genes in barley produces amylose-only starch granules. *BMC Plant Biol,* 12**,** 223.

CARDELLE-COBAS, A., FERNANDEZ, M., SALAZAR, N., MARTINEZ-VILLALUENGA, C., VILLAMIEL, M., RUAS-MADIEDO, P. & DE LOS REYES-GAVILAN, C. G. 2009. Bifidogenic effect and stimulation of short chain fatty acid production in human faecal slurry cultures by oligosaccharides derived from lactose and lactulose. *J Dairy Res,* 76**,** 317-25.

CARLSON, J., ESPARZA, J., SWAN, J., TAUSSIG, D., COMBS, J. & SLAVIN, J. 2016. In vitro analysis of partially hydrolyzed guar gum fermentation differences between six individuals. *Food Funct,* 7**,** 1833-8.

CASIRAGHI, M. C., ZANCHI, R., CANZI, E., PAGANI, M. A., VIARO, T., BENINI, L. & D'EGIDIO, M. G. 2011. Prebiotic potential and gastrointestinal effects of immature wheat grain (IWG) biscuits. *Antonie Van Leeuwenhoek,* 99**,** 795-805.

CHAMBERS, E. S., VIARDOT, A., PSICHAS, A., MORRISON, D. J., MURPHY, K. G., ZAC-VARGHESE, S. E. K., MACDOUGALL, K., PRESTON, T., TEDFORD, C., FINLAYSON, G. S., BLUNDELL, J. E., BELL, J. D., THOMAS, E. L., MT-ISA, S., ASHBY, D., GIBSON, G. R., KOLIDA, S., DHILLO, W. S., BLOOM, S. R., MORLEY, W., CLEGG, S. & FROST, G. 2014. Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults.

CHEN, J., LIANG, R. H., LIU, W., LI, T., LIU, C. M., WU, S. S. & WANG, Z. J. 2013. Pectic-oligosaccharides prepared by dynamic high-pressure microfluidization and their in vitro fermentation properties. *Carbohydr Polym,* 91**,** 175-82.

CHEN, W., LI, W., YANG, Y., YU, H., ZHOU, S., FENG, J., LI, X. & LIU, Y. 2015. Analysis and evaluation of tasty components in the pileus and stipe of Lentinula edodes at different growth stages. *J Agric Food Chem,* 63**,** 795-801.

CHERBUT, C., AUBE, A. C., MEKKI, N., DUBOIS, C., LAIRON, D. & BARRY, J. L. 1997. Digestive and metabolic effects of potato and maize fibres in human subjects. *Br J Nutr,* 77**,** 33-46.

CHERBUT, C., SALVADOR, V., BARRY, J. L., DOULAY, F. & DELORT-LAVAL, J. 1991. Dietary fibre effects of intestinal transit in man: involvement of their physicochemical and fermentative properties. *Food hydrocolloids (USA),* 5**,** 15-22.

CHILD, M. W., KENNEDY, A., WALKER, A. W., BAHRAMI, B., MACFARLANE, S. & MACFARLANE, G. T. 2006. Studies on the effect of system retention time on bacterial populations colonizing a three-stage continuous culture model of the human large gut using FISH techniques. *FEMS Microbiol Ecol,* 55**,** 299-310.

CHRISTIAN, M. T., EDWARDS, C. A., PRESTON, T., JOHNSTON, L., VARLEY, R. & WEAVER, L. T. 2003. Starch fermentation by faecal bacteria of infants, toddlers and adults: importance for energy salvage. *Eur J Clin Nutr,* 57**,** 1486-91.

CHRISTL, S. U., BARTRAM, H. P., RUCKERT, A., SCHEPPACH, W. & KASPER, H. 1995. Influence of starch fermentation on bile acid metabolism by colonic bacteria. *Nutr Cancer,* 24**,** 67-75.

CHRISTL, S. U., KATZENMAIER, U., HYLLA, S., KASPER, H. & SCHEPPACH, W. 1997. In vitro fermentation of high-amylose cornstarch by a mixed population of colonic bacteria. *JPEN J Parenter Enteral Nutr,* 21**,** 290-5.

CLEGG, M. E. & THONDRE, P. S. 2014. Molecular weight of barley beta-glucan does not influence satiety or energy intake in healthy male subjects. *Appetite,* 83**,** 167-72.

COHEN, M. A., ELLIS, S. M., LE ROUX, C. W., BATTERHAM, R. L., PARK, A., PATTERSON, M., FROST, G. S., GHATEI, M. A. & BLOOM, S. R. 2003. Oxyntomodulin suppresses appetite and reduces food intake in humans. *J Clin Endocrinol Metab,* 88**,** 4696-701.

CONSORTIUM, T. I. 2015. Dietary fibre and incidence of type 2 diabetes in eight European countries: the EPIC-InterAct Study and a meta-analysis of prospective studies. *Diabetologia,* 58**,** 1394-408.

CORNALL, L. M., MATHAI, M. L., HRYCIW, D. H. & MCAINCH, A. J. 2011. Diet-induced obesity up-regulates the abundance of GPR43 and GPR120 in a tissue specific manner. *Cell Physiol Biochem,* 28**,** 949-58.

CUMMINGS, D. E., WEIGLE, D. S., FRAYO, R. S., BREEN, P. A., MA, M. K., DELLINGER, E. P. & PURNELL, J. Q. 2002. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *N Engl J Med,* 346**,** 1623-30.

CUMMINGS, J. H., POMARE, E. W., BRANCH, W. J., NAYLOR, C. P. & MACFARLANE, G. T. 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut,* 28**,** 1221-7.

DARWICHE, G., OSTMAN, E. M., LILJEBERG, H. G., KALLINEN, N., BJORGELL, O., BJORCK, I. M. & ALMER, L. O. 2001. Measurements of the gastric emptying rate by use of ultrasonography: studies in humans using bread with added sodium propionate. *Am J Clin Nutr,* 74**,** 254-8.

DARZI, J., FROST, G. S. & ROBERTSON, M. D. 2011. Do SCFA have a role in appetite regulation? *Proc Nutr Soc.* England.

DARZI, J., FROST, G. S. & ROBERTSON, M. D. 2012. Effects of a novel propionate-rich sourdough bread on appetite and food intake. *Eur J Clin Nutr.* England.

DARZI, J., FROST, G. S., SWANN, J. R., COSTABILE, A. & ROBERTSON, M. D. 2015. L-Rhamnose as a source of colonic propionate inhibits insulin secretion but does not influence measures of appetite or food intake. *Appetite*.

DASS, N. B., JOHN, A. K., BASSIL, A. K., CRUMBLEY, C. W., SHEHEE, W. R., MAURIO, F. P., MOORE, G. B., TAYLOR, C. M. & SANGER, G. J. 2007. The relationship between the effects of short-chain fatty acids on intestinal motility in vitro and GPR43 receptor activation. *Neurogastroenterol Motil,* 19**,** 66-74.

DAUD, N. M., ISMAIL, N. A., THOMAS, E. L., FITZPATRICK, J. A., BELL, J. D., SWANN, J. R., COSTABILE, A., CHILDS, C. E., PEDERSEN, C., GOLDSTONE, A. P. & FROST, G. S. 2014. The impact of oligofructose on stimulation of gut hormones, appetite regulation and adiposity. *Obesity (Silver Spring),* 22**,** 1430-8.

DAVID, L. A., MAURICE, C. F., CARMODY, R. N., GOOTENBERG, D. B., BUTTON, J. E., WOLFE, B. E., LING, A. V., DEVLIN, A. S., VARMA, Y., FISCHBACH, M. A., BIDDINGER,

S. B., DUTTON, R. J. & TURNBAUGH, P. J. 2014. Diet rapidly and reproducibly alters the human gut microbiome. *Nature,* 505**,** 559-63.

DE FILIPPO, C., CAVALIERI, D., DI PAOLA, M., RAMAZZOTTI, M., POULLET, J. B., MASSART, S., COLLINI, S., PIERACCINI, G. & LIONETTI, P. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci U S A,* 107**,** 14691-6.

DE MUNTER, J. S., HU, F. B., SPIEGELMAN, D., FRANZ, M. & VAN DAM, R. M. 2007. Whole grain, bran, and germ intake and risk of type 2 diabetes: a prospective cohort study and systematic review. *PLoS Med,* 4**,** e261.

DE PRETER, V., FALONY, G., WINDEY, K., HAMER, H. M., DE VUYST, L. & VERBEKE, K. 2010. The prebiotic, oligofructose-enriched inulin modulates the faecal metabolite profile: an in vitro analysis. *Mol Nutr Food Res,* 54**,** 1791-801.

DE VADDER, F., KOVATCHEVA-DATCHARY, P., GONCALVES, D., VINERA, J., ZITOUN, C., DUCHAMPT, A., BACKHED, F. & MITHIEUX, G. 2014. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell,* 156**,** 84-96.

DE VADDER, F., PLESSIER, F., GAUTIER-STEIN, A. & MITHIEUX, G. 2015. Vasoactive intestinal peptide is a local mediator in a gut-brain neural axis activating intestinal gluconeogenesis. *Neurogastroenterol Motil,* 27**,** 443-8.

DEN BESTEN, G., LANGE, K., HAVINGA, R., VAN DIJK, T. H., GERDING, A., VAN EUNEN, K., MULLER, M., GROEN, A. K., HOOIVELD, G. J., BAKKER, B. M. & REIJNGOUD, D. J. 2013a. Gut-derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids. *Am J Physiol Gastrointest Liver Physiol,* 305**,** G900-10.

DEN BESTEN, G., VAN EUNEN, K., GROEN, A. K., VENEMA, K., REIJNGOUD, D. J. & BAKKER, B. M. 2013b. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res,* 54**,** 2325-40.

DENNY, A. L., J. 2008. Mycoprotein and health. *Nutrition Bulletin,* 33**,** 298-310.

DEVILLE, C., GHARBI, M., DANDRIFOSSE, G., PEULEN, O. 2007. Study on the effects of laminarin, a polysaccharide from seaweed, on gut characteristics. *Journal of the Science of Food and Agriculture,* 87**,** 1717-1725.

DONGOWSKI, G. & LORENZ, A. 1998. Unsaturated oligogalacturonic acids are generated by in vitro treatment of pectin with human faecal flora. *Carbohydr Res,* 314**,** 237-44.

DONGOWSKI, G., LORENZ, A. & ANGER, H. 2000. Degradation of pectins with different degrees of esterification by Bacteroides thetaiotaomicron isolated from human gut flora. *Appl Environ Microbiol,* 66**,** 1321-7.

DU, H., VAN DER, A. D., BOSHUIZEN, H. C., FOROUHI, N. G., WAREHAM, N. J., HALKJAER, J., TJONNELAND, A., OVERVAD, K., JAKOBSEN, M. U., BOEING, H., BUIJSSE, B., MASALA, G., PALLI, D., SORENSEN, T. I., SARIS, W. H. & FESKENS, E. J. 2010. Dietary fiber and subsequent changes in body weight and waist circumference in European men and women. *Am J Clin Nutr,* 91**,** 329-36.

DUCA, F. A. & COVASA, M. 2012. Current and emerging concepts on the role of peripheral signals in the control of food intake and development of obesity. *Br J Nutr.* England.

DUNCAN, S. H., HOLTROP, G., LOBLEY, G. E., CALDER, A. G., STEWART, C. S. & FLINT, H. J. 2004. Contribution of acetate to butyrate formation by human faecal bacteria. *Br J Nutr,* 91**,** 915-23.

ECKBURG, P. B., BIK, E. M., BERNSTEIN, C. N., PURDOM, E., DETHLEFSEN, L., SARGENT, M., GILL, S. R., NELSON, K. E. & RELMAN, D. A. 2005. Diversity of the Human Intestinal Microbial Flora. *Science,* 308**,** 1635-8.

EDHOLM, T., DEGERBLAD, M., GRYBACK, P., HILSTED, L., HOLST, J. J., JACOBSSON, H., EFENDIC, S., SCHMIDT, P. T. & HELLSTROM, P. M. 2010. Differential incretin effects of GIP and GLP-1 on gastric emptying, appetite, and insulin-glucose homeostasis. *Neurogastroenterol Motil,* 22**,** 1191-200, e315.

EDWARDS, C. A., ADIOTOMRE, J. & EASTWOOD, M. A. 1992. Dietary fibre: The use of in-vitro and rat models to predict action on stool output in man. *Journal of the Science of Food and Agriculture,* 59**,** 257-260.

EDWARDS, C. A., DUERDEN, B. I. & READ, N. W. 1985. The effects of pH on colonic bacteria grown in continuous culture. *J Med Microbiol,* 19**,** 169-80.

EDWARDS, C. A. & EASTWOOD, M. A. 1992. Comparison of the effects of ispaghula and wheat bran on rat caecal and colonic fermentation. *Gut,* 33**,** 1229-33.

EDWARDS, C. A. & EASTWOOD, M. A. 1995. Caecal and faecal short-chain fatty acids and stool output in rats fed on diets containing non-starch polysaccharides. *Br J Nutr,* 73**,** 773-81.

EDWARDS, C. A., GIBSON, G., CHAMP, M., JENSEN, B. B., TJELE, D., MATHERS, J. C., NAGENGAST, F., RUMNEY, C. & QUEHL, A. 1996. In Vitro Method for Quantification of the Fermentation of Starch by Human Faecal Bacteria. *Journal of the Science of Food and Agriculture,* 71**,** 209-217.

EL OUFIR, L., FLOURIE, B., BRULEY DES VARANNES, S., BARRY, J. L., CLOAREC, D., BORNET, F. & GALMICHE, J. P. 1996. Relations between transit time, fermentation products, and hydrogen consuming flora in healthy humans. *Gut,* 38**,** 870-7.

ELLIS, P. R., DAWOUD, F. M. & MORRIS, E. R. 1991. Blood glucose, plasma insulin and sensory responses to guar-containing wheat breads: effects of molecular weight and particle size of guar gum. *Br J Nutr,* 66**,** 363-79.

ENGLAND, P. H. 2014. Adult obesity and type 2 diabetes.

FA1005, C. A. 2015. *The Impact of Food Bio-Actives on Gut Health: In Vitro and Ex Vivo Models*, Springer international publishing.

FADDEN, K., HILL, M. J. & OWEN, R. W. 1997. Effect of fibre on bile acid metabolism by human faecal bacteria in batch and continuous culture. *Eur J Cancer Prev,* 6**,** 175-94.

FALLINGBORG, J., CHRISTENSEN, L. A., INGEMAN-NIELSEN, M., JACOBSEN, B. A., ABILDGAARD, K. & RASMUSSEN, H. H. 1989. pH-profile and regional transit times of the normal gut measured by a radiotelemetry device. *Aliment Pharmacol Ther,* 3**,** 605-13.

FARDET, A., GUILLON, F., HOEBLER, C. & BARRY, J. L. 1997. In vitro fermentation of beet fibre and barley bran, of their insoluble residues after digestion and of ileal effluents. *Journal of the Science of Food and Agriculture,* 75**,** 315-325.

FÄSSLER, C., ARRIGONI, E., VENEMA, K., BROUNS, F. & AMADÒ, R. 2006. In vitro fermentability of differently digested resistant starch preparations. *Molecular Nutrition and Food Research,* 50**,** 1220-1228.

FASSLER, C., GILL, C. I., ARRIGONI, E., ROWLAND, I. & AMADO, R. 2007. Fermentation of resistant starches: influence of in vitro models on colon carcinogenesis. *Nutr Cancer,* 58**,** 85-92.

FASTINGER, N. D., KARR-LILIENTHAL, L. K., SPEARS, J. K., SWANSON, K. S., ZINN, K. E., NAVA, G. M., OHKUMA, K., KANAHORI, S., GORDON, D. T. & FAHEY, G. C., JR. 2008.

A novel resistant maltodextrin alters gastrointestinal tolerance factors, fecal characteristics, and fecal microbiota in healthy adult humans. *J Am Coll Nutr,* 27**,** 356-66.

FAVA, F., GITAU, R., GRIFFIN, B. A., GIBSON, G. R., TUOHY, K. M. & LOVEGROVE, J. A. 2013. The type and quantity of dietary fat and carbohydrate alter faecal microbiome and short-chain fatty acid excretion in a metabolic syndrome 'at-risk' population. *Int J Obes (Lond),* 37**,** 216-23.

FERGUSON, M. J. & JONES, G. P. 2000. Production of short-chain fatty acids following in vitro fermentation of saccharides, saccharide esters, fructo-oligosaccharides, starches, modified starches and non-starch polysaccharides. *Journal of the Science of Food and Agriculture,* 80**,** 166-170.

FERNANDES, J., RAO, A. V. & WOLEVER, T. M. 2000. Different substrates and methane producing status affect short-chain fatty acid profiles produced by In vitro fermentation of human feces. *J Nutr,* 130**,** 1932-6.

FINLEY, J. W., BURRELL, J. B. & REEVES, P. G. 2007. Pinto bean consumption changes SCFA profiles in fecal fermentations, bacterial populations of the lower bowel, and lipid profiles in blood of humans. *J Nutr,* 137**,** 2391-8.

FLEISCHER, J., BUMBALO, R., BAUTZE, V., STROTMANN, J. & BREER, H. 2015. Expression of odorant receptor Olfr78 in enteroendocrine cells of the colon. *Cell Tissue Res,* 361**,** 697-710.

FLEMING , S. E., CHOI, S. Y. & FITCH, M. D. 1991. Absorption of Short-Chain Fatty Acids from the Rat Cecum in Vivo. *American Institute of Nutrition,* 121.

FLINT, H. J., BAYER, E. A., RINCON, M. T., LAMED, R. & WHITE, B. A. 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Micro,* 6**,** 121-131.

FROST, G., SLEETH, M. L., SAHURI-ARISOYLU, M., LIZARBE, B., CERDAN, S., BRODY, L., ANASTASOVSKA, J., GHOURAB, S., HANKIR, M., ZHANG, S., CARLING, D., SWANN, J. R., GIBSON, G., VIARDOT, A., MORRISON, D., THOMAS, E. L. & BELL, J. D. 2014. The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. *Nature Communications,* 5.

FROST, G. S., BRYNES, A. E., DHILLO, W. S., BLOOM, S. R. & MCBURNEY, M. I. 2003. The effects of fiber enrichment of pasta and fat content on gastric emptying, GLP-1, glucose, and insulin responses to a meal. *Eur J Clin Nutr,* 57**,** 293-8.

FUENTES, S., VAN NOOD, E., TIMS, S., HEIKAMP-DE JONG, I., TER BRAAK, C. J., KELLER, J. J., ZOETENDAL, E. G. & DE VOS, W. M. 2014. Reset of a critically disturbed microbial ecosystem: faecal transplant in recurrent Clostridium difficile infection. *Isme j,* 8**,** 1621-33.

FUJII, H., IWASE, M., OHKUMA, T., OGATA-KAIZU, S., IDE, H., KIKUCHI, Y., IDEWAKI, Y., JOUDAI, T., HIRAKAWA, Y., UCHIDA, K., SASAKI, S., NAKAMURA, U. & KITAZONO, T. 2013. Impact of dietary fiber intake on glycemic control, cardiovascular risk factors and chronic kidney disease in Japanese patients with type 2 diabetes mellitus: the Fukuoka Diabetes Registry. *Nutr J,* 12**,** 159.

FURUSAWA, Y., OBATA, Y., FUKUDA, S., ENDO, T. A., NAKATO, G., TAKAHASHI, D., NAKANISHI, Y., UETAKE, C., KATO, K., KATO, T., TAKAHASHI, M., FUKUDA, N. N., MURAKAMI, S., MIYAUCHI, E., HINO, S., ATARASHI, K., ONAWA, S., FUJIMURA, Y., LOCKETT, T., CLARKE, J. M., TOPPING, D. L., TOMITA, M., HORI, S., OHARA, O., MORITA, T., KOSEKI, H., KIKUCHI, J., HONDA, K., HASE, K. & OHNO, H. 2013.

Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature,* 504**,** 446-50.

GE, H., LI, X., WEISZMANN, J., WANG, P., BARIBAULT, H., CHEN, J. L., TIAN, H. & LI, Y. 2008. Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids. *Endocrinology,* 149**,** 4519-26.

GELISSEN, I. C. & EASTWOOD, M. A. 1995. Taurocholic acid adsorption during non-starch polysaccharide fermentation: an in vitro study. *Br J Nutr,* 74**,** 221-8.

GIBSON, G. R., BEATTY, E. R., WANG, X. & CUMMINGS, J. H. 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. *Gastroenterology,* 108**,** 975-82.

GIBSON, G. R. & ROBERFROID, M. B. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr,* 125**,** 1401-12.

GIETL, E., MENGERINK, W., SLEGTE, J. D., GIBSON, G., RASTALL, R., HEUVEL, E. V. D., DEN HEUVEL, E. V., DE SLEGTE, J. & VAN DEN HEUVEL, E. 2012. Factors involved in the in vitro fermentability of short carbohydrates in static faecal batch cultures. *International Journal of Carbohydrate Chemistry,* 2012**,** 197809-Article ID 197809.

GLEI, M., HOFMANN, T., KÜSTER, K., HOLLMANN, J., LINDHAUER, M. G. & POOL-ZOBEL, B. L. 2006. Both Wheat (Triticum aestivum) Bran Arabinoxylans and Gut Flora-Mediated Fermentation Products Protect Human Colon Cells from Genotoxic Activities of 4-Hydroxynonenal and Hydrogen Peroxide. *Journal of Agricultural and Food Chemistry,* 54**,** 2088-2095.

GOIRI, I., OREGUI, L. M. & GARCIA-RODRIGUEZ, A. 2009. Use of chitosans to modulate ruminal fermentation of a 50:50 forage-to-concentrate diet in sheep. *J Anim Sci,* 88**,** 749-55.

GRANITO, M., FOOD ANALYSIS LABORATORY, S. B. U., A CARACAS, VENEZUELA, FOOD ANALYSIS LABORATORY, S. B. U., A CARACAS, VENEZUELA, CHAMP, M., FUNCTIONS, D., HUMAN NUTRITION LABORATORY, N. I. F. A. R., F-44316 NANTES CEDEX 03, FRANCE, DAVID, A., FUNCTIONS, D., HUMAN NUTRITION LABORATORY, N. I. F. A. R., F-44316 NANTES CEDEX 03, FRANCE, BONNET, C., FUNCTIONS, D., HUMAN NUTRITION LABORATORY, N. I. F. A. R., F-44316 NANTES CEDEX 03, FRANCE, GUERRA, M. & FOOD ANALYSIS LABORATORY, S. B. U., A CARACAS, VENEZUELA 2001. Identification of gas-producing components in different varieties of Phaseolus vulgaris by in vitro fermentation. *Journal of the Science of Food and Agriculture,* 81**,** 543-550.

GRUNERT, S. C., MULLERLEILE, S., DE SILVA, L., BARTH, M., WALTER, M., WALTER, K., MEISSNER, T., LINDNER, M., ENSENAUER, R., SANTER, R., BODAMER, O. A., BAUMGARTNER, M. R., BRUNNER-KRAINZ, M., KARALL, D., HAASE, C., KNERR, I., MARQUARDT, T., HENNERMANN, J. B., STEINFELD, R., BEBLO, S., KOCH, H. G., KONSTANTOPOULOU, V., SCHOLL-BURGI, S., VAN TEEFFELEN-HEITHOFF, A., SUORMALA, T., SPERL, W., KRAUS, J. P., SUPERTI-FURGA, A., SCHWAB, K. O. & SASS, J. O. 2013. Propionic acidemia: clinical course and outcome in 55 pediatric and adolescent patients. *Orphanet J Rare Dis,* 8**,** 6.

GUILLON, F., BARRY, J. L. & THIBAULT, J. F. 1992. Effect of autoclaving sugar-beet fibre on its physico-chemical properties and its in-vitro degradation by human faecal bacteria. *Journal of the Science of Food and Agriculture,* 60**,** 69-79.

GUILLON, F. & CHAMP, M. M. 2002. Carbohydrate fractions of legumes: uses in human nutrition and potential for health. *Br J Nutr,* 88 Suppl 3**,** S293-306.

GULFI, M., ARRIGONI, E. & AMADO, R. 2007. In vitro fermentability of a pectin fraction rich in hairy regions. *Carbohydrate Polymers,* 67**,** 410-416.

GULFI, M., ARRIGONI, E. & AMADÒ, R. 2005. Influence of structure on in vitro fermentability of commercial pectins and partially hydrolysed pectin preparations. *Carbohydrate Polymers,* 59**,** 247-255.

GULLÓN, B., GULLÓN, P., SANZ, Y., ALONSO, J. L. & PARAJÓ, J. C. 2011. Prebiotic potential of a refined product containing pectic oligosaccharides. *LWT - Food Science and Technology,* 44**,** 1687-1696.

HA, A. W., KIM, W. K., KIM, J. H. & KANG, N. E. 2015. The supplementation effects of peanut sprout on reduction of abdominal fat and health indices in overweight and obese women. *Nutr Res Pract,* 9**,** 249-55.

HABIB, A. M., RESEARCH, C. I. F. M., RICHARDS, P., RESEARCH, C. I. F. M., ROGERS, G. J., RESEARCH, C. I. F. M., REIMANN, F., RESEARCH, C. I. F. M., FR222@CAM.AC.UK, GRIBBLE, F. M., RESEARCH, C. I. F. M. & FMG23@CAM.AC.UK 2013. Co-localisation and secretion of glucagon-like peptide 1 and peptide YY from primary cultured human L cells. *Diabetologia,* 56**,** 1413-1416.

HADJIAGAPIOU, C., SCHMIDT, L., DUDEJA, P. K., LAYDEN, T. J. & RAMASWAMY, K. 2000. Mechanism(s) of butyrate transport in Caco-2 cells: role of monocarboxylate transporter 1. *Am J Physiol Gastrointest Liver Physiol,* 279**,** G775-80.

HANDAYANI, D., MEYER, B. J., CHEN, J., BROWN, S. H. J., MITCHELL, T. W. & HUANG, X. F. 2014. A High-Dose Shiitake Mushroom Increases Hepatic Accumulation of Triacylglycerol in Rats Fed a High-Fat Diet: Underlying Mechanism. *Nutrients,* 6**,** 650-62.

HARTZELL, A. L., MALDONADO-GÓMEZ, M. X., HUTKINS, R. W. & ROSE, D. J. 2013. Synthesis and in vitro digestion and fermentation of acylated inulin. *Bioactive Carbohydrates and Dietary Fibre,* 1**,** 81-88.

HEIMANN, E., NYMAN, M. & DEGERMAN, E. 2015. Propionic acid and butyric acid inhibit lipolysis and de novo lipogenesis and increase insulin-stimulated glucose uptake in primary rat adipocytes. *Adipocyte,* 4**,** 81-8.

HEINE, H. 2016. *Universitat Dusseldorf: G*Power* [Online]. Contact. Available: http://www.gpower.hhu.de/en.html [Accessed].

HERNANDEZ-HERNANDEZ, O., CÔTÉ, G. L., KOLIDA, S., RASTALL, R. A. & SANZ, M. L. 2011. In Vitro Fermentation of Alternansucrase Raffinose-Derived Oligosaccharides by Human Gut Bacteria.

HERNANDEZ-SALAZAR, M., OSORIO-DIAZ, P., LOARCA-PINA, G., REYNOSO-CAMACHO, R., TOVAR, J. & BELLO-PEREZ, L. A. 2010. In vitro fermentability and antioxidant capacity of the indigestible fraction of cooked black beans (Phaseolus vulgaris L.), lentils (Lens culinaris L.) and chickpeas (Cicer arietinum L.). *J Sci Food Agric,* 90**,** 1417-22.

HINNEBUSCH, B. F., MENG, S., WU, J. T., ARCHER, S. Y. & HODIN, R. A. 2002. The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *J Nutr,* 132**,** 1012-7.

HIRASAWA, A., TSUMAYA, K., AWAJI, T., KATSUMA, S., ADACHI, T., YAMADA, M., SUGIMOTO, Y., MIYAZAKI, S. & TSUJIMOTO, G. 2005. Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat Med,* 11**,** 90-4.

HOFMANN, A. F. 1999. The continuing importance of bile acids in liver and intestinal disease. *Arch Intern Med,* 159**,** 2647-58.

HOPKINS, M. J., ENGLYST, H. N., MACFARLANE, S., FURRIE, E., MACFARLANE, G. T. & MCBAIN, A. J. 2003. Degradation of cross-linked and non-cross-linked arabinoxylans by the intestinal microbiota in children. *Appl Environ Microbiol,* 69**,** 6354-60.

HOSSEINI, E., GROOTAERT, C., VERSTRAETE, W. & VAN DE WIELE, T. 2011. Propionate as a health-promoting microbial metabolite in the human gut. *Nutr Rev,* 69**,** 245-58.

HUGHES, S. A., SHEWRY, P. R., GIBSON, G. R., MCCLEARY, B. V. & RASTALL, R. A. 2008. In vitro fermentation of oat and barley derived beta-glucans by human faecal microbiota. *FEMS Microbiol Ecol,* 64**,** 482-93.

HUGHES, S. A., SHEWRY, P. R., LI, L., GIBSON, G. R., SANZ, M. L. & RASTALL, R. A. 2007. In vitro fermentation by human fecal microflora of wheat arabinoxylans. *J Agric Food Chem,* 55**,** 4589-95.

HURST, N. R., KENDIG, D. M., MURTHY, K. S. & GRIDER, J. R. 2014. The short chain fatty acids, butyrate and propionate, have differential effects on the motility of the guinea pig colon. *Neurogastroenterol Motil,* 26**,** 1586-96.

IVARSSON, E., ANDERSSON, R. & LINDBERG, J. E. 2012. Molecular weight distribution of soluble fiber fractions and short chain fatty acids in ileal digesta of growing pigs. *J Anim Sci,* 90 Suppl 4**,** 65-7.

JENKINS, C. L. D., LEWIS, D., BUSHELL, R., BELOBRAJDIC, D. P. & BIRD, A. R. 2011. Chain length of cereal fructans isolated from wheat stem and barley grain modulates in vitro fermentation. *Journal of Cereal Science,* 53**,** 188-191.

JIANG, J., QIU, H., ZHAO, G., ZHOU, Y., ZHANG, Z., ZHANG, H., JIANG, Q., SUN, Q., WU, H., YANG, L., RUAN, X. & XU, W. H. 2012. Dietary Fiber Intake Is Associated with HbA1c Level among Prevalent Patients with Type 2 Diabetes in Pudong New Area of Shanghai, China. *PLoS One,* 7.

JIANG, T. & SAVAIANO, D. A. 1997. Modification of colonic fermentation by bifidobacteria and pH in vitro. Impact on lactose metabolism, short-chain fatty acid, and lactate production. *Dig Dis Sci,* 42**,** 2370-7.

JONATHAN, M. C., VAN DEN BORNE, J. J. G. C., VAN WIECHEN, P., SOUZA DA SILVA, C., SCHOLS, H. A. & GRUPPEN, H. 2012. In vitro fermentation of 12 dietary fibres by faecal inoculum from pigs and humans. *Food Chemistry,* 133**,** 889-897.

JOUET, P., MOUSSATA, D., DUBOC, H., BOSCHETTI, G., ATTAR, A., GORBATCHEF, C., SABATE, J. M., COFFIN, B. & FLOURIE, B. 2013. Effect of short-chain fatty acids and acidification on the phasic and tonic motor activity of the human colon. *Neurogastroenterol Motil,* 25**,** 943-9.

KAJI, I., KARAKI, S., TANAKA, R. & KUWAHARA, A. 2011. Density distribution of free fatty acid receptor 2 (FFA2)-expressing and GLP-1-producing enteroendocrine L cells in human and rat lower intestine, and increased cell numbers after ingestion of fructo-oligosaccharide. *J Mol Histol,* 42**,** 27-38.

KARAKI, S., TAZOE, H., HAYASHI, H., KASHIWABARA, H., TOOYAMA, K., SUZUKI, Y. & KUWAHARA, A. 2008. Expression of the short-chain fatty acid receptor, GPR43, in the human colon. *J Mol Histol,* 39**,** 135-42.

KARPPINEN, S., KIILIÄINEN, K., LIUKKONEN, K., FORSSELL, P. & POUTANEN, K. 2001. Extraction and in vitro Fermentation of Rye Bran Fractions. *Journal of Cereal Science,* 34**,** 269-278.

KARPPINEN, S., LIUKKONEN, K., AURA, A. M., FORSSELL, P. & POUTANEN, K. 2000. In vitro fermentation of polysaccharides of rye, wheat and oat brans and inulin by human faecal bacteria. *Journal of the Science of Food and Agriculture,* 80**,** 1469-1476.

KAUR, A., ROSE, D. J., RUMPAGAPORN, P., PATTERSON, J. A. & HAMAKER, B. R. 2011. In vitro batch fecal fermentation comparison of gas and short-chain fatty acid production using "slowly fermentable" dietary fibers. *J Food Sci,* 76**,** H137-42.

KEDIA, G., VAZQUEZ, J. A., CHARALAMPOPOULOS, D. & PANDIELLA, S. S. 2009. In vitro fermentation of oat bran obtained by debranning with a mixed culture of human fecal bacteria. *Curr Microbiol,* 58**,** 338-42.

KETTLE, H., LOUIS, P., HOLTROP, G., DUNCAN, S. H. & FLINT, H. J. 2015. Modelling the emergent dynamics and major metabolites of the human colonic microbiota. *Environ Microbiol,* 17**,** 1615-30.

KHALIL, N. A., WALTON, G. E., GIBSON, G. R., TUOHY, K. M. & ANDREWS, S. C. 2014. In vitro batch cultures of gut microbiota from healthy and ulcerative colitis (UC) subjects suggest that sulphate-reducing bacteria levels are raised in UC and by a protein-rich diet. *Int J Food Sci Nutr,* 65**,** 79-88.

KHAN, K. M. & EDWARDS, C. A. 2002. Effect of Substrate Concentration on Short Chain Fatty Acid Production in In vitro Cultures of Human Faeces with Lactulose, a Rapidly Fermented Carbohydrate. *http://dx.doi.org/10.1080/089106002320644348*.

KHAN, K. M. & EDWARDS, C. A. 2005. In vitro fermentation characteristics of a mixture of Raftilose and guar gum by human faecal bacteria. *Eur J Nutr,* 44**,** 371-6.

KIM, H. J. & WHITE, P. J. 2009. In Vitro Fermentation of Oat Flours from Typical and High beta-Glucan Oat Lines. *Journal of Agricultural and Food Chemistry,* 57**,** 7529-7536.

KIM, H. J. & WHITE, P. J. 2010. In vitro bile-acid binding and fermentation of high, medium, and low molecular weight beta-glucan. *J Agric Food Chem,* 58**,** 628-34.

KIM, H. J. & WHITE, P. J. 2011a. Molecular Weight of β-Glucan Affects Physical Characteristics, In Vitro Bile Acid Binding, and Fermentation of Muffins. *http://dx.doi.org/10.1094/CCHEM-06-10-0084*.

KIM, H. J. & WHITE, P. J. 2011b. Optimizing the molecular weight of oat beta-glucan for in vitro bile acid binding and fermentation. *J Agric Food Chem,* 59**,** 10322-8.

KIM, H. J. & WHITE, P. J. 2011c. Optimizing the Molecular Weight of Oat β-Glucan for in Vitro Bile Acid Binding and Fermentation.

KIM, M. H., KANG, S. G., PARK, J. H., YANAGISAWA, M. & KIM, C. H. 2013. Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. *Gastroenterology,* 145**,** 396-406.e1-10.

KIM, Y. & JE, Y. 2014. Dietary fiber intake and total mortality: a meta-analysis of prospective cohort studies. *Am J Epidemiol,* 180**,** 565-73.

KINROSS, J. M., DARZI, A. W. & NICHOLSON, J. K. 2011. Gut microbiome-host interactions in health and disease. *Genome Med,* 3**,** 14.

KISSILEFF, H. R., PI-SUNYER, F. X., THORNTON, J. & SMITH, G. P. 1981. C-terminal octapeptide of cholecystokinin decreases food intake in man. *Am J Clin Nutr,* 34**,** 154-60.

KNAPP, B. K., BAUER, L. L., SWANSON, K. S., TAPPENDEN, K. A., FAHEY, G. C., JR. & DE GODOY, M. R. 2013. Soluble fiber dextrin and soluble corn fiber supplementation modify indices of health in cecum and colon of Sprague-Dawley rats. *Nutrients,* 5**,** 396-410.

KOLIDA, S., TUOHY, K. & GIBSON, G. R. 2002. Prebiotic effects of inulin and oligofructose. *Br J Nutr.* England.

KOTCHARIANA, A. K., H. DONGOWSKIB, G 2004. The influence of variety on the enzymatic degradation of carrots and on functional and physiological properties of the cell wall materials. 87**,** 231–245.

KRUSE, H. P., KLEESSEN, B. & BLAUT, M. 1999. Effects of inulin on faecal bifidobacteria in human subjects. *Br J Nutr,* 82**,** 375-82.

KUDA, T., YANO, T., MATSUDA, N. & NISHIZAWA, M. 2005. Inhibitory effects of laminaran and low molecular alginate against the putrefactive compounds produced by intestinal microflora in vitro and in rats. *Food Chemistry,* 91**,** 745-749.

LADIRAT, S. E., SCHOLS, H. A., NAUTA, A., SCHOTERMAN, M. H. C., SCHUREN, F. H. J. & GRUPPEN, H. 2014. In vitro fermentation of galacto-oligosaccharides and its specific size-fractions using non-treated and amoxicillin-treated human inoculum. *Bioactive Carbohydrates and Dietary Fibre,* 3**,** 59-70.

LAHAYE, M., MICHEL, C. & BARRY, J. L. 1993. Chemical, physicochemical and in-vitro fermentation characteristics of dietary fibres from Palmaria palmata (L.) Kuntze. *Food Chemistry,* 47**,** 29-36.

LARSSON, S. C. & WOLK, A. 2014. Dietary fiber intake is inversely associated with stroke incidence in healthy Swedish adults. *J Nutr,* 144**,** 1952-5.

LAURENTIN, A. & EDWARDS, C. A. 2004. Differential fermentation of glucose-based carbohydrates in vitro by human faecal bacteria--a study of pyrodextrinised starches from different sources. *Eur J Nutr,* 43**,** 183-9.

LE CHATELIER, E., NIELSEN, T., QIN, J., PRIFTI, E., HILDEBRAND, F., FALONY, G., ALMEIDA, M., ARUMUGAM, M., BATTO, J. M., KENNEDY, S., LEONARD, P., LI, J., BURGDORF, K., GRARUP, N., JORGENSEN, T., BRANDSLUND, I., NIELSEN, H. B., JUNCKER, A. S., BERTALAN, M., LEVENEZ, F., PONS, N., RASMUSSEN, S., SUNAGAWA, S., TAP, J., TIMS, S., ZOETENDAL, E. G., BRUNAK, S., CLEMENT, K., DORE, J., KLEEREBEZEM, M., KRISTIANSEN, K., RENAULT, P., SICHERITZ-PONTEN, T., DE VOS, W. M., ZUCKER, J. D., RAES, J., HANSEN, T., BORK, P., WANG, J., EHRLICH, S. D. & PEDERSEN, O. 2013. Richness of human gut microbiome correlates with metabolic markers. *Nature,* 500**,** 541-6.

LE POUL, E., LOISON, C., STRUYF, S., SPRINGAEL, J. Y., LANNOY, V., DECOBECQ, M. E., BREZILLON, S., DUPRIEZ, V., VASSART, G., VAN DAMME, J., PARMENTIER, M. & DETHEUX, M. 2003. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J Biol Chem,* 278**,** 25481-9.

LEBET, V., ARRIGONI, E. & AMADÒ, R. 1998. Measurement of Fermentation Products and Substrate Disappearance During Incubation of Dietary Fibre Sources with Human Faecal Flora. *LWT - Food Science and Technology,* 31**,** 473-479.

LEI, F., YIN, Y., WANG, Y., DENG, B., YU, H. D., LI, L., XIANG, C., WANG, S., ZHU, B. & WANG, X. 2012. Higher-level production of volatile fatty acids in vitro by chicken gut microbiotas than by human gut microbiotas as determined by functional analyses. *Appl Environ Microbiol,* 78**,** 5763-72.

LEVRAT, M. A., MOUNDRAS, C., YOUNES, H., MORAND, C., DEMIGNE, C. & REMESY, C. 1996. Effectiveness of resistant starch, compared to guar gum, in depressing plasma cholesterol and enhancing fecal steroid excretion. *Lipids,* 31**,** 1069-75.

LEY, R. E., TURNBAUGH, P. J., KLEIN, S. & GORDON, J. I. 2006. Microbial ecology: human gut microbes associated with obesity. *Nature,* 444**,** 1022-3.

LILJEBERG, H. G. & BJORCK, I. M. 1996. Delayed gastric emptying rate as a potential mechanism for lowered glycemia after eating sourdough bread: studies in humans

and rats using test products with added organic acids or an organic salt. *Am J Clin Nutr,* 64**,** 886-93.

LILJEBERG, H. G., LONNER, C. H. & BJORCK, I. M. 1995. Sourdough fermentation or addition of organic acids or corresponding salts to bread improves nutritional properties of starch in healthy humans. *J Nutr,* 125**,** 1503-11.

LIN, H. V., FRASSETTO, A., KOWALIK JR, E. J., NAWROCKI, A. R., LU, M. M., KOSINSKI, J. R., HUBERT, J. A., SZETO, D., YAO, X., FORREST, G. & MARSH, D. J. 2012. Butyrate and Propionate Protect against Diet-Induced Obesity and Regulate Gut Hormones via Free Fatty Acid Receptor 3-Independent Mechanisms. *PLoS One,* 7.

LIU, S., WILLETT, W. C., MANSON, J. E., HU, F. B., ROSNER, B. & COLDITZ, G. 2003. Relation between changes in intakes of dietary fiber and grain products and changes in weight and development of obesity among middle-aged women. *Am J Clin Nutr,* 78**,** 920-7.

LOMBARD, V., GOLACONDA RAMULU, H., DRULA, E., COUTINHO, P. M. & HENRISSAT, B. 2014. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res,* 42**,** D490-5.

LOUIS, P., HOLD, G. L. & FLINT, H. J. 2014. The gut microbiota, bacterial metabolites and colorectal cancer. *Nature Reviews Microbiology,* 12**,** 661-672.

LOUIS, P., YOUNG, P., HOLTROP, G. & FLINT, H. J. 2010. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environ Microbiol,* 12**,** 304-14.

LTD, M. F. 2016. *What is Mycoprotein?* [Online]. Available: http://www.mycoprotein.org/ [Accessed].

LYLY, M., LIUKKONEN, K. H., SALMENKALLIO-MARTTILA, M., KARHUNEN, L., POUTANEN, K. & LAHTEENMAKI, L. 2009. Fibre in beverages can enhance perceived satiety. *Eur J Nutr,* 48**,** 251-8.

LYNCH, M. B., SWEENEY, T., CALLAN, J. J., O'SULLIVAN, J. T. & O'DOHERTY, J. V. 2010. The effect of dietary Laminaria-derived laminarin and fucoidan on nutrient digestibility, nitrogen utilisation, intestinal microflora and volatile fatty acid concentration in pigs. *J Sci Food Agric,* 90**,** 430-7.

MACFARLANE, G. T., MACFARLANE, S. & GIBSON, G. R. 1998. Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon. *Microbial Ecology,* 35**,** 180-187.

MACFARLANE, G. T., MEDICAL RESEARCH COUNCIL, D. C. N. C., CAMBRIDGE, UK, GIBSON, G. R., MEDICAL RESEARCH COUNCIL, D. C. N. C., CAMBRIDGE, UK, CUMMINGS, J. H. & MEDICAL RESEARCH COUNCIL, D. C. N. C., CAMBRIDGE, UK 1992. Comparison of fermentation reactions in different regions of the human colon. *Journal of Applied Bacteriology,* 72**,** 57-64.

MACFARLANE, S. & MACFARLANE, G. T. 2003. Regulation of short-chain fatty acid production. *Proc Nutr Soc,* 62**,** 67-72.

MAGEE, E. A., RICHARDSON, C. J., HUGHES, R. & CUMMINGS, J. H. 2000. Contribution of dietary protein to sulfide production in the large intestine: an in vitro and a controlled feeding study in humans. *Am J Clin Nutr,* 72**,** 1488-94.

MAKIVUOKKO, H., NURMI, J., NURMINEN, P., STOWELL, J. & RAUTONEN, N. 2005. In vitro effects on polydextrose by colonic bacteria and caco-2 cell cyclooxygenase gene expression. *Nutr Cancer,* 52**,** 94-104.

MALLILLIN, A. C., TRINIDAD, T. P., RATERTA, R., DAGBAY, K. & LOYOLA, A. S. 2008. Dietary fibre and fermentability characteristics of root crops and legumes. *Br J Nutr,* 100**,** 485-8.

MARTENS, E. C., KELLY, A. G., TAUZIN, A. S. & BRUMER, H. 2014. The Devil Lies in the Details: How Variations in Polysaccharide Fine-Structure Impact the Physiology and Evolution of Gut Microbes. *J Mol Biol,* 426**,** 3851-3865.

MARTENS, E. C., LOWE, E. C., CHIANG, H., PUDLO, N. A., WU, M., MCNULTY, N. P., ABBOTT, D. W., HENRISSAT, B., GILBERT, H. J., BOLAM, D. N. & GORDON, J. I. 2011. Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts. *PLoS Biol,* 9**,** e1001221.

MARTÍN BERNABÉ, A., SRIKAEO, K. & SCHLÜTER, M. 2011. Resistant Starch Content, Starch Digestibility and the Fermentation of Some Tropical Starches In Vitro. *Food Digestion,* 2**,** 37-42.

MARTINEZ, I., KIM, J., DUFFY, P. R., SCHLEGEL, V. L. & WALTER, J. 2010. Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects. *PLoS One,* 5**,** e15046.

MASLOWSKI, K. M., VIEIRA, A. T., NG, A., KRANICH, J., SIERRO, F., YU, D., SCHILTER, H. C., ROLPH, M. S., MACKAY, F., ARTIS, D., XAVIER, R. J., TEIXEIRA, M. M. & MACKAY, C. R. 2009a. Regulation of inflammatory responses by gut microbiota and chemoattractant. *Nature,* 461**,** 1282-6.

MASLOWSKI, K. M., VIEIRA, A. T., NG, A., KRANICH, J., SIERRO, F., YU, D., SCHILTER, H. C., ROLPH, M. S., MACKAY, F., ARTIS, D., XAVIER, R. J., TEIXEIRA, M. M. & MACKAY, C. R. 2009b. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature,* 461**,** 1282-6.

MATHUS-VLIEGEN, E. M. & DE GROOT, G. H. 2013. Fasting and meal-induced CCK and PP secretion following intragastric balloon treatment for obesity. *Obes Surg,* 23**,** 622-33.

MCBURNEY, M. I., CUFF, D. J. & THOMPSON, L. U. 1990. Rates of fermentation and short chain fatty acid and gas production of six starches by human faecal microbiota. *Journal of the Science of Food and Agriculture,* 50**,** 79-88.

MCBURNEY, M. I. & THOMPSON, L. U. 1989. Effect of human faecal donor on in vitro fermentation variables. *Scand J Gastroenterol,* 24**,** 359-67.

MCBURNEY, M. I. & THOMPSON, L. U. 1990. Fermentative characteristics of cereal brans and vegetable fibers. *Nutr Cancer,* 13**,** 271-80.

MCBURNEY, M. I., THOMPSON, L. U., CUFF, D. J. & JENKINS, D. J. 1988. Comparison of ileal effluents, dietary fibers, and whole foods in predicting the physiological importance of colonic fermentation. *Am J Gastroenterol,* 83**,** 536-40.

MCBURNEY, M. I., THOMPSON, LILIAN U. 1989. In Vitro Fermentabilities of Purified Fiber Supplements. *Journal of Food Science,* 54**,** 347-350.

MCCRORY, M. A., HAMAKER, B. R., LOVEJOY, J. C. & EICHELSDOERFER, P. E. 2010. Pulse consumption, satiety, and weight management. *Adv Nutr,* 1**,** 17-30.

MHURCHU, C. N., POPPITT, S. D., MCGILL, A. T., LEAHY, F. E., BENNETT, D. A., LIN, R. B., ORMROD, D., WARD, L., STRIK, C. & RODGERS, A. 2004. The effect of the dietary supplement, Chitosan, on body weight: a randomised controlled trial in 250 overweight and obese adults. *Int J Obes Relat Metab Disord,* 28**,** 1149-56.

MICHEL, C., KRAVTCHENKO, T. P., DAVID, A., GUENEAU, S., KOZLOWSKI, F. & CHERBUT, C. 1998. In vitro prebiotic effects of Acacia gums onto the human intestinal

microbiota depends on both botanical origin and environmental pH. *Anaerobe,* 4**,** 257-66.

MICHEL, C., LAHAYE, M., BONNET, C., MABEAU, S. & BARRY, J. L. 1996. In vitro fermentation by human faecal bacteria of total and purified dietary fibres from brown seaweeds. *Br J Nutr,* 75**,** 263-80.

MINEKUS, M., MARTEAU, P., HAVENAAR, R. & HUIS IN'T VELD, J. H. 1995. A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. ATLA Altern Lab Anim

Alternatives to laboratory animals, 23**,** 197-209.

MINEKUS, M., SMEETS-PEETERS, M., BERNALIER, A., MAROL-BONNIN, S., HAVENAAR, R., MARTEAU, P., ALRIC, M., FONTY, G. & HUIS IN'T VELD, J. H. 1999. A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. *Appl Microbiol Biotechnol,* 53**,** 108-14.

MOHER, D., LIBERATI, A., TETZLAFF, J. & ALTMAN, D. G. 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Ann Intern Med,* 151**,** 264-9, w64.

MOLLER, J. 2011. Dietary Fibre- Codex definition and methods of analysis. *In:* FOSS, W. P. B. (ed.). Denmark: FOSS.

MOLLY, K., VANDE WOESTYNE, M. & VERSTRAETE, W. 1993. Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. *Appl Microbiol Biotechnol,* 39**,** 254-8.

MORRISON, C. D., XI, X., WHITE, C. L., YE, J. & MARTIN, R. J. 2007. Amino acids inhibit Agrp gene expression via an mTOR-dependent mechanism. *Am J Physiol Endocrinol Metab,* 293**,** E165-71.

MORRISON, D. J., MACKAY, W. G., EDWARDS, C. A., PRESTON, T., DODSON, B. & WEAVER, L. T. 2006. Butyrate production from oligofructose fermentation by the human faecal flora: what is the contribution of extracellular acetate and lactate? *Br J Nutr,* 96**,** 570-7.

MORTENSEN, P. B., HOLTUG, K., BONNEN, H. & CLAUSEN, M. R. 1990. The degradation of amino acids, proteins, and blood to short-chain fatty acids in colon is prevented by lactulose. *Gastroenterology,* 98**,** 353-60.

MORTENSEN, P. B., HOLTUG, K. & RASMUSSEN, H. S. 1988. Short-chain fatty acid production from mono- and disaccharides in a fecal incubation system: implications for colonic fermentation of dietary fiber in humans. *J Nutr,* 118**,** 321-5.

MORTENSEN, P. B., HOVE, H., CLAUSEN, M. R. & HOLTUG, K. 1991. Fermentation to short-chain fatty acids and lactate in human faecal batch cultures. Intra- and inter-individual variations versus variations caused by changes in fermented saccharides. *Scand J Gastroenterol,* 26**,** 1285-94.

MORTENSEN, P. B. & NORDGAARD-ANDERSEN, I. 1993. The dependence of the in vitro fermentation of dietary fibre to short-chain fatty acids on the contents of soluble non-starch polysaccharides. *Scand J Gastroenterol,* 28**,** 418-22.

MULLE, J. G., SHARP, W. G. & CUBELLS, J. F. 2013. The Gut Microbiome: A New Frontier in Autism Research. *Curr Psychiatry Rep,* 15**,** 337.

MÜLLER, J., AESCHBACHER, R. A., WINGLER, A., BOLLER, T. & WIEMKEN, A. 2001. Trehalose and Trehalase in Arabidopsis. *Plant physiology*.

MUNJAL, U., GLEI, M., POOL-ZOBEL, B. L. & SCHARLAU, D. 2009. Fermentation products of inulin-type fructans reduce proliferation and induce apoptosis in human colon tumour cells of different stages of carcinogenesis. *Br J Nutr,* 102**,** 663-71.

MUNOZ-TAMAYO, R., LAROCHE, B., WALTER, E., DORE, J., DUNCAN, S. H., FLINT, H. J. & LECLERC, M. 2011. Kinetic modelling of lactate utilization and butyrate production by key human colonic bacterial species. *FEMS Microbiol Ecol,* 76**,** 615-24.

NIEMI, P., AURA, A. M., MAUKONEN, J., SMEDS, A. I., MATTILA, I., NIEMELA, K., TAMMINEN, T., FAULDS, C. B., BUCHERT, J. & POUTANEN, K. 2013. Interactions of a lignin-rich fraction from brewer's spent grain with gut microbiota in vitro. *J Agric Food Chem,* 61**,** 6754-62.

NILSSON, N. E., KOTARSKY, K., OWMAN, C. & OLDE, B. 2003. Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. *Biochem Biophys Res Commun,* 303**,** 1047-52.

NOACK, J., TIMM, D., HOSPATTANKAR, A. & SLAVIN, J. 2013. Fermentation profiles of wheat dextrin, inulin and partially hydrolyzed guar gum using an in vitro digestion pretreatment and in vitro batch fermentation system model. *Nutrients,* 5**,** 1500-10.

NORDGAARD, I., MORTENSEN, P. B. & LANGKILDE, A. M. 1995. Small intestinal malabsorption and colonic fermentation of resistant starch and resistant peptides to short-chain fatty acids. *Nutrition,* 11**,** 129-37.

NORDLUND, E., AURA, A. M., MATTILA, I., KOSSO, T., ROUAU, X. & POUTANEN, K. 2012. Formation of phenolic microbial metabolites and short-chain fatty acids from rye, wheat, and oat bran and their fractions in the metabolical in vitro colon model. *J Agric Food Chem,* 60**,** 8134-45.

NORDLUND, E., KATINA, K., AURA, A.-M. & POUTANEN, K. 2013. Changes in bran structure by bioprocessing with enzymes and yeast modifies the in vitro digestibility and fermentability of bran protein and dietary fibre complex. *Journal of Cereal Science,* 58**,** 200-208.

NUTTALL, F. Q., NGO, A. & GANNON, M. C. 2008. Regulation of hepatic glucose production and the role of gluconeogenesis in humans: is the rate of gluconeogenesis constant? *Diabetes Metab Res Rev,* 24**,** 438-58.

O'KEEFE, S. J. D., LI, J. V., LAHTI, L., OU, J., CARBONERO, F., MOHAMMED, K., POSMA, J. M., KINROSS, J., WAHL, E., RUDER, E., VIPPERLA, K., NAIDOO, V., MTSHALI, L., TIMS, S., PUYLAERT, P. G. B., DELANY, J., KRASINSKAS, A., BENEFIEL, A. C., KASEB, H. O., NEWTON, K., NICHOLSON, J. K., DE VOS, W. M., GASKINS, H. R. & ZOETENDAL, E. G. 2015. Fat, fibre and cancer risk in African Americans and rural Africans. *Nat Commun,* 6.

OHASHI Y, H. K., TOKUNAGA M, ISHIHARA N, OKUBO T, OGASAWARA T, JUEJA LR, FUJISAWA T 2012. Faecal fermentation of partially hydrolyzed guar gum. 4**,** 398–402.

OLANO-MARTIN, E., MOUNTZOURIS, K. C., GIBSON, G. R. & RASTALL, R. A. 2000. In vitro fermentability of dextran, oligodextran and maltodextrin by human gut bacteria. *Br J Nutr,* 83**,** 247-55.

OUFIR, L. E., BARRY, J. L., FLOURIE, B., CHERBUT, C., CLOAREC, D., BORNET, F. & GALMICHE, J. P. 2000. Relationships between transit time in man and in vitro fermentation of dietary fiber by fecal bacteria. *Eur J Clin Nutr,* 54**,** 603-9.

PARKAR, S. G., ROSENDALE, D., PATURI, G., HERATH, T. D., STOKLOSINSKI, H., PHIPPS, J. E., HEDDERLEY, D. & ANSELL, J. 2012. In vitro Utilization of Gold and Green Kiwifruit

Oligosaccharides by Human Gut Microbial Populations. *Plant Foods for Human Nutrition,* 67**,** 200-207.

PARKAR, S. G., TROWER, T. M. & STEVENSON, D. E. 2013. Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe,* 23**,** 12-19.

PARNELL, J. A. & REIMER, R. A. 2009. Weight loss during oligofructose supplementation is associated with decreased ghrelin and increased peptide YY in overweight and obese adults.

PAYNE, A. N., ZIHLER, A., CHASSARD, C. & LACROIX, C. 2012. Advances and perspectives in in vitro human gut fermentation modeling. *Trends Biotechnol,* 30**,** 17-25.

PEDERSEN, C., LEFEVRE, S., PETERS, V., PATTERSON, M., GHATEI, M. A., MORGAN, L. M. & FROST, G. S. 2013. Gut hormone release and appetite regulation in healthy non-obese participants following oligofructose intake. A dose-escalation study. *Appetite,* 66**,** 44-53.

PENHOAT, A., MUTEL, E., AMIGO-CORREIG, M., PILLOT, B., STEFANUTTI, A., RAJAS, F. & MITHIEUX, G. 2011. Protein-induced satiety is abolished in the absence of intestinal gluconeogenesis. *Physiol Behav,* 105**,** 89-93.

PERIC-HASSLER, L., HANSEN, H. S., BARON, R. & HUNENBERGER, P. H. 2010. Conformational properties of glucose-based disaccharides investigated using molecular dynamics simulations with local elevation umbrella sampling. *Carbohydr Res,* 345**,** 1781-801.

PERRY, R. J., PENG, L., BARRY, N. A., CLINE, G. W., ZHANG, D., CARDONE, R. L., PETERSEN, K. F., KIBBEY, R. G., GOODMAN, A. L. & SHULMAN, G. I. 2016. Acetate mediates a microbiome-brain-beta-cell axis to promote metabolic syndrome. *Nature,* 534**,** 213-7.

PETERS, S. G., POMARE, E. W. & FISHER, C. A. 1992. Portal and peripheral blood short chain fatty acid concentrations after caecal lactulose instillation at surgery. *Gut,* 33**,** 1249-52.

PICKARDT, C., DONGOWSKI, G. & KUNZEK, H. 2004. The influence of mechanical and enzymatic disintegration of carrots on the structure and properties of cell wall materials. *European Food Research and Technology,* 219**,** 229-239.

PLUZNICK, J. L., PROTZKO, R. J., GEVORGYAN, H., PETERLIN, Z., SIPOS, A., HAN, J., BRUNET, I., WAN, L. X., REY, F., WANG, T., FIRESTEIN, S. J., YANAGISAWA, M., GORDON, J. I., EICHMANN, A., PETI-PETERDI, J. & CAPLAN, M. J. 2013. Olfactory receptor responding to gut microbiota-derived signals plays a role in renin secretion and blood pressure regulation. *Proc Natl Acad Sci U S A,* 110**,** 4410-5.

POUTEAU, E., VAHEDI, K., MESSING, B., FLOURIE, B., NGUYEN, P., DARMAUN, D. & KREMPF, M. 1998. Production rate of acetate during colonic fermentation of lactulose: a stable-isotope study in humans. *Am J Clin Nutr,* 68**,** 1276-83.

PSICHAS, A., SLEETH, M. L., MURPHY, K. G., BROOKS, L., BEWICK, G. A., HANYALOGLU, A. C., GHATEI, M. A., BLOOM, S. R. & FROST, G. 2014. The short chain fatty acid propionate stimulates GLP-1 and PYY secretion via free fatty acid receptor 2 in rodents. *International Journal of Obesity,* 39**,** 424-429.

PYLKAS, A. M., JUNEJA, L. R. & SLAVIN, J. L. 2005. Comparison of different fibers for in vitro production of short chain fatty acids by intestinal microflora. *J Med Food,* 8**,** 113-6.

QIN, J., LI, R., RAES, J., ARUMUGAM, M., BURGDORF, K. S., MANICHANH, C., NIELSEN, T., PONS, N., LEVENEZ, F., YAMADA, T., MENDE, D. R., LI, J., XU, J., LI, S., LI, D., CAO, J., WANG, B., LIANG, H., ZHENG, H., XIE, Y., TAP, J., LEPAGE, P., BERTALAN, M.,

BATTO, J. M., HANSEN, T., LE PASLIER, D., LINNEBERG, A., NIELSEN, H. B., PELLETIER, E., RENAULT, P., SICHERITZ-PONTEN, T., TURNER, K., ZHU, H., YU, C., JIAN, M., ZHOU, Y., LI, Y., ZHANG, X., QIN, N., YANG, H., WANG, J., BRUNAK, S., DORE, J., GUARNER, F., KRISTIANSEN, K., PEDERSEN, O., PARKHILL, J., WEISSENBACH, J., BORK, P. & EHRLICH, S. D. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature,* 464**,** 59-65.

QUEENAN, K. M., STEWART, M. L., SMITH, K. N., THOMAS, W., FULCHER, R. G. & SLAVIN, J. L. 2007. Concentrated oat beta-glucan, a fermentable fiber, lowers serum cholesterol in hypercholesterolemic adults in a randomized controlled trial. *Nutr J,* 6**,** 6.

QUORN, M. F. L. 2016. *What is Mycoprotein?* [Online]. Available: http://www.mycoprotein.org/ [Accessed].

RAMASAMY, U. S., VENEMA, K., SCHOLS, H. A. & GRUPPEN, H. 2014. Effect of soluble and insoluble fibers within the in vitro fermentation of chicory root pulp by human gut bacteria. *J Agric Food Chem,* 62**,** 6794-802.

RAMNANI, P., CHITARRARI, R., TUOHY, K., GRANT, J., HOTCHKISS, S., PHILP, K., CAMPBELL, R., GILL, C. & ROWLAND, I. 2012. In vitro fermentation and prebiotic potential of novel low molecular weight polysaccharides derived from agar and alginate seaweeds. *Anaerobe,* 18**,** 1-6.

RASMUSSEN, H. S., HOLTUG, K. & MORTENSEN, P. B. 1988. Degradation of amino acids to short-chain fatty acids in humans. An in vitro study. *Scand J Gastroenterol,* 23**,** 178-82.

RAVI KUMAR, M. N. V. 2000. A review of chitin and chitosan applications. *Reactive and Functional Polymers,* 46**,** 1-27.

READ, N. W., MILES, C. A., FISHER, D., HOLGATE, A. M., KIME, N. D., MITCHELL, M. A., REEVE, A. M., ROCHE, T. B. & WALKER, M. 1980. Transit of a meal through the stomach, small intestine, and colon in normal subjects and its role in the pathogenesis of diarrhea. *Gastroenterology,* 79**,** 1276-82.

RECHKEMMER, G. & VON ENGELHARDT, W. 1988. Concentration- and pH-dependence of short-chain fatty acid absorption in the proximal and distal colon of guinea pig (Cavia porcellus). *Comp Biochem Physiol A Comp Physiol,* 91**,** 659-63.

REICHARDT, N., DUNCAN, S. H., YOUNG, P., BELENGUER, A., MCWILLIAM LEITCH, C., SCOTT, K. P., FLINT, H. J. & LOUIS, P. 2014. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. *Isme j,* 8**,** 1323-35.

REMELY, M., AUMUELLER, E., MEROLD, C., DWORZAK, S., HIPPE, B., ZANNER, J., POINTNER, A., BRATH, H. & HASLBERGER, A. G. 2014. Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity. *Gene,* 537**,** 85-92.

RIDAURA, V. K., FAITH, J. J., REY, F. E., CHENG, J., DUNCAN, A. E., KAU, A. L., GRIFFIN, N. W., LOMBARD, V., HENRISSAT, B., BAIN, J. R., MUEHLBAUER, M. J., ILKAYEVA, O., SEMENKOVICH, C. F., FUNAI, K., HAYASHI, D. K., LYLE, B. J., MARTINI, M. C., URSELL, L. K., CLEMENTE, J. C., TREUREN, W. V., WALTERS, W. A., KNIGHT, R., NEWGARD, C. B., HEATH, A. C. & GORDON, J. I. 2013. Gut Microbiota from Twins Discordant for Obesity Modulate Metabolism in Mice.

ROBERFROID, M., GIBSON, G. R., HOYLES, L., MCCARTNEY, A. L., RASTALL, R., ROWLAND, I., WOLVERS, D., WATZL, B., SZAJEWSKA, H., STAHL, B., GUARNER, F., RESPONDEK, F., WHELAN, K., COXAM, V., DAVICCO, M. J., LEOTOING, L., WITTRANT, Y.,

DELZENNE, N. M., CANI, P. D., NEYRINCK, A. M. & MEHEUST, A. 2010. Prebiotic effects: metabolic and health benefits. *Br J Nutr,* 104 Suppl 2**,** S1-63.

RODES, L., PAUL, A., COUSSA-CHARLEY, M., AL-SALAMI, H., TOMARO-DUCHESNEAU, C., FAKHOURY, M. & PRAKASH, S. 2011. Transit time affects the community stability of Lactobacillus and Bifidobacterium species in an in vitro model of human colonic microbiotia. *Artif Cells Blood Substit Immobil Biotechnol,* 39**,** 351-6.

ROEDIGER, W. E. 1980. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut,* 21**,** 793-8.

ROGERS, T. E., PUDLO, N. A., KOROPATKIN, N. M., BELL, J. S., MOYA BALASCH, M., JASKER, K. & MARTENS, E. C. 2013. Dynamic responses of Bacteroides thetaiotaomicron during growth on glycan mixtures. *Mol Microbiol,* 88**,** 876-90.

ROSE, D. J., PATTERSON, J. A. & HAMAKER, B. R. 2010. Structural differences among alkali-soluble arabinoxylans from maize (Zea mays), rice (Oryza sativa), and wheat (Triticum aestivum) brans influence human fecal fermentation profiles. *J Agric Food Chem,* 58**,** 493-9.

ROSENDALE, D. I., BLATCHFORD, P. A., SIMS, I. M., PARKAR, S. G., CARNACHAN, S. M., HEDDERLEY, D. & ANSELL, J. 2012. Characterizing kiwifruit carbohydrate utilization in vitro and its consequences for human faecal microbiota. *J Proteome Res,* 11**,** 5863-75.

RUIJSCHOP, R. M. A. J., BOELRIJK, A. E. M. & TE GIFFEL, M. C. 2008. Satiety effects of a dairy beverage fermented with propionic acid bacteria. *International Dairy Journal,* 18**,** 945-950.

RUMPAGAPORN, P., KAUR, A., CAMPANELLA, O. H., PATTERSON, J. A. & HAMAKER, B. R. 2012. Heat and pH stability of alkali-extractable corn arabinoxylan and its xylanase-hydrolyzate and their viscosity behavior. *J Food Sci,* 77**,** H23-30.

RUXTON, C. H. S. & MCMILLAN, B. 2010. The impact of mycoprotein on blood cholesterol levels: a pilot study. *British Food Journal,* 112**,** 1092-1101.

RYCROFT, C. E., JONES, M. R., GIBSON, G. R. & RASTALL, R. A. 2001a. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *J Appl Microbiol,* 91**,** 878-87.

RYCROFT, C. E., JONES, M. R., GIBSON, G. R. & RASTALL, R. A. 2001b. Fermentation properties of gentio-oligosaccharides. *Lett Appl Microbiol,* 32**,** 156-61.

SALAZAR, N., GUEIMONDE, M., MARIA HERNANDEZ-BARRANCO, A., RUAS-MADIEDO, P. & REYES-GAVILAN, C. G. D. L. 2008. Exopolysaccharides produced by intestinal Bifidobacterium strains act as fermentable substrates for human intestinal bacteria. *Applied and Environmental Microbiology,* 74**,** 4737-4745.

SALVADOR, V., CHERBUT, C., BARRY, J. L., BERTRAND, D., BONNET, C. & DELORT-LAVAL, J. 1993. Sugar composition of dietary fibre and short-chain fatty acid production during in vitro fermentation by human bacteria. *Br J Nutr,* 70**,** 189-97.

SAMUEL, B. S., SHAITO, A., MOTOIKE, T., REY, F. E., BACKHED, F., MANCHESTER, J. K., HAMMER, R. E., WILLIAMS, S. C., CROWLEY, J., YANAGISAWA, M. & GORDON, J. I. 2008. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. *Proc Natl Acad Sci U S A,* 105**,** 16767-72.

SANZ, M. L., COTE, G. L., GIBSON, G. R. & RASTALL, R. A. 2006a. Influence of glycosidic linkages and molecular weight on the fermentation of maltose-based oligosaccharides by human gut bacteria. *J Agric Food Chem,* 54**,** 9779-84.

SANZ, M. L., COTE, G. L., GIBSON, G. R. & RASTALL, R. A. 2006b. Selective fermentation of gentiobiose-derived oligosaccharides by human gut bacteria and influence of molecular weight. *FEMS Microbiol Ecol,* 56**,** 383-8.

SANZ, M. L., GIBSON, G. R. & RASTALL, R. A. 2005a. Influence of disaccharide structure on prebiotic selectivity in vitro. *J Agric Food Chem,* 53**,** 5192-9.

SANZ, M. L., GIBSON, G. R. & RASTALL, R. A. 2005b. Influence of Disaccharide Structure on Prebiotic Selectivity in Vitro.

SANZ, M. L., GLENN R. GIBSON, A. & RASTALL*, R. A. 2005c. Influence of Disaccharide Structure on Prebiotic Selectivity in Vitro.

SARBINI, S. R., KOLIDA, S., NAEYE, T., EINERHAND, A., BRISON, Y., REMAUD-SIMEON, M., MONSAN, P., GIBSON, G. R. & RASTALL, R. A. 2011. In vitro fermentation of linear and alpha-1,2-branched dextrans by the human fecal microbiota. *Appl Environ Microbiol,* 77**,** 5307-15.

SAYAR, S., JANNINK, J. L. & WHITE, P. J. 2007. Digestion residues of typical and high-beta-glucan oat flours provide substrates for in vitro fermentation. *J Agric Food Chem,* 55**,** 5306-11.

SELLIN, J. H. 1999. SCFAs: The Enigma of Weak Electrolyte Transport in the Colon. *News Physiol Sci,* 14**,** 58-64.

SILJESTROM, M., BJORCK, I. & WESTERLUND, E. 1989. TRANSGLYCOSIDATION REACTIONS FOLLOWING HEAT-TREATMENT OF STARCH - EFFECTS ON ENZYMIC DIGESTIBILITY. *Starch-Starke,* 41**,** 95-100.

SINGH, N., GURAV, A., SIVAPRAKASAM, S., BRADY, E., PADIA, R., SHI, H., THANGARAJU, M., PRASAD, P. D., MANICASSAMY, S., MUNN, D. H., LEE, J. R., OFFERMANNS, S. & GANAPATHY, V. 2014. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity,* 40**,** 128-39.

SLAVIN, J. 2013. Fiber and Prebiotics: Mechanisms and Health Benefits. *Nutrients,* 5**,** 1417-35.

SLEETH, M. L., THOMPSON, E. L., FORD, H. E., ZAC-VARGHESE, S. E. & FROST, G. 2010. Free fatty acid receptor 2 and nutrient sensing: a proposed role for fibre, fermentable carbohydrates and short-chain fatty acids in appetite regulation. *Nutr Res Rev,* 23**,** 135-45.

SMITH, E. A. & MACFARLANE, G. T. 1997. Dissimilatory amino Acid metabolism in human colonic bacteria. *Anaerobe,* 3**,** 327-37.

SPADAFRANCA, A., RINELLI, S., RIVA, A., MORAZZONI, P., MAGNI, P., BERTOLI, S. & BATTEZZATI, A. 2013. Phaseolus vulgaris extract affects glycometabolic and appetite control in healthy human subjects. *Br J Nutr,* 109**,** 1789-95.

SPILLER, G. A., CHERNOFF, M. C., HILL, R. A., GATES, J. E., NASSAR, J. J. & SHIPLEY, E. A. 1980. Effect of purified cellulose, pectin, and a low-residue diet on fecal volatile fatty acids, transit time, and fecal weight in humans. *Am J Clin Nutr,* 33**,** 754-9.

SQUIRES, P. E., RUMSEY, R. D., EDWARDS, C. A. & READ, N. W. 1992. Effect of short-chain fatty acids on contractile activity and fluid flow in rat colon in vitro. *Am J Physiol,* 262**,** G813-7.

STEVENS, J., LEVITSKY, D. A., VANSOEST, P. J., ROBERTSON, J. B., KALKWARF, H. J. & ROE, D. A. 1987. Effect of psyllium gum and wheat bran on spontaneous energy intake. *Am J Clin Nutr,* 46**,** 812-7.

STEVENSON, A., BUCHANAN, C. J., ABIA, R. & EASTWOOD, M. A. 1997. A Simple In Vitro Fermentation System for Polysaccharidesâ”The Effects of Fermenter Fluid

Surface Area/Fluid Volume Ratio and Amount of Substrate. *Journal of the Science of Food and Agriculture,* 73**,** 101-105.

STEWART, M. L. & SLAVIN, J. L. 2006. Molecular weight of guar gum affects short-chain fatty acid profile in model intestinal fermentation. *Mol Nutr Food Res,* 50**,** 971-6.

STEWART, M. L. & SLAVIN, J. L. 2009. Particle size and fraction of wheat bran influence short-chain fatty acid production in vitro. *Br J Nutr,* 102**,** 1404-7.

STEWART, M. L., TIMM, D. A. & SLAVIN, J. L. 2008. Fructooligosaccharides exhibit more rapid fermentation than long-chain inulin in an in vitro fermentation system. *Nutr Res,* 28**,** 329-34.

SUNVOLD, G. D., HUSSEIN, H. S., FAHEY, G. C., JR., MERCHEN, N. R. & REINHART, G. A. 1995. In vitro fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and ruminal fluid from cattle. *J Anim Sci,* 73**,** 3639-48.

TANG, C., AHMED, K., GILLE, A., LU, S., GRONE, H. J., TUNARU, S. & OFFERMANNS, S. 2015. Loss of FFA2 and FFA3 increases insulin secretion and improves glucose tolerance in type 2 diabetes. *Nat Med,* 21**,** 173-7.

TARINI, J. & WOLEVER, T. M. 2010. The fermentable fibre inulin increases postprandial serum short-chain fatty acids and reduces free-fatty acids and ghrelin in healthy subjects. *Appl Physiol Nutr Metab,* 35**,** 9-16.

TEDELIND, S., WESTBERG, F., KJERRULF, M. & VIDAL, A. 2007. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. *World J Gastroenterol,* 13**,** 2826-32.

THANE, C. W., STEPHEN, A. M. & JEBB, S. A. 2009. Whole grains and adiposity: little association among British adults. *Eur J Clin Nutr,* 63**,** 229-37.

THANGARAJU, M., CRESCI, G. A., LIU, K., ANANTH, S., GNANAPRAKASAM, J. P., BROWNING, D. D., MELLINGER, J. D., SMITH, S. B., DIGBY, G. J., LAMBERT, N. A., PRASAD, P. D. & GANAPATHY, V. 2009. GPR109A is a G-protein-coupled receptor for the bacterial fermentation product butyrate and functions as a tumor suppressor in colon. *Cancer Res,* 69**,** 2826-32.

THOMPSON, L. U., MANINGAT, C. C., WOO, K. & SEIB, P. A. 2011. In Vitro Digestion of RS4-Type Resistant Wheat and Potato Starches, and Fermentation of Indigestible Fractions. *Cereal Chemistry,* 88**,** 72-79.

THREAPLETON, D. E., GREENWOOD, D. C., EVANS, C. E., CLEGHORN, C. L., NYKJAER, C., WOODHEAD, C., CADE, J. E., GALE, C. P. & BURLEY, V. J. 2013. Dietary fibre intake and risk of cardiovascular disease: systematic review and meta-analysis. *Bmj,* 347**,** f6879.

TITGEMEYER, E. C., BOURQUIN, L. D., FAHEY, G. C., JR. & GARLEB, K. A. 1991. Fermentability of various fiber sources by human fecal bacteria in vitro. *Am J Clin Nutr,* 53**,** 1418-24.

TODESCO, T., RAO, A. V., BOSELLO, O. & JENKINS, D. J. 1991. Propionate lowers blood glucose and alters lipid metabolism in healthy subjects. *Am J Clin Nutr,* 54**,** 860-5.

TOLHURST, G., HEFFRON, H., LAM, Y. S., PARKER, H. E., HABIB, A. M., DIAKOGIANNAKI, E., CAMERON, J., GROSSE, J., REIMANN, F. & GRIBBLE, F. M. 2012. Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. *Diabetes,* 61**,** 364-71.

TOPPING, D. L. & CLIFTON, P. M. 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol Rev,* 81**,** 1031-64.

TRINIDAD, T. P., WOLEVER, T. M. & THOMPSON, L. U. 1996. Availability of calcium for absorption in the small intestine and colon from diets containing available and unavailable carbohydrates: an in vitro assessment. *Int J Food Sci Nutr,* 47**,** 83-8.

TROMPETTE, A., GOLLWITZER, E. S., YADAVA, K., SICHELSTIEL, A. K., SPRENGER, N., NGOM-BRU, C., BLANCHARD, C., JUNT, T., NICOD, L. P., HARRIS, N. L. & MARSLAND, B. J. 2014. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med,* 20**,** 159-66.

TURNBULL, W. H., BESSEY, D., WALTON, J. & LEEDS, A. R. 1991. *THE EFFECT OF MYCO-PROTEIN ON HUNGER, SATIETY AND SUBSEQUENT FOOD-CONSUMPTION*.

TURNBULL, W. H., LEEDS, A. R. & EDWARDS, D. G. 1992. Mycoprotein reduces blood lipids in free-living subjects. *Am J Clin Nutr,* 55**,** 415-9.

TURNBULL, W. H., LEEDS, A. R. & EDWARDS, G. D. 1990. EFFECT OF MYCOPROTEIN ON BLOOD-LIPIDS. *American Journal of Clinical Nutrition,* 52**,** 646-650.

TURNBULL, W. H., WALTON, J. & LEEDS, A. R. 1993a. ACUTE EFFECTS OF MYCOPROTEIN ON SUBSEQUENT ENERGY-INTAKE AND APPETITE VARIABLES. *American Journal of Clinical Nutrition,* 58**,** 507-512.

TURNBULL, W. H., WALTON, J. & LEEDS, A. R. 1993b. Acute effects of mycoprotein on subsequent energy intake and appetite variables. *Am J Clin Nutr,* 58**,** 507-12.

TURNBULL, W. H. & WARD, T. 1995. MYCOPROTEIN REDUCES GLYCEMIA AND INSULINEMIA WHEN TAKEN WITH AN ORAL GLUCOSE-TOLERANCE TEST. *American Journal of Clinical Nutrition,* 61**,** 135-140.

TURNBULL, W. H., WARD, T. & WALTON, J. 1994. Mycoprotein reduces glycemia, insulinemia, energy intake, and appetite. *International Journal of Obesity,* 18**,** 136-136.

TYAGI, S., VENUGOPALAKRISHNAN, J., RAMASWAMY, K. & DUDEJA, P. K. 2002. Mechanism of n-butyrate uptake in the human proximal colonic basolateral membranes. *Am J Physiol Gastrointest Liver Physiol,* 282**,** G676-82.

UDALL, J. N., LO, C. W., YOUNG, V. R. & SCRIMSHAW, N. S. 1984. The tolerance and nutritional value of two microfungal foods in human subjects. *Am J Clin Nutr,* 40**,** 285-92.

UHE, A. M., COLLIER, G. R. & O'DEA, K. 1992. A comparison of the effects of beef, chicken and fish protein on satiety and amino acid profiles in lean male subjects. *J Nutr,* 122**,** 467-72.

VALJAMAE, P., SILD, V., PETTERSSON, G. & JOHANSSON, G. 1998. The initial kinetics of hydrolysis by cellobiohydrolases I and II is consistent with a cellulose surface-erosion model. *Eur J Biochem,* 253**,** 469-75.

VAN DEN ABBEELE, P., GERARD, P., RABOT, S., BRUNEAU, A., EL AIDY, S., DERRIEN, M., KLEEREBEZEM, M., ZOETENDAL, E. G., SMIDT, H., VERSTRAETE, W., VAN DE WIELE, T. & POSSEMIERS, S. 2011. Arabinoxylans and inulin differentially modulate the mucosal and luminal gut microbiota and mucin-degradation in humanized rats. *Environ Microbiol,* 13**,** 2667-80.

VAN DEN ABBEELE, P., VENEMA, K., VAN DE WIELE, T., VERSTRAETE, W. & POSSEMIERS, S. 2013. Different human gut models reveal the distinct fermentation patterns of Arabinoxylan versus inulin. *J Agric Food Chem,* 61**,** 9819-27.

VEERIAH, S., HOFMANN, T., GLEI, M., DIETRICH, H., WILL, F., SCHREIER, P., KNAUP, B. & POOL-ZOBEL, B. L. 2007. Apple polyphenols and products formed in the gut differently inhibit survival of human cell lines derived from colon adenoma (LT97) and carcinoma (HT29). *J Agric Food Chem,* 55**,** 2892-900.

VELDHORST, M. A., WESTERTERP-PLANTENGA, M. S. & WESTERTERP, K. R. 2009. Gluconeogenesis and energy expenditure after a high-protein, carbohydrate-free diet. *Am J Clin Nutr,* 90**,** 519-26.

VERBEKE, K., FERCHAUD-ROUCHER, V., PRESTON, T., SMALL, A. C., HENCKAERTS, L., KREMPF, M., WANG, H., VONK, R. J. & PRIEBE, M. G. 2010. Influence of the type of indigestible carbohydrate on plasma and urine short-chain fatty acid profiles in healthy human volunteers. *Eur J Clin Nutr,* 64**,** 678-84.

VERBEKE, K. A., BOOBIS, A. R., CHIODINI, A., EDWARDS, C. A., FRANCK, A., KLEEREBEZEM, M., NAUTA, A., RAES, J., VAN TOL, E. A. & TUOHY, K. M. 2015. Towards microbial fermentation metabolites as markers for health benefits of prebiotics. *Nutr Res Rev,* 28**,** 42-66.

VERDICH, C., FLINT, A., GUTZWILLER, J. P., NASLUND, E., BEGLINGER, C., HELLSTROM, P. M., LONG, S. J., MORGAN, L. M., HOLST, J. J. & ASTRUP, A. 2001. A meta-analysis of the effect of glucagon-like peptide-1 (7-36) amide on ad libitum energy intake in humans. *J Clin Endocrinol Metab,* 86**,** 4382-9.

VERNIA, P., CITTADINI, M., CAPRILLI, R. & TORSOLI, A. 1995. Topical treatment of refractory distal ulcerative colitis with 5-ASA and sodium butyrate. *Dig Dis Sci,* 40**,** 305-7.

VITAGLIONE, P., LUMAGA, R. B., STANZIONE, A., SCALFI, L. & FOGLIANO, V. 2009. beta-Glucan-enriched bread reduces energy intake and modifies plasma ghrelin and peptide YY concentrations in the short term. *Appetite,* 53**,** 338-44.

VOGT, J. A., ISHII-SCHRADE, K. B., PENCHARZ, P. B., JONES, P. J. & WOLEVER, T. M. 2006. L-rhamnose and lactulose decrease serum triacylglycerols and their rates of synthesis, but do not affect serum cholesterol concentrations in men. *J Nutr,* 136**,** 2160-6.

VOGT, J. A., ISHII-SCHRADE, K. B., PENCHARZ, P. B. & WOLEVER, T. M. 2004a. L-Rhamnose increases serum propionate after long-term supplementation, but lactulose does not raise serum acetate. *Am J Clin Nutr,* 80**,** 1254-61.

VOGT, J. A., PENCHARZ, P. B. & WOLEVER, T. M. 2004b. L-Rhamnose increases serum propionate in humans. *Am J Clin Nutr,* 80**,** 89-94.

VOGT, J. A. & WOLEVER, T. M. 2003. Fecal acetate is inversely related to acetate absorption from the human rectum and distal colon. *J Nutr,* 133**,** 3145-8.

WALDECKER, M., KAUTENBURGER, T., DAUMANN, H., BUSCH, C. & SCHRENK, D. 2008a. Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon. *J Nutr Biochem,* 19**,** 587-93.

WALDECKER, M., KAUTENBURGER, T., DAUMANN, H., VEERIAH, S., WILL, F., DIETRICH, H., POOL-ZOBEL, B. L. & SCHRENK, D. 2008b. Histone-deacetylase inhibition and butyrate formation: Fecal slurry incubations with apple pectin and apple juice extracts. *Nutrition,* 24**,** 366-74.

WALKER, A. W., DUNCAN, S. H., MCWILLIAM LEITCH, E. C., CHILD, M. W. & FLINT, H. J. 2005. pH and Peptide Supply Can Radically Alter Bacterial Populations and Short-Chain Fatty Acid Ratios within Microbial Communities from the Human Colon. *Appl Environ Microbiol,* 71**,** 3692-700.

WALKER, A. W., INCE, J., DUNCAN, S. H., WEBSTER, L. M., HOLTROP, G., ZE, X., BROWN, D., STARES, M. D., SCOTT, P., BERGERAT, A., LOUIS, P., MCINTOSH, F., JOHNSTONE, A. M., LOBLEY, G. E., PARKHILL, J. & FLINT, H. J. 2011. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *Isme j,* 5**,** 220-30.

WANDERS, A. J., VAN DEN BORNE, J. J., DE GRAAF, C., HULSHOF, T., JONATHAN, M. C., KRISTENSEN, M., MARS, M., SCHOLS, H. A. & FESKENS, E. J. 2011. Effects of dietary fibre on subjective appetite, energy intake and body weight: a systematic review of randomized controlled trials. *Obes Rev,* 12**,** 724-39.

WANG, X. & GIBSON, G. R. 1993. Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *J Appl Bacteriol,* 75**,** 373-80.

WANG, Y. T., MOHAMMED, S. D., FARMER, A. D., WANG, D., ZARATE, N., HOBSON, A. R., HELLSTROM, P. M., SEMLER, J. R., KUO, B., RAO, S. S., HASLER, W. L., CAMILLERI, M. & SCOTT, S. M. 2015. Regional gastrointestinal transit and pH studied in 215 healthy volunteers using the wireless motility capsule: influence of age, gender, study country and testing protocol. *Aliment Pharmacol Ther,* 42**,** 761-72.

WANNET, W. J., OP DEN CAMP, H. J., WISSELINK, H. W., VAN DER DRIFT, C., VAN GRIENSVEN, L. J. & VOGELS, G. D. 1998. Purification and characterization of trehalose phosphorylase from the commercial mushroom Agaricus bisporus. *Biochim Biophys Acta,* 1425**,** 177-88.

WEAVER, G. A., KRAUSE, J. A., MILLER, T. L. & WOLIN, M. J. 1989. Constancy of glucose and starch fermentations by two different human faecal microbial communities. *Gut,* 30**,** 19-25.

WEAVER, G. A., KRAUSE, J. A., MILLER, T. L. & WOLIN, M. J. 1992. Cornstarch fermentation by the colonic microbial community yields more butyrate than does cabbage fiber fermentation; cornstarch fermentation rates correlate negatively with methanogenesis. *Am J Clin Nutr,* 55**,** 70-7.

WEICKERT, M. O., ARAFAT, A. M., BLAUT, M., ALPERT, C., BECKER, N., LEUPELT, V., RUDOVICH, N., MOHLIG, M. & PFEIFFER, A. F. 2011. Changes in dominant groups of the gut microbiota do not explain cereal-fiber induced improvement of whole-body insulin sensitivity. *Nutr Metab (Lond),* 8**,** 90.

WEIGLE, D. S., CUMMINGS, D. E., NEWBY, P. D., BREEN, P. A., FRAYO, R. S., MATTHYS, C. C., CALLAHAN, H. S. & PURNELL, J. Q. 2003. Roles of leptin and ghrelin in the loss of body weight caused by a low fat, high carbohydrate diet. *J Clin Endocrinol Metab,* 88**,** 1577-86.

WHO 2015. WHO | Obesity and overweight. *WHO*.

WIKOFF, W. R., ANFORA, A. T., LIU, J., SCHULTZ, P. G., LESLEY, S. A., PETERS, E. C. & SIUZDAK, G. 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc Natl Acad Sci U S A,* 106**,** 3698-703.

WILLIAMS, C. F., WALTON, G. E., JIANG, L., PLUMMER, S., GARAIOVA, I. & GIBSON, G. R. 2015. Comparative analysis of intestinal tract models. *Annu Rev Food Sci Technol,* 6**,** 329-50.

WILLIAMSON, D. A., GEISELMAN, P. J., LOVEJOY, J., GREENWAY, F., VOLAUFOVA, J., MARTIN, C. K., ARNETT, C. & ORTEGO, L. 2006. Effects of consuming mycoprotein, tofu or chicken upon subsequent eating behaviour, hunger and safety. *Appetite,* 46**,** 41-8.

WILLIS, H. J., ELDRIDGE, A. L., BEISEIGEL, J., THOMAS, W. & SLAVIN, J. L. 2009. Greater satiety response with resistant starch and corn bran in human subjects. *Nutr Res,* 29**,** 100-5.

WILTROUT, D. W. & SATTER, L. D. 1972. Contribution of propionate to glucose synthesis in the lactating and nonlactating cow. *J Dairy Sci,* 55**,** 307-17.

WINDEY, K., DE PRETER, V. & VERBEKE, K. 2012. Relevance of protein fermentation to gut health. *Mol Nutr Food Res,* 56**,** 184-96.

WOLEVER, T. M., SPADAFORA, P. & ESHUIS, H. 1991. Interaction between colonic acetate and propionate in humans. *Am J Clin Nutr,* 53**,** 681-7.

WOLEVER, T. M., SPADAFORA, P. J., CUNNANE, S. C. & PENCHARZ, P. B. 1995. Propionate inhibits incorporation of colonic [1,2-13C]acetate into plasma lipids in humans. *Am J Clin Nutr,* 61**,** 1241-7.

WOLIN, M. J., MILLER, T. L., YERRY, S., ZHANG, Y., BANK, S. & WEAVER, G. A. 1999. Changes of fermentation pathways of fecal microbial communities associated with a drug treatment that increases dietary starch in the human colon. *Appl Environ Microbiol,* 65**,** 2807-12.

WONG, J. M., DE SOUZA, R., KENDALL, C. W., EMAM, A. & JENKINS, D. J. 2006. Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol,* 40**,** 235-43.

WONG, K. H., WONG, K. Y., KWAN, H. S. & CHEUNG, P. C. 2005. Dietary fibers from mushroom sclerotia: 3. In vitro fermentability using human fecal microflora. *J Agric Food Chem,* 53**,** 9407-12.

WOOD, P. J., ARRIGONI, E., MILLER, S. S. & AMADÒ, R. 2002. Fermentability of Oat and Wheat Fractions Enriched in β-Glucan Using Human Fecal Inoculation. *Cereal Chemistry Journal,* 79**,** 445-454.

WREN, A. M., SEAL, L. J., COHEN, M. A., BRYNES, A. E., FROST, G. S., MURPHY, K. G., DHILLO, W. S., GHATEI, M. A. & BLOOM, S. R. 2001. Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab,* 86**,** 5992.

WRIGHT, R. S., ANDERSON, J. W. & BRIDGES, S. R. 1990. Propionate inhibits hepatocyte lipid synthesis. *Proc Soc Exp Biol Med,* 195**,** 26-9.

XIONG, Y., MIYAMOTO, N., SHIBATA, K., VALASEK, M. A., MOTOIKE, T., KEDZIERSKI, R. M. & YANAGISAWA, M. 2004. Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. *Proc Natl Acad Sci U S A,* 101**,** 1045-50.

YANG, J., MALDONADO-GOMEZ, M. X., HUTKINS, R. W. & ROSE, D. J. 2014. Production and in vitro fermentation of soluble, non-digestible, feruloylated oligo- and polysaccharides from maize and wheat brans. *J Agric Food Chem,* 62**,** 159-66.

YAP, I. K. S., LI, J. V., SARIC, J., MARTIN, F.-P., DAVIES, H., WANG, Y., WILSON, I. D., NICHOLSON, J. K., UTZINGER, J., MARCHESI, J. R. & HOLMES, E. 2008. Metabonomic and Microbiological Analysis of the Dynamic Effect of Vancomycin-Induced Gut Microbiota Modification in the Mouse. *Journal of Proteome Research,* 7**,** 3718-3728.

YATSUNENKO, T., REY, F. E., MANARY, M. J., TREHAN, I., DOMINGUEZ-BELLO, M. G., CONTRERAS, M., MAGRIS, M., HIDALGO, G., BALDASSANO, R. N., ANOKHIN, A. P., HEATH, A. C., WARNER, B., REEDER, J., KUCZYNSKI, J., CAPORASO, J. G., LOZUPONE, C. A., LAUBER, C., CLEMENTE, J. C., KNIGHTS, D., KNIGHT, R. & GORDON, J. I. 2012. Human gut microbiome viewed across age and geography. *Nature,* 486**,** 222-7.

YE, Z., ARUMUGAM, V., HAUGABROOKS, E., WILLIAMSON, P. & HENDRICH, S. 2015. Soluble dietary fiber (Fibersol-2) decreased hunger and increased satiety hormones in humans when ingested with a meal. *Nutr Res,* 35**,** 393-400.

YU, S., WEAVER, V., MARTIN, K. & CANTORNA, M. T. 2009. The effects of whole mushrooms during inflammation. *BMC Immunology,* 10**,** 1-13.

YU, Z. T., LIU, B., MUKHERJEE, P. & NEWBURG, D. S. 2013. Trametes versicolor extract modifies human fecal microbiota composition in vitro. *Plant Foods Hum Nutr,* 68**,** 107-12.

ZAMBELL, K. L., FITCH, M. D. & FLEMING, S. E. 2003. Acetate and butyrate are the major substrates for de novo lipogenesis in rat colonic epithelial cells. *J Nutr,* 133**,** 3509-15.

ZAYED, H. 2015. Propionic acidemia in the Arab World. *Gene,* 564**,** 119-24.

ZE, X., DUNCAN, S. H., LOUIS, P. & FLINT, H. J. 2012. Ruminococcus bromii is a keystone species for the degradation of resistant starch in the human colon. *Isme j,* 6**,** 1535-43.

ZENG, W., PIRZGALSKA, R. M., PEREIRA, M. M., KUBASOVA, N., BARATEIRO, A., SEIXAS, E., LU, Y. H., KOZLOVA, A., VOSS, H., MARTINS, G. G., FRIEDMAN, J. M. & DOMINGOS, A. I. 2015. Sympathetic neuro-adipose connections mediate leptin-driven lipolysis. *Cell,* 163**,** 84-94.

ZHANG, H., XU, X. & JIN, Z. 2012. Fermentation characteristics of resistant starch from maize prepared by the enzymatic method in vitro. *Int J Biol Macromol,* 51**,** 1185-8.

ZHANG, X., ZHU, X., SUN, Y., HU, B., SUN, Y., JABBAR, S. & ZENG, X. 2013. Fermentation in vitro of EGCG, GCG and EGCG3 '' Me isolated from Oolong tea by human intestinal microbiota. *Food Research International,* 54**,** 1589-1595.

ZHAO, J. & CHEUNG, P. C. 2011. Fermentation of beta-glucans derived from different sources by bifidobacteria: evaluation of their bifidogenic effect. *J Agric Food Chem,* 59**,** 5986-92.

ZHAO, X. H. & LIN, Y. 2009. Resistant starch prepared from high-amylose maize starch with citric acid hydrolysis and its simulated fermentation in vitro. *European Food Research and Technology,* 228**,** 1015-1021.

ZHOU, J., MARTIN, R. J., TULLEY, R. T., RAGGIO, A. M., MCCUTCHEON, K. L., SHEN, L., DANNA, S. C., TRIPATHY, S., HEGSTED, M. & KEENAN, M. J. 2008. Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents. *Am J Physiol Endocrinol Metab,* 295**,** E1160-6.

ZHOU, Z., CAO, X. & ZHOU, J. Y. H. 2013. Effect of resistant starch structure on short-chain fatty acids production by human gut microbiota fermentation in vitro. *Starch - Stärke,* 65**,** 509-516.

ZHU, X., ZHANG, X., SUN, Y., SU, D., HU, B. & ZENG, X. 2013. Purification and fermentation in vitro of sesaminol triglucoside from sesame cake by human intestinal microbiota. *J Agric Food Chem,* 61**,** 1868-77.