The Human Gut Microbiota, Dietary Fibre and Production of Short Chain Fatty Acids in Inflammatory Bowel Disease

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Abstract

Research must be implemented to further our current understanding of the pathogenesis of Inflammatory Bowel Diseases (IBD) in order to improve the clinical treatment of these idiopathic conditions. Current evidence suggests that the aetiology of both Crohn's Disease (CD) and Ulcerative Colitis (UC) is multifactorial and with advancements in microbiological techniques, there has been much interest in the role of the gut microbiota in these diseases.

The indigenous gut community play many vital roles within the colon, one of which is the fermentation of dietary fibre which produces various metabolites, including the health-promoting organic anions, short chain fatty acids (SCFA). SCFA, particularly butyrate, provide up to 70% of colonocyte energy and play a vital role in gut immunoregulation. Some studies have reported improvements in clinical IBD symptoms in response to dietary fibre supplementation however others do not support a therapeutic effect. The medicinal properties of a fibre-free diet used to successfully treat paediatric CD suggests that fibre may not be as important as we think for colonic health and on the contrary may aggravate inflammation in active disease, and this may be mediated by either the profile of resident bacteria, and/or their functional capacity. Indeed, there is some evidence to suggest that the bacteria of IBD patients have a reduced capacity to utilise dietary fibre and efficiently produce SCFA, which may either initiate or perpetuate colonic inflammation.

The studies within this thesis aimed to investigate the interaction between dietary fibre and the human gut microbiota of IBD patients. The first study aimed to quantify the *in vitro* production of SCFA by the human gut microbiota of adult UC and CD patients in remission in response to the anaerobic fermentation of various fibres, and compare these values to those of matched healthy controls Another aspect of this study was to investigate SCFA production by the gut bacteria of newly diagnosed, treatment-naïve paediatric CD patients, comparing it to that of matched CD patients on concomitant medication and to healthy controls. The second study within this thesis aimed to characterise the baseline bacterial profile of a subset of adult UC patients, CD patients and healthy controls, and to assess differences in the bacterial response to various fibrous stimuli between groups.

Patients of this study were recruited in gasteroenterology clinics in Glasgow Royal Infirmary and at the Royal Hospital for Sick Children Yorkhill. Healthy controls were

matched to patients in terms of age, gender and body mass index. All participants were asked to donate a single stool sample which was used in the well-established batch culture fermentation model employed by this study. A further short methodological study was conducted with the objection of quantifying the production of *in vitro* SCFA in association with substrate availability, to gain insight into potential of product inhibition with increasing fibre.

The results of the first study implied that IBD patients, particularly UC patients, displayed an overall tendency of reduced *in vitro* total SCFA and butyrate production compared to matched healthy controls. In particular, butyrate production was significantly less in UC patients compared to healthy controls when fermented with hi-maize, a known butyrogenic fibre (median (IQR) HC; 58.87 (17.69) vs. UC; 44.92 (21.49) p=0.02). Both CD and UC patients displayed a significantly lower concentration of total SCFA compared to healthy controls (median (IQR) HC; 51.76 (22.02) vs. CD; 41.12 (23.28) vs. UC; 41.94 (14.72) p=0.02). The proportional contribution of butyrate to total SCFA following fermentation with mixed fibre was also signficiantly higher for healthy controls compared to UC patients (median (IQR) HC; 10.76 (8.26) vs. CD; 8.75 (4.74) p=0.049) but not CD patients. In no cases were there any significant differences between the SCFA concentration or relative contribution in CD or UC patients ($p>0.05$). There was a clear tendancy for total SCFA, acetate, propionate and butyrate concentrations to be highest in healthy children for most substrates; however these differences did not reach statistical significance ($p>0.05$). The exceptions to this was total SCFA and acetate concentration in response to fermentation of hi-maize, which was significantly higher in healthy controls compared to newly diagnosed patients (total SCFA; mean ± SEM, healthy controls; 57.99 ± 5.11 vs newly diagnosed; 38.89 ± 3.23, p=0.01, acetate; mean ± SEM, healthy controls; 32.93 ± 2.93 vs newly diagnosed; 21.41 ± 2.48, p=0.02) but not those who were on medication. Although not significant, there was a clear trend for total SCFA concentration to be the highest in healthy children, lower in patients who had already been on treatment, and lowest in newly diagnosed patients for all substrates.

The second study of this project reported that the baseline microbial diveristy of CD patients was lower than that of healthy controls, however there was not sufficient power to conduct statistical analysis. Fermentation with all fermentable fibres signficantly reduced microbial diversity in healthy participants ($p<0.05$), however showed little statistically relevant changes CD patients and none in UC patients. Baseline community structure of UC patients was statistically different to that of healthy controls. However the structure of all participants groups responded similarly to fermentation with all fibres, and

these changes were not dependant on participant type (interaction = 1.00). In terms of the relative abundance of bacterial phyla, there was evidence of an increased abundance of in unidentified species and those within the Proteobacteria phylum in CD patients at baseline, although increased power is necessary to assert statistical significance on this observation.

The results of these studies shed some further light on the interaction between dietary fibre and the gut bacteria of IBD patients, implying that there is a reduced ability to efficiently utilise dietary fibre to produce important SCFA *in vitro* in the disease state. As the prime SCFA involved in colonic health, the tendency for reduced butyrate production in the disease state is potentially fundamental to disease pathogenesis as this acid has important immunoregulation properties within the colon. The obvious trend of reduced total and individual SCFA production in newly diagnosed, treatment naïve paediatric CD patients compared to both healthy controls and CD children on medication suggests that the severity of disease activity is associated with SCFA production. This study implies that in a reduced *in vitro* production is an underlying issue despite the management of inflammation and clinical symptoms. This study, particularly the paediatric cohort, would benefit greatly from increased power to statistically ascertain the trends observed.

The reduced microbial diversity of CD patients observed in the baseline samples of this study are in coherence with multiple other studies in this area. Microbial diversity is important in gut immune function, the regulation of pathogenic bacteria, and the breakdown of vital nutrients and thus this reduction is extremely likely to play a role in IBD. Nonetheless, the *in vitro* fermentation of a solitary dietary fibre is likely to benefit specific bacteria and thus a reduction in diversity during such experiments, as shown in the healthy controls in this study, would be expected. However, neither CD nor UC patients reduced diversity to the same extent as healthy controls, implying that the baseline bacteria of patients could not optimally utilise the fibres provided. This may indicate a reduced functional capacity of the innate bacteria, or indeed a difference in baseline microbial profile. Indeed, the increase in unidentified bacteria and those within the Proteobacteria phylum in CD are in line with other studies. Nonetheless, the community structure of both patients and healthy controls responded in the same way to fermentation despite differences at baseline, indicated that to some extent, dietary fibre can modulate the gut community of IBD patients to be more like that of a healthy GIT.

In conclusion, this thesis ascertains previous beliefs that there is an abnormal gut bacterial response to dietary fibre in IBD. It is likely that the subdued response in IBD exacerbates inflammation, but whether it is the initial cause of disease is unlikely. The implications of this study highlight that the intake of dietary fibre in remission is important

in order to overcome innate inabilities to utilise these compounds to the same extent as healthy controls, and regain colonic homeostasis.

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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.

Mhairi McGowan, BSc

I certify that the work reported in this thesis has been performed by Mhairi McGowan and that during the period of study he has fulfilled the conditions of the ordinances and regulations governing the Degree of Master by Research, University of Glasgow

Dr Konstantinos Gerasimidis, BSc, MSc, PhD, FHEA

Abbreviation List

IBD	**Inflammatory Bowel Disease**
CD	**Crohn's Disease**
UC	**Ulcerative Colitis**
pCD	**Paediatric Crohn's Disease**
GIT	**Gastrointestinal Tract**
EEN	**Exclusive Enteral Nutrition**
SCFA	**Short Chain fatty Acid**
BCFA	**Branched Chain Fatty Acid**
BMI	**Body Mass Index**

Chapter 1 : Inflammatory Bowel Disease, Dietary Fibre and the Human Gut Microbiota

1.1 Inflammatory Bowel Disease

The term inflammatory bowel disease (IBD) encompasses a collection of conditions typically characterised by chronic, relapsing inflammation which manifests in the gastrointestinal tract (GIT). The predominant forms of IBD are Crohn's Disease (CD) and Ulcerative Colitis (UC), conditions which exhibit similar characteristics and symptoms making differential diagnosis hard. However, where CD patients suffer from transmural granulomatous inflammation throughout the GIT, UC inflammation is restricted to the colon and rectum [1]. Additionally, whilst inflammation in CD often occurs in a discontinuous manner, UC patients suffer from continuous inflammation which is confined to the mucosal layer [2]. IBD patients suffer from flare up episodes of varying severity, followed by ambiguous periods of remission. However, a minority of sufferers of both conditions experience chronic, continuous symptoms [3, 4].

IBD triggers symptoms that include, but are not restricted to, abdominal pain, bloody diarrhoea and weight loss [5]. Paediatric sufferers of IBD are at a particularly high risk of malnutrition, which can cause nutritional deficiencies such as anaemia, as well as negatively impacting growth and bone health [6]. Quality of life is commonly reduced for IBD sufferers [4], who often experience psychological issues such as depression and anxiety as a direct result of their condition [7].

The pathology and etiology of IBD are extremely complex, thus both CD and UC remain as idiopathic, lifelong conditions. Although treatment is available, there is no current cure for these organic diseases, leaving health care systems around the world financially challenged [8] as the incidence of IBD rapidly increases globally [4].

1.1.1 Epidemiology of IBD

1.1.1.1 Incidence and Prevalence

Despite being most common in North America [9] and Northern Europe[10], the international incidence of IBD is growing steadily [4], particularly in low income countries such as those in Eastern Europe, South East Asia, South America and Africa [11]. Furthermore, incidence has increased in developed countries such as South Korea, Japan

and Singapore - areas which previously enjoyed low incidence rates [12]. In most countries, UC has a higher incidence rate in adults than CD [4, 13] and its growing incidence preceded that of CD's by approximately 10 years [4]. However, where UC's growing incidence has stabilised in the previous decades, that of CD's has continuously risen [4]. In terms of paediatric disease, CD tends to have a higher prevalence than UC [14].

1.1.1.2 Age of Presentation

IBD can be encountered at any point in the lifecycle; however the peak age at which CD occurs is within the third decade [13], whilst UC typically presents itself later in life, between the ages of 20 and 50 [1]. The incidence of paediatric IBD is rising particularly quickly [10], accounting for 7-20% of all cases [4].

1.1.1.3 Gender

Epidemiological research implies that there is a slight male predominance in UC (60%) whilst in high incidence areas approximately 30-40% more females suffer CD [4]. However with regards to low incidence countries, some reports suggest that CD occurs more frequently in males, whilst others imply that there is no gender bias in these areas [4]**.** Conversely, paediatric UC is more common in females whilst juvenile CD is more frequently diagnosed in males [15].

1.1.2 Aetiology of IBD

IBD is more common in genetically susceptible individuals. Furthermore, immunological factors appear to be perturbed in IBD patients and there has been much interest in recent years in the involvement of the intestinal microbiome in IBD. However, the increased incidence of IBD in coherence with industrialisation in the developing world suggests that it is likely that environmental factors, including the Western lifestyle, also influence the onset of these conditions. Thus, the aetiology of IBD is complex and it is unlikely that there is a single causative factor. Rather, IBD is probably multifactorial and research is currently aimed at investigating the relationship between genetic, immunological, microbial and environmental factors.

1.1.2.1 Genetic Determinants

There is strong evidence supporting a familial occurrence of IBD, especially in first degree relatives [16]. Twins studies report that monozygotic twins have a higher concordance rate of CD (~45%) than dizygotic twins [13]. However this figure is markedly lower with regards to UC (~16%)[17], which generally appears to have a lower heritability than CD [1].

In the past few decades, a variety of candidate genes have been proposed to play a role in the aetiology of IBD. The first gene associated with CD was nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and it is reported that mutations in this gene occurs in about one-third of CD patients[18]. As an innate recognition receptor, NOD2 plays a vital role in bacterial recognition and acts as a defence mechanism against invading pathogens [19], and a defection in this gene is likely to therefore cause inflammation.

A recent meta-analysis reviewing 15 genome-wide association studies identified 163 loci associated with IBD susceptibility- approximately two-thirds of which were associated with both CD and UC [20]. The genes that overlapped both conditions were predominately involved in the interleukin-23 signalling pathway, which is important in maintaining the Th17 cell population and therefore, the antimicrobial immune response. However there were a number of disease specific genes identified for both conditions [20]. In particular, genes identified to play a role in UC were involved in epithelial integrity (HNF4A, CDH1, LAMB1, ECM1), immune regulatory function (HLA-region, IL-10, BTNL2, IFN$_\gamma$-IL25, NKX2-3), cellular homeostasis (ORMDL3) and innate immune function (PLA2G2E, CARD9). Regarding genes associated with CD, there is strong evidence to suggest that genes involved in autophagy (ATG16L1, IRGM), which involves the degradation of a cell's own components, are linked to this disease.

1.1.2.2 Immunological Factors

Alongside its role in nutrient absorption, the GIT is additionally the largest immune organ of the human body [21]. The majority of the susceptibility genes identified to be related to IBD are involved in immunological pathways, indicating abnormal immune function in IBD patients. Numerous aspects of the immune system have been indicated to play a role in the onset or perpetuation of IBD, incorporating both the innate and adaptive immune response.

In a healthy GIT, the intestinal epithelial cells provide a single-layered physical barrier which protects intestinal tissues from luminal contaminants. Its complex structure, constructed with columnar cells adjoined by intracellular junctions, is designed to be selectively permeable, allowing the transport of essential nutrients into the blood stream whilst excluding harmful microorganisms. It is proposed that IBD patients have an aberrant barrier function, and thus are exposed to detrimental pathogens. Studies have shown that impaired tight junctions and increased permeability are common in the intestinal epithelium of IBD patients [22], particularly in areas of the GIT which have suffered either acute inflammation or chronic damage [23]. Enteric permeability is reportedly higher in not only CD patients, but also in first degree relatives of the disease, implying that a combination of genetic and immunological defaults underlies the pathology of IBD [22]. It is likely that the compromised intestinal integrity is regulated by pro-inflammatory cytokines such as interferon $_{\gamma}$ (IFN-$_{\gamma}$) and tumor necrosis factor κ(TNF-κ), both of which are involved in the disassembly of tight junctions [22]. In a healthy GIT, epithelial cells are buffered from luminal contents by intestinal mucus which is locally produced by goblet cells; however, there is evidence to suggest that this mucus production is impaired in IBD. The removal of mucin 2 (MUC2), the gene partly responsible for the production of mucus, reportedly causes intestinal inflammation in mouse models [24], and alterations in the equivalent gene in humans (MUC19) has been noted in CD patients [25]. Thus IBD patients may suffer from increased epithelial exposure to luminal contents, which may induce an inflammatory response.

Cytokines additionally play a central role in the immunological aetiology of IBD. The equilibrium upheld between pro- and anti-inflammatory cytokines in normal immune responses appears to be disrupted in IBD patients, favouring inflammation [26]. Pro-inflammatory markers which have been detected in high levels in IBD patients include IL-6, IL-1β, TNF-α and IL-8 [27] . Highlighted as a candidate gene involved in CD, NOD2 is responsible for the binding of intracellular peptidoglycan present in the cell wall of bacteria, activating NFκB which is responsible for the rapid transcription of pro-inflammatory molecules. NFκB is additionally activated via Toll-like receptors (TLRs) which reside on the surface of sentinel cells and play a central role in the recognition of microbial structures. In particular, TLR4 binds lipopolysaccharide (LPS), a molecule expressed in the outer membrane of gram-negative bacteria, activating NFκB and inducing a pro-inflammatory response. Both NOD2 and TLR4 receptors are reportedly upregulated in IBD patients [28]. Colitis has been induced in gene-knockout mice deficient for IL-2 and IL-10, implicating that these regulatory cytokines play a vital role in IBD

pathophysiology [29]. Further investigation in mice models report that the administration of anti-inflammatory drugs both prevented and treated colonic inflammation [30].

In a healthy GIT, a delicate balance is maintained between the defence against pathogenic bacteria and tolerance towards harmless bacteria; a relationship that appears to be disrupted in IBD patients. This particular aspect is explored later in this chapter (see Section 1.4.1

1.1.2.3 Gut Microbiome

Interest in the role of the gut microbiome in the aetiology of IBD has been heightened in previous decades. A comprehensive review of this ongoing research is provided later in the introduction of this thesis (see Section 1.4).

1.1.2.4 Environmental Factors

The rapid increase of CD and UC, including their emergence in countries that previously had low incidence, indicates that environmental factors must be influential in the aetiology of IBD, as it is unlikely that the human genome could be so drastically altered in such a small time frame. Epidemiological research implies that appendectomy, drugs, socioeconomic status, diet and stress have an association with IBD [31]. Interestingly, although smoking is protective against UC, CD risk is enhanced with smoking [4].

1.1.3 Diet and IBD

As diseases of the GIT, it is logical to assume that nutrition plays an important role in CD and UC. Although evidence is not sufficient to provide a clear relationship between pre-illness diet and IBD, various studies have indicated an association between certain dietary components and both disease onset and perpetuation.

There is epidemiological evidence to suggest that IBD may be linked to high levels of total fat intake [32]. A major prospective cohort study, The European Investigation into Cancer and Nutrition (EPIC), reported that UC is associated with enhanced intakes of α-linoelic acid (n-6 PUFA) [33]. This is also evident in studies of murine models [34]. However, the relationship between dietary fat and IBD is not entirely clear cut, as the EPIC study additionally suggested a protective effect of the n-3 PUFA, docosahexaenoic acid, on UC [33]. It was concluded that an increased n-6: n-3 PUFA ratio enhances UC risk [33].

Although the evidence is weaker, there are similar findings with regards to fat intake and CD risk [35].

Increased total and saturated fat intakes are characteristics of the Western diet. The increasing emergence of IBD in both developed and developing nations appears to correlate with this shift from traditional diets, highlighting a plausible role of diet in IBD aetiology. The Western diet is also typically high in refined carbohydrates and processed, sugary foods- dietary components which have been associated with an increased IBD risk [36-38]. Again associated with modern diets, increased meat intake tends to be associated with increased risk of IBD, with slightly more convincing evidence for UC development [35, 39].

On the other hand, a diet rich in fruit and vegetables may be protective against CD and UC [35, 40], decreasing the risk of IBD by up to 40% [33]. In particular, vitamin C, and thus citrus fruits are associated with a lesser risk of IBD, especially UC [36].

Establishing a relationship between diet and IBD is difficult due to issues regarding the study design of research in this field. Epidemiological research is resourceful in providing association between dietary components and disease state, yet it is hard to denote a true cause-effect relationship. Additionally, such studies cannot truly investigate the impact of single dietary components and the effect of the food matrix must be considered when interpreting results. Clinical studies provide a more insightful representation of the impact of diet on gut health, yet they suffer from ethical restraints and may be conflicted by medical therapies undertaken by IBD patients. Thus, although there is evidence to suggest a link between IBD and diet, further investigation is needed using a variety of study designs, including credible *in vitro* experiments.

A nutritional component which has received a plethora of scientific interest with regards to its protective role against IBD is dietary fibre, and thus will be discussed in further detail in this thesis.

As aforementioned, the aetiology of IBD cannot be explained by any one single element and is more likely to be the result of a combination of complex factors. The current theory is that IBD affects genetically susceptible individuals, whose immune system is activated by the bacteria present within the gut, and produces an exaggerated immune response which is exacerbated by environmental factors.

1.2 Dietary Fibre

Although its definition has been revised throughout the last few decades, dietary fibre is currently described as the edible component of plants (or equivalent carbohydrate) that resists digestion and absorption by the enzymes of the small intestine, yet may undergo full or partial fermentation in the colon, conferring beneficial physiological effects on the host [41, 42]. It should be noted that not all dietary fibres can be fermented and the degree of fermentability is dependent on the structural properties of the fibre [43]. The term dietary fibre encompasses resistant starch, resistant oligosaccharides and non-starch polysaccharides of which all have distinctive features and have been associated with improvements in various aspects of health. As they mediate the health-promoting effects of dietary fibre, it is important to briefly outline the structural and functional properties of these food components.

1.2.1 Structural and functional properties of dietary fibre

1.2.1.1 General structure of the plant cell wall

The physiological function of dietary fibre is dependent on the properties of the cell wall, which provides an insoluble barrier in which nutrients are trapped [44]. However, this term is extremely broad due to ambiguity regarding both the type of plant and the specific type of cell involved. Furthermore, fibre within our diet can be sourced from many areas of a single plant, be it seeds, stems, leaves, roots or fruits. However although these large variations do not allow for an easy structural definition of fibre, some generalisations can be made.

When considering the structure of the plant cell wall, it is appropriate to first outline the molecular structure of the polysaccharides which contributes to its formation. The properties of polysaccharides are reliant on the constituent monosaccharides, which are classified by the number of carbon atoms present. It is at this level at which the most important attribute of dietary fibre is consolidated; the presence of non-alpha-glucosidic bonds between monosaccharides, which inhibit hydrolysis by the enzymes in the upper GIT [45]. Although all fibres have a central backbone chain, most of which are comprised of a single monosaccharide [46], there is variation in side-chain and branch formation. The bonding involved in such substructures influences overall form of the polysaccharide. Similar to the structure of protein, if the bonds of side-chains are constructed in an alpha configuration, a helical form is adopted whilst a beta-configuration causes a flat, ribbon-

like structure. The configuration determines the extent of both intermolecular associations between polysaccharides and also the ability to bind proteins within the cell wall [45], factors which influence overall solubility. This partly explains the insolubility of cellulose, which is a linear D-glucose polymer linked by β-D (1-4) glycosidic bonds, giving rise to the formation of many intermolecular hydrogen bonds.

There are generally three structural layers in the plant cell wall; the primary cell wall, the secondary cell wall, and the middle lamella. Responsible for the conjunction of adjacent cells, the middle lamella is the first structure to develop during cytokinesis and is rich in pectin, a polysaccharide which has a high concentration of uronic acid. The primary wall is then formed next to the middle lamella, made up of polymer chains known as cellulose fibrils packed closely together in a random matrix via hemicellulose and hydrogen bonds. These fibrils resist enzymatic attack in the upper GIT, and often incorporate various glycoproteins [47]. Finally, once everything else has developed, the thicker secondary wall is formed in which the cellulose fibrils are arranged in parallel sheets. Its function is to strengthen the structure of the cell wall, and depending on the cell type multiple secondary cells walls may be developed, each one reinforcing the strength. On the other hand, some cells do not develop a secondary cell wall at all.

1.2.1.2 Non-starch polysaccharides

Non-starch polysaccharides (NSPs) refer to long polymeric carbohydrate chains containing at least ten monosaccharides which resist digestion in the small intestine. Polysaccharides differ with regards to type and number of monosaccharides present, as well as the type of bonding between them. Examples of NSPs can be found in Table 1.1.

1.2.1.3 Resistant oligosaccharides

Unlike NSPs, resistant oligosaccharides contain a fairly low degree of polymerisation, with a maximum of ten monomeric units. Perhaps the most important oligosaccharides are fructo-oligosaccarides (FOS) and galacto-oligosaccarides (GOS), both of which are known prebiotics. Prebiotics refers to dietary substances which are known to resist digestion in the upper GIT and thus advance to the colon where they are fermented by the resident gut bacteria, promoting the activity and number of certain beneficial strains of bacteria and therefore enhancing colonic health [48].

1.2.1.4 Resistant starch

Although much of dietary starch is hydrolysed in the upper GIT by amylase, there are sub-categories of starch that resist this digestion due to their chemical structure, aptly referred to as resistant starch (RS). There are four independent categories of RS; RS1, RS2, RS3 and RS4. RS1 encompasses starch that is physically inaccessible to small intestinal enzymes and is found in many seeds, legumes and some chewed cereals. The conformation of RS2, such as raw potato and unripe banana, inhibits digestion by pancreatic amylase as it is surrounded by larger starch granules. RS3 refers to retrograded starch, which occurs when starch is cooked and then cooled as in potato or pasta. Finally, RS4 includes chemically modified resistant starches which have been produced with the aim of resisting digestion. RS4 is often produced in the form of prebiotics in order to positively manipulate the composition of the gut microbiota.

1.2.1.5 Solubility of dietary fibre

The solubility of dietary fibre is an important determinant of the health benefits conferred upon the host. Soluble dietary fibre is commonly associated with enhancing viscosity in the small intestine, thus slowing the rate of glucose absorption and avoiding a spike in insulin response [49]. Furthermore, soluble fibre improves the ratio of low density lipoprotein to high density lipoprotein, which benefits total cholesterol levels [50]. Therefore, such fibres have been linked to a reduction in risk of type 2 diabetes mellitus and cardiovascular related diseases [51]. Whilst soluble fibres slow down gastric emptying, insoluble dietary fibre improves efficiency of bowel emptying, increasing faecal mass and regularity [52]. Most dietary fibres are composed of a mixture of soluble and insoluble fibres. One-third of whole grains such as rye, barley and oats is soluble fibre, whilst wheat has a much higher proportion of insoluble constituents than most whole grains [53]. Refined foods have gone through a process which strips much of the available dietary fibre, particularly that of the insoluble nature [54].

1.2.2 Colonic health benefits of dietary fibre

The consumption of fibre has been linked to many health promoting effects, not least in the GIT and the recommended dietary fibre intake in the United Kingdom is currently 18g per day [55]. Although fibre does not exert many nutritional benefits in the upper GIT, its impact on the digestion and metabolism of other nutrients has been linked to numerous

health claims that include a reduction in risk of coronary heart disease [56], certain types of cancer [57] and insulin-sensitivity [58]. Fibre is known to have a protective effect on obesity [59, 60], and has a positive relationship with satiety [61], with a suggestion that a 14g per day increase in fibre intake reduces energy intake by 10%. However, it is out with the realms of this thesis to provide a comprehensive review of the extensive range of health-promoting effects of dietary fibre. Rather, it is important to hone in on the relationship between dietary fibre and IBD in this thesis. The intact transit of dietary fibre to the large intestine provides numerous effects on colonic function including changes in whole gut transit time, gastric emptying, stool bulking and short chain fatty acid (SCFA) production [62]. It is therefore plausible that dietary fibre can subdue, or exacerbate, the symptoms of IBD. The production of SCFA in response to dietary fibre is of particular interest, as these anions have multiple medicinal properties within the colon, which will be discussed later in this chapter. The production of SCFA is mediated by the resident gut microbiota, which utilise fibre as fuel for anaerobic fermentation and thus play an invaluable part in colonic health. In order to comprehend and interpret the role of the gut microbiota in IBD, it is important to first outline the background of this complex eco-system.

Table 1.1 Food sources, physiological characteristics and health benefits of non-starch polysaccharides

Non-starch polysaccharide	Food Source	Solubility	Fermentability	Physiological Impact
Cellulose	Cruciferous vegetables, tubers, some brans	Insoluble	Low	Increases stool output and regularity, enhances satiety [63]
Hemicellulose	Cereals	Soluble	Partial	Slows glucose absorption, enhances satiety [64]
Pectin	Fruits, vegetables	Soluble	High	Slows glucose absorption, enhances satiety, promotes bacterial fermentation [65]
Guar Gum	Seaweed extracts, plant extracts, legumes	Soluble	High	Slows glucose absorption, enhances satiety, promotes regular bowel movements [66]
Inulin	Chicory, artichoke, onions	Soluble	High	Promotes growth of beneficial gut bacteria, reduces low-density lipoprotein cholesterol [67]

1.3 The Human Microbiome

In the past decade, extensive research has aimed at characterising the indigenous microbial species that inhabit the GIT of human beings The average healthy gut microbiome is comprised of at least 100 trillion diverse microbes [68], most of which are anaerobic bacteria. The number and variety of microorganisms residing in the GIT are progressively enhanced as the GIT descends, thus, as an ideal habitat for bacterial ecosystems, the large intestine is the most densely populated area of the GIT with up to 10^{12} colony forming units per gram of luminal content [69]. The intestinal bacteria are in fact so numerous and important to host health, they are often referred to as a "*metabolic organ"*. Understanding of these complex communities has been greatly enhanced in the last few decades due to vast improvements in scientific techniques such as next genome sequencing and metagenomics[68], allowing the identification of previously undisclosed genera. Major projects such as the Human Microbiome Project [70] and MetaHIT [71] have permitted the establishment of a critical metagenomic database, supporting subsequent research aimed at characterising bacterial communities. Such advances have thrusted gut bacteria into the scientific limelight, heightening interest and research in this area, particularly with regards to the implications that the gut microbiome may have on human health. Studies have indicated that the gut flora may not only influence colonic health, but also impact obesity [59], the metabolic syndrome [72], and even mental health [73]. Many of the health-promoting effects of the bacteria occur via the production of SCFA, which will be discussed later in this review. However, although such scientific advances have improved comprehension of the microbes within the GIT and their contribution towards human well-being, there is still a lot to learn about this complex eco-system and its role in health and disease.

1.3.1 Bacterial colonisation of the gastrointestinal tract

Although it has been generally accepted for decades that the foetal GIT is sterile, recent research suggests that bacterial colonisation may in fact be initiated in the foetal intestine via swallowing of amniotic fluid [74]. However, this is preliminary evidence and it is not yet clear what effect this prenatal colonisation has on future bacterial composition. It cannot be contested that the vast majority of native bacterial species are acquired during birth from maternal vaginal, intestinal or skin populations [75], highlighting that microbial composition is somewhat hereditary.

The next critical period of bacterial colonisation of the GIT occurs immediately after birth and is heavily dependent on infant feeding practises. The bacterial population of breast-fed infants is dominated by *bifidobacteria* and *lactobacilli* and whilst although formula-fed infants have a more diverse range of bacteria within their GIT, they are also more prone to pathogenic organisms [76]. Weaning is another significant period of colonisation and it has been noted that formula-fed babies enhance their repertoire of microorganisms quicker than breast-fed infants [76]. By the age of 1 year, the gut flora have developed and matured to that of an adult and from this point the microbiota remain generally stable throughout life [69, 76]. However, although the human intestinal ecosystem is populated by permanent indigenous species which are very hard to alter after initial colonisation, the external environment continuously introduces transient species into the GIT[69] in various manners such as illness, drug use, and diet. Reports suggest that environmental factors can alter up to 20% of faecal bacterial composition [69]. A review on the impact of diet on the gut microbiota can be found later in this section.

1.3.2 Composition of the normal human microbiome

Although dozens of bacteria and archaea phyla live on Earth, less than 10 have found a habitat within the GIT [71]. However within these phyla, over 1,000 bacterial species [71] and 7,000 strains [77] reside in this eco-system, the majority of which belong to the gram-positive phylum of *Firmicutes* [43] which constitutes 40-65% of total faecal bacterial population from healthy people. It is reported that 95% of *Firmicutes* within stool belongs to the *Clostridia* class[78], the predominant species of which is *Faecalibacterium prausnitzii,* a significant producer of the important SCFA, butyrate. Further butyrate producers *Roseburia* spp. and *Eubacterium rectale,* which belong to the *Clostridial Cluster XIVa* of the *Firmicutes* phylum, contribute to 7% of total faecal bacterial population of healthy subjects according to research employing 16S rRNA sequencing. *Bacteriodetes,* which are gram-negative, represent the second most abundant phylum present within the intestine, making up 25% of human colonic bacteria [79]. It is proposed that this phylum are the most significant producers of the SCFA propionate [79]. A review by Duncan et al suggests that over 80% of gut bacteria belong to the Firmicutes or Bacteriodes phyla [79]. Further important, yet less abundant phyla identified within the human gut microbiome are *Proteobacteria, Actinobacteria, Fusobacteria and Verrucomirobia* [73].

1.3.3 Functions of the human gut microbiota

In healthy conditions, a mutualistic relationship exists between the gut flora and the host in which the bacteria confer many health benefits to the host whilst benefiting from the lucrative environment created within the GIT. The bacterial genome seriously outweighs that of the host, providing a whole new repertoire of functions that the host simply does not have the genetic material to conduct independently, incorporating innate and adaptive immunity, nutrient absorption, and gut motility [73]. These roles are often strain-related [75] and make an important contribution to the maintenance of intestinal homeostasis. Largely occurring in the proximal colon [80], the microbiota hydrolyse previously undigested fibre producing a range of end products, principally SCFA. This anaerobic fermentation plays a vital role in the maintenance of epithelial health as, amongst other important functions, the SCFA produced provide more than 70% of colonocyte energy.

1.3.3.1 Saccharolytic breakdown

The human genome is not equipped to breakdown all dietary components, resulting in the intact transit of such food components to the colon. The gut microbiota thrives on such dietary components, namely non-digestible carbohydrates and protein, utilising them as fuel. The vast diversity of dietary fibre and the complexity of their molecular structure require a range of saccharolytic enzymes. Bacterial enzymatic capacity is determined by the presence of carbohydrate-active enzymes (CAZymes), which are responsible for the degradation of dietary fibre [81]. There are currently four recognised classes of CAZymes: glycoside hydrolases (GH), polysaccharide lyases (PL), carbohydrate esters (CE), and auxiliary activities (AA) which are a newly identified class thought to be involved in the breakdown of lignin. GH are responsible for the cleavage of glycosidic linkages between two sugars whilst ester linkages, which are prominent in pectin and some arabinoxylans, are catalysed by CE and acid sugars are degraded by PL. There is still a lot to learn about the members and activities of CAZymes, but it is clear that bacteria possess an extensive collection compared to the limited range in the human genome. According to the CAZyme database, 3,841 CAZymes are found in bacteria whilst only 181 have been found in eukaryotes [81].

1.3.3.2 Gut fermentation and SCFA production

Dietary fibre has been associated with the improvement of gut health via colonic fermentation, promoting the growth of certain beneficial bacteria in the large intestine. These health-promoting effects are largely mediated via the production of SCFA – the main end-products of fibrous fermentation. These important organic compounds are produced via numerous metabolic pathways, such as the Embden-Meyerhof pathway. Acetate, propionate and butyrate are the predominant SCFA, produced in a ratio of 3:1:1 respectively. The acidic nature of SCFA decreases the intestinal pH, enhancing water and nutrient absorption whilst preventing the overgrowth of some pathogenic bacteria [80] and inhibiting harmful colonic cell proliferation [82].

Produced in the largest concentrations, acetate is the predominant SCFA to reach the systematic circulation where it contributes to 10% of total energy used by the host [83]. It is proposed that acetate enhances de novo lipogenesis by promoting the production of acetyl coenzyme A and fatty acid synthase [84]. Furthermore, both acetate and propionate have been linked to enhancing satiety via G-protein coupled receptors 41 and 43 which are linked to the release of appetite regulating hormones, leptin and polypeptide Y [85]. In particular, there has been much interest in the role of propionate as a possible treatment of the current obesity epidemic [85].

1.3.3.3 Colonic health promoting benefits of butyrate

Evidently, SCFA interact with many aspects of human health and are not restricted to their site of production. However, the most important SCFA in terms of colonic health is butyrate. As the principal energy source of colonocytes, butyrate provides up to 70% of the energy used by these cells. Furthermore, it enhances the activation of NFκB whilst degrading the enzyme IκB [86], inhibiting the expression of pro-inflammatory cytokines in the epithelial mucosa. As a known histone deacetylase inhibitor, butyrate also regulates gene expression for hyperacetylation, a process which promotes cell proliferation [87]. Thus, butyrate production has been associated with a decreased risk of colitis [88] and colorectal cancer [89] and it can be inferred that butyrate-producing bacteria, such as those in the phylum *Firmicutes*, are beneficial to host health.

Relative SCFA production is regulated not only by the type of bacteria present, but also varies with substrate availability. Wheat bran [90], resistant starch [91] and oat bran [92] reportedly enhance butyrate production, whilst guar gum [90] and ispaghula [93] are associated with increased propionate. Thus, it is proposed that by carefully selecting the

type of fibre consumed within the diet, the type of bacteria in the gut and therefore the subsequent production of SCFA can be modulated. Table 1.2 displays the differential production of SCFA in response to difference fibrous substrates, as measured during *in vitro* fermentation studies.

Table 1.2 Production of SCFA following 24 hours in vitro fermentation of different fibrous substrates by human gut microbiota

			Production of SCFA ***(mM)(Relative SCFA production (%))***			
Author	**Experimental Design**	**Fibre**	**Total SCFA**	**Acetate**	**Propionate**	**Butyrate**
Vince at al, 1990 [94]	Batch culture fermentations of faecal samples of 5 healthy participants	Cellulose	77.00	45.00 (58)	15.00 (19)	14.00 (18)
		Pectin	121.00	82.00 (68)	16.00 (13)	25.00 (21)
		Arabino-galactose	124.00	83.00 (67)	31.00 (25)	24.00 (19)
Nordland et al, 2012 [95]	*In vitro* colon digestion model used to ferment different substrates alongside faecal samples collected from 6 healthy volunteers. Results shown after 24 hours of fermentation	Rye Bran	92.00	57.00 (62)	19.00 (20)	16.00 (20)
		Wheat Bran	69.00	39.00(57)	15.00 (23)	14.00 (17)
Barry et al, 1995 [96]	This inter-laboratory study was conducted to investigate the use of a batch culture *in-vitro* fermentations. 5 European laboratories used mixed faecal inoculate from 3 different human volunteers. Results shown after 24 hours of fermentation	Pectin	67.70	50.37 (74.4)	6.02 (8.9)	11.3.016.7)
		Sugar Beet	44.7	31.00(70)	8.00(18)	5.68 (12.7)
		Soya Bean	63.6	41.50 (65.2)	13.37(21)	8.78(13.8)

1.3.3.4 Dietary manipulation of human gut microbiota and subsequent SCFA production

Variation in monosaccharide composition, linkage and chain length affects the utilisation of dietary fibre by the gut flora [97], and some bacteria are better suited than others in the breakdown of certain fibres. Therefore, in order to accommodate the wide variety of fibre entering the gut, a range of bacterial species is necessary. "Generalist" bacteria, such as *Bacteroides cellulosilyticus* (*vide supra*), *Bacteroides thetaiotaomicron* and *Bacteroides ovatus* [43], are able to utilise numerous polysaccharides, allowing for meal-to-meal alterations in fibre intake. This is reflected in a high presence of various CAZymes in these species genome [43]. On the other hand, "specialist" bacteria are more selective about their substrates and can utilise only specific carbohydrates. The composition of the microbial community can therefore be dictated by the substrates that are presented in the colon, with some species thriving more than others depending on the food sources available and their capacity to utilise this substance. This provides an interesting avenue of manipulating gut health via selective growth of specific bacteria in response to diet.

Epidemiological studies researching the interaction between fibre intake and microbiota are often conducted in a cross-sectional fashion, during which the faecal bacteria of a population known to have a high dietary intake of fibre is compared to that of a community with low dietary fibre [98-100]. De Fillipo et al [98] used 16S rRNA sequencing to study differences in the faecal microbiota in children from a rural African community who consumed a plant-based diet to age-matched children from Europe who consumed the typical Western diet; high in animal fat, processed carbohydrates and low in dietary fibre. It was found that the African children had a higher abundance of Actinobacteria and Bacteriodes, whilst European children had increased Proteobacteria. Some species of Proteobacteria, such as adherent-invasive *E.coli,* have been linked to inflammation [101] and thus are not associated with a healthy colonic environment. In De Filippo's study, Firmicutes counts of European stool samples were over twice that of their African counterparts. Overall, the African children had a much richer microbial diversity as well as a significantly higher production of SCFA, specifically butyrate and propionate ($p<0.001$). However, other studies [100, 102] of similar design have reported an increase in the prevalence of Bacteriodes in children on a Western diet compared to those on a plant based diet.

Although such epidemiological research is valuable in indicating a relationship between diet and the microbial community, intervention studies give a clearer indication of the direct impact of specific dietary fibres on the gut microbiota. However, alterations in

type and amount of fermentable biomass is associated with changes in abundance and composition of gut microbiota and thus subsequently, SCFA production [103, 104]. When compared to normal integrative diet, counts of important butyrate-producers *Faecalibacterium prausnitzii* and *Roseburia spp* are significantly reduced following a fibre-free diet in healthy individuals [103]. A study in obese individuals reported that decreasing carbohydrate intake from 399g per day to 24 grams per day reduced SCFA production by 50%, which correlated with reduced counts of *Roseburia spp.* and *Eubacterium rectale* related species [105]. A further study, again in overweight and obese participants (n=91) found that *Bifidobacteria* numbers were significantly reduced after 8 weeks on a low carbohydrate diet ($p<0.001$), as were total SCFA ($p<0.04$) and butyrate ($p=0.001$) faecal concentrations [106]. One study [107] found that both *Roseburia* spp and *Eubacterium rectale* are related to carbohydrate intake in a dose-dependent manner ($p<0.001$). Thus, availability of fermentable carbohydrate in the colon plays a central role in SCFA production and selective bacterial growth.

Selective modulation of the gut microbiota can also be achieved via the type of dietary fibre. *Clostridium Cluster XIVA* and the *Roseburia* genus are major butyrate producers and play an important role in gut fermentation [108]. These species, alongside total bacterial population, increased during *in vitro* fermentations of faecal samples supplemented with 14grams per day of wheat dextrin. The addition of wheat dextrin also enhanced SCFA production, particularly butyrate [108]. Studies indicate that both acute and chronic alteration of fibre dietary intake can modulate bacterial composition and activity [109]. Table 1.3 gives an overview of recent studies investigating the influence of both type and amount of dietary fibre on the composition of gut microbiota and subsequent SCFA production. .

Table 1.3 Influence of dietary fibre on gut microbiota of healthy human subjects using information from both intervention studies and *in vitro* fermentation studies

Author	Purpose	Population	Exposure	Outcome	Results/Comments
Intervention Studies					
Benus et al, 2010 [103]	Influence of a fibre-free vs fibre-supplemented diet on *F. prausnitzii* compared to a free living diet	10 Healthy subjects (6 men, 4 women)	Subjects followed 3 dietary phases as follows: a) normal diet, b) EEN (fibre-free), and c) fibre supplemented EEN (14g/L) Each diet was followed for 14 days.	Faecal samples taken before and after interventions. Faecal microbiota analysed using fluorescent *in situ* hybridisation (FiSH) and SCFA using gas chromatography.	*Faecalibacterium prausnitzii* and *Roseburia spp* groups decreased following fibre-free and fibre-supplement diet ($p<0.01$). SCFA decreased following the fibre free diet ($p<0.05$) Strong correlation between *F. prausnitzii* and butyrate production on normal diet
Francois et al , 2013[110]	Investigation of Wheat Bran Extract (WBE) on gastrointestinal health	58 healthy volunteers	Subjects consumed 3 diets supplemented with 0g, 3g or 10g of WBE.	Faecal samples analysed for SCFA production, pH and bacteria at end of each treatment period.	SCFA production and faecal *bifidobacteria* levels significantly higher following supplementation with 10g/d WBE, whilst faecal pH was decreased.

Author	Purpose	Population	Exposure	Outcome	Results/Comments
Duncan et al, 2007 [79]	Impact of a diet low in fermentable carbohydrate on microbiota	19 overweight and obese, yet otherwise healthy volunteers	Subjects consumed 3 dietary phases as follows: a) control diet (52% carbohydrate), b) high protein (30%), moderate carbohydrate (35%) diet, and c) high protein, low carbohydrate (4%) diet.	Faecal samples required at the end of each diet. Specific 16s rRNA FiSH probes used to identify differences in bacterial populations between diets. Gas chromatography was used to analyse SCFA concentrations	Reductions in *Roseburia spp, Eubacterium rectale* ($p<0.001$) and *Bifidobacterium* spp ($p<0.05$) abundance, and total SCFA and relative butyrate production ($p<0.001$) on low carbohydrate diet.
Russell et al, 2011 [107]	Effect of low-fibre diet on microbiota and metabolites	17 obese male volunteers	Randomised cross-over trial.1 week control diet (85 g protein, 116 g fat, and 360 g carbohydrate/d) followed by 4 weeks on a moderate (181g/day) or low carbohydrate (22g/day) diet.	Stools samples collected at the end of all dietary periods. Stool SCFA concentrations measured using gas chomatography and bacterial cell counts taken via FiSH	Reduced butyrate concentrations, *Roseburia* spp and *Eubacterium rectale* abundance on the low carbohydrate diet ($p<0.001$).

Author	Purpose	Population	Exposure	Outcome	Results/Comments
Brinkworth et al, 2009 [106]	Low carbohydrate (LC) vand high carbohydrate (HC) diet in overweight and obese individuals	91 overweight and obese, yet otherwise healthy volunteers	Subjects randomly assigned to isoenergetic LC (4% CHO) or HC (46% CHO) diet for 8 weeks	Stool samples collected pre- and post-intervention Specific bacteria investigated using selective plating methods	Significant reduction in the abundance of Bifidobacteria, total SCFA and butyrate faecal concentrations on LC diet ($p<0.001$).
Gibson et al, 1995 [111]	Investigative the microbial impact of dietary prebiotics, oligofructose and inulin	8 healthy human subjects	3 dietary phases as follows: a) control diet, b) 15g/d sucrose of control diet replaced with 15g/d oligofructose, c) 15g/d sucrose of control diet replaced with 15g/d inulin.	Faecal samples collected at end of each dietary period. Bacteria enumerated using specific plating methods for certain bacteria.	Bifidobacterial counts significantly increased with oligofructose and inulin ($p<0.01$). Bacteriodes, fusobacteria ($p<0.01$) and clostridia ($p<0.05$) significantly reduced during oligofructose diet. Total counts of cocci reduced with inulin

(p<0.001).

Author	Purpose	Population	Exposure	Outcome	Results/Comments
Boler et al, 2011[112]	Impact of soluble dietary fibre on gastrointestinal outcomes	21 healthy male volunteers	Participants consumed snack bar containing polydextrose (PDX), soluble corn flour (SCF) or a no fibre control (NFC) daily for 21 days	Faecal samples collected at end of each diet. Faecal pH, ammonia and SCFA recorded. Quantitative PCR enumerated E. coli, Bifidobacterium genus and Lactobacillus genus.	PDX and SCF decreased faecal ammonia and BCFA compared to NFC (*p<0.01).* SCFA production reduced with PDX compared to NFC (*p<0.05).* Compared to NFC, *Bifidobacteria* levels were significantly higher with SCF.
Holscher et al, 2015 [113]	Further investigation of the bacterial changes observed with administration of dietary fibre seen in Boler et	21 healthy male volunteers	Participants consumed snack bar containing polydextrose (PDX), soluble corn flour (SCF) or a no fibre control (NFC) daily for 21 days.	Faecal samples requested at end of each diet. Whole-genome shotgun 454 pyrosequencing used to identify bacterial composition and functional capacity.	PDX and SCF altered the Bacteriodes: Firmicutes ratio, by enhancing Bacteriodes, notably the saccorlytic species *Parabacteriodes*.

	al's study (2011) [112]				

Author	Purpose	Population	Exposure	Outcome	Results/Comments
Costabile et al, 2010[114]	To compare the microbial effects of dietary very long chain inulin (VLCI) and a maltodextrin placebo	32 healthy adult volunteers	Participants consumed both 10g/d VLCI or maltodextrin placebo for 21 days seperately.	Faecal samples collected before, during and after each diet. FiSH/ 16S rRNA was used to enumerate bacteria populations	VLCI consumption increased *Bifidobacterium* spp. and Lactobacilli levels ($p<0.05$). Bacteriodes - Prevotella counts reduced on VLCI ($p<0.05$).
Tuohy et al, 2002[115]	To monitor the colonic effects of dietary supplementation of the prebiotic, lactulose	20 healthy human volunteers	Participants were randomly assigned to consume a lactose supplement (10g) or placebo (5g lactulose, 5g glucose) daily for 26-33 days	Faecal samples collected before, during and one month after dietary interventions. Faecal bacteria analysed using both specific plating methods and FiSH/ 16S rRNA.	Lactulose supplement significantly increased *Bifidobacterium* spp. levels compared to both baseline levels and the placebo treatment ($p<0.01$). These levels fell back to pre-treatment levels

					following 1 month back on normal diet.

***In-vitro* fermentation studies**					
Author	**Purpose**	**Population**	**Exposure**	**Outcome**	**Results/Comments**
Hobden et al, 2013[108]	Impact of wheat dextrin on the composition and metabolic activity of gut microbiota	3 healthy human subjects	Three-stage continuous culture gut model used for *in vitro* fermentation of faecal samples. Fermentations carried out for 18 days (7g of wheat dextrin being added twice daily).	16S rRNA-based fluorescence in situ hybridisation used to analyse microbial composition	14g/d wheat dextrin increased total bacterial population, particularly *Clostridium cluster XIVa* and the *Roseburia* genus. SCFA production (especially butyrate) also increased
Connolly et al, 2010 [104]	The influence of fibre size on in vitro fermentation capacity	3 healthy human subjects	*In vitro* fermentation with oat flakes sizes of 0.53–0.63 mm and 0.85–1.0 mm, compared with oligosaccharide and cellulose, using faecal slurry from subjects.	Measurements of SCFA and DNA were taken after 0, 5hr, 10hr and 24hrs of fermentation.	Total bacterial population increased after 24hours of fermentation with larger oat flakes and oligosaccharide *($p<0.05$).* Increased *Bifidobacterium* and butyrate production seen in latter

					stages of fermentation with larger oats.
Leitch et al, 2007[116]	To determine whether different insoluble substrates are colonised by specific gut bacteria	4 healthy human participants	Faecal slurry fermented alongside 3 different insoluble substrates (wheat bran, high amylose starch and porcine gastric mucin) using an anaerobic continuous flow fermenter.	16S rRNA sequencing and fluorescent in situ hybridization analysis used to characterise and quantify tbacteria.	Clear association of specific bacterial species with the different substrates. Wheat bran associated with Clostridial Cluster XIVa. *Bifidobacterium adolescentis* and *E. Rectale* associated with the amylose starch. *Bifidobacterium bifidum* and relatives of Ruminococcus lactaris were affiliated with mucin.

1.4 Microbial and metabolic perturbations in IBD

1.4.1 IBD and gut microbiota

Animal studies have provided solid evidence highlighting the role of the intestinal bacteria in IBD. It has been shown previously that animals kept in "germ-free" conditions from birth maintain a healthy GIT however, on exposure to bacteria the animals are more likely to develop intestinal inflammation or colitis [117-119], highlighting that bacterial presence is necessary in the induction of colonic inflammation. It is likely that the symbiotic relationship between the gut microbiome and host is disrupted in IBD patients, and this may be mediated by a microbial dysbiosis.

UC and CD patients tend to have altered composition and diversity of intestinal bacteria [120, 121], with decreased numbers of beneficial bacteria such as lactic acid bacteria, *Faecalibacterium prausnitzii* and *Bifidobacteria* [103, 122], yet increased harmful pathogens including species from *Clostridium* [123], *Escherichia coli*, and those within the phylum Bacteriodetes [124]. Pathogenic strains of Proteobacteria have also been found in higher counts in IBD patients [68]. A study using a metagenomic approach to investigate the faecal microbial diversity of CD patients in remission reported that whilst 43 Firmicutes species were identified in healthy controls, only 12 were noted in patients [86], indicating a decrease in microbial complexity in IBD. In particular, *Clostridium leptum* and *Clostridium coccoides*, both butyrogenic species, were reduced in these CD patients. Furthermore, this study illustrated that only 13% of the operational taxonomic units (OTU) found in faecal samples of CD patients were completely unknown, compared to 69% in healthy people, again stressing a reduction of microbial diversity in CD patients. A recent study by Khalil and colleagues [125] investigated the differences between bacterial growth and SCFA production in UC patients compared to healthy controls, using an *in vitro* batch culture fermentation model. They reported that after 48 hours of fermentation with starch and peptone, UC patients had significantly less bacterial growth than healthy controls, with major reductions in 4 bacterial species (*Atopobium Lactobacillus, Clostridium histolyticum* and *Eubacterium rectale–Clostridium coccoides)*. However, there was a noted increase in sulphate-reducing bacteria in UC patients a species group which produces cytotoxic hydrogen sulphide and may perpetuate inflammation [123], further highlighting microbial dysbiotic relationships in these patients. However, despite an apparent reduction in microbial complexity in IBD, other studies have implied that both CD [126] and UC [126, 127] patients have an increased bacterial concentration both in faecal and mucosal samples compared to healthy people. It is plausible that this

may be caused by the thinner mucosal layer seen in IBD patients [24], which may support bacterial cell proliferation and penetration. This reduction in mucus production alongside an increased bacterial load may enhance the exposure of antigens to epithelial cells, inducing the immune response typical of IBD.

Aside from comparing the bacterial composition of IBD patients compared to healthy controls, it is also interesting to investigate discrepancies between patients with active disease and those in remission, as this may allude to underlying issues in IBD that are not directly associated with inflammation. In 2013, Kumari et al [128] used 16S rRNA sequencing to distinguish differences between the faecal microbial composition of UC patients with severe, moderate and quiescent disease. It was reported that *Clostridium coccoides* and *Clostridium leptum* were significantly reduced during severe and moderate disease, but restored to some extent in remission. A similar case-control study conducted in CD patients reported that the butyrate-producing bacterium *Faecalibacterium prausnitzii* was reduced in patients, and this reduction is directly associated with increased disease severity [129]. These studies imply that the bacterial dysbiosis displayed by IBD patients is not inherent but is in fact either caused by the inflammation experienced during active disease or by an external factor such as diet. The apparent association of bacterial perturbation with disease severity suggests that dysbiosis can be rectified to an extent, and that this might be associated with improvements in disease activity. However, an alternative study by Bibiloni et al (2006) [126] did not reveal differences between the microbial composition of non-inflamed and inflamed mucosal tissue of newly diagnosed IBD patients despite recognising differential bacterial patterns between CD and UC biopsies. These findings may suggest that bacterial imbalance in IBD is not confined to areas of inflammation, which would imply that inflammation is not the direct cause of dysbiosis. A further clinical study which challenges the role of microbiota in health disease reported that *Faecalibacterium prausnitzii*, which is largely regarded as health-promoting, was significantly reduced in paediatric CD patients whilst on treatment on EEN and this was accompanied by a significant reduction in faecal butyrate concentration [122]. The majority of the children in this study reached clinical remission in response to EEN despite the decrease in these beneficial parameters. These values returned to pre-treatment levels when the patients returned to their normal diet. As a fibre-free treatment, it is likely that the colonic bacteria were starved whilst on EEN. However the results of this study provide paradoxical evidence regarding the perceived benefits of *Faecalibacterium prausnitzii*. Thus, although there is an apparent bacterial contribution to both the onset and

treatment of IBD, the involvement of individual species is not yet well understood and there is currently no clear bacterial profile for either disease.

Whilst microbial composition is of interest, the functional capacity of the present bacteria offers an insight into the implications of bacterial perturbations of IBD patients. As mentioned, colonic fermentation is dependent on both the number and composition of available bacteria**.** The dysbiosis displayed by IBD patients' likely impacts colonic fermentation and its important medicinal end-products, SCFA.

1.4.2 SCFA production and IBD

Reduced faecal butyrate concentration has been found in studies in both CD [130, 131] and UC [125, 128, 131-133] patients. One study noted that although all CD patients did have a lower faecal butyrate concentration than healthy controls, this difference was significant only for those with mild to moderately active CD and not those in remission. Similarly, Galecka et al [129] and Kumari et al [128] respectively found that the concentration of SCFA measured in stool was inversely linked to disease activity in 34 CD patients and 26 UC patients with varying disease severity. As previously discussed, these studies also linked disease severity with reduced counts of *Faecalibacterium prausnitzi* and species from the *Clostridium* genus respectively. These studies imply that a reduction in SCFA production in IBD is linked to increasing inflammation, and that production of these beneficial anions is somewhat restored with improvements in colonic lesions. It is not yet clear whether inflammation reduces the capacity of bacteria to produce SCFA, or if in fact a reduction in SCFA precedes, or even provokes, the inflammatory response. Elucidating the direction of this relationship may help to uncover the pathophysiology of IBD. However, although these studies indicate an association between disease activity and faecal SCFA concentrations, it is hard to infer the actual production of these organic ions within the colon from such studies as the majority of SCFA are absorbed from the luminal environment prior to excretion [134]. Furthermore, the concentration of SCFA in faecal samples of IBD patients may be compromised due to the dilution effect of diarrhoea, of which is a common symptom of both UC and CD [135]. Nevertheless, the aforementioned studies that report reduced faecal SCFA concentrations do provide an insight into potential discrepancies in the production of these beneficial anions between those with a healthy GIT and IBD patients, and this area should be investigated further.

In an attempt to overcome this apparent decrease in butyrate production, there have been a number of clinical trials investigating the effect of butyrate enemas, more so in UC

patients than in CD patients. However, there is inconsistency in the results of these studies. Whilst some report that UC disease activity is improved with butyrate enemas [136-138], others have not found any clinical benefit to this type of treatment [139-141]. However, there is evidence to suggest that IBD patients cannot metabolise butyrate as efficiently as healthy counterparts, particularly with regards to UC. A study reflecting on the ability of substrate oxidation in mucosal biopsies taken from patients with mild UC or from those in remission (n=15) compared to those with healthy colonic cells (n=28) found that, although butyrate was the preferred fuel source in both healthy and UC colonocytes, UC patients had a significant reduction in rate of butyrate oxidation compared to controls (p=0.016) [142]. This study was similar to one performed previous in 1980 by Roediger et al [143] who found that butyrate oxidation was reduced in patients with both active and quiescent UC. A more up-to-date study by De Preter et al [144] again mirrored these results, adding that increasing the availability of butyrate to mucosal biopsies of active UC patients did not increase its oxidation to that of healthy counterparts.

Although the relationship between SCFA and IBD is not entirely clear, studies investigating faecal concentrations of these anions have identified impairments in IBD patients, particularly in the case of butyrate. Whether these faecal concentrations are representative of colonic SCFA production needs to be further elucidated, and the use of *in vitro* methods, which will be discussed later in this chapter, are invaluable in this field. Despite concerns that butyrate utilisation is not optimal in UC patients, the manipulation of SCFA production via dietary means remains to be an area of interest in this field.

1.4.3 Therapeutic potential of dietary fibre in IBD

As discussed, the fermentation of fibre in the large intestine is known to be beneficial to the colonic environment and thus there is reason to believe that dietary fibre may be important in IBD aetiology and therapy. Whilst there is substantial epidemiological research highlighting an association between intake of dietary fibre and improvements in colonic health in the general population, it is necessary to study the link between fibre substrates and IBD using both animal studies and randomised-control studies in humans in order to fully elucidate a cause-effect relationship. However, as some dietary fibres can cause bloating and abdominal discomfort, some IBD patients avoid the intake of such substrates [145]. It is therefore important to consider not only the physiological benefits of fibre intake, but also any detrimental effect it may have on health and in the context of IBD.

1.4.3.1 Studies in animal models

The consumption of non-digestible carbohydrates has been shown to have anti-colitic properties in murine and rodent models [34, 80], much of which was associated with enhanced butyrate production [146]. Dietary supplementation of plantago ovata has been shown to improve colonic health in both HLA-B27 transgenic rats [147] and rats in which colitis has been induced by means of trinitrobenzenesulfonic acid (TNBS) [148]**.** The supplementation of these seeds for two weeks prior to, and one week after the induction of colitis with TNBS, and for thirteen weeks in the HLA-B27 rats significantly decreased the production of TNF-α and the activity of nitric oxide synthase (NOS) which stimulate an immune response typical of colitis. These responses were linked to an enhancement in SCFA production, particularly butyrate and propionate.

Oral supplementation of the prebiotic inulin (400mg/day) in dextran sodium sulphate (DSS) colitis rats has been reported to improve mucosal lesions and decrease production of pro-inflammatory mediators [149]. This was associated with a decrease in pH, which may be indicative of enhanced SCFA production. Furthermore, a decrease in colonic pH is known to improve conditions for favourable bacteria whilst inhibiting the growth of detrimental bacteria, and this study noted that dietary inulin enhanced the presence of lactic acid bacteria which is known to be beneficial to colonic health.

Again using rodent models, FOS supplementation has also been linked to a decrease in release of pro-inflammatory cytokines, NOS activity and colonic damage, whilst increasing the beneficial bacteria, bifidobacteria and lactobacillus [150, 151]. However, another study conducted by Moreau et al [152] did not find any benefit of oral FOS supplementation (30g per litre of water) over the course of 7 or 14 days in DSS rats. On the other hand, these researchers found that 50g of resistant starch per litre of water did enhance colonic butyrate production and instigated mucosal healing.

1.4.3.2 Studies in humans

Research in the 1970's by Davies and Rhodes assessed the impact of dietary supplementation of oat-bran in patients with quiescent UC [153]. They reported that, although generally well tolerated by patients, the daily supplementation of 25g oat-bran alongside increasing general fibre intake via foodstuffs did not enhance remission period compared to a standard treatment drug. However, as an insoluble fibre, oat-bran is not fermentable and thus should not be expected to greatly enhance colonic environment. Therefore, a similar study was conducted by Hallart et al [154] using the soluble plantago

ovato husk and in this instance, it was reported that the functional symptoms of UC patients in remission were significantly decreased following 4-months of dietary supplementation of this fibre when compared to a placebo.

Plantago ovato seeds were used in another study comparing its impact on quiescent UC maintenance against the standard treatment drug, mesalamine [155]. Patients (n=105) were randomly allocated into one of three following treatment groups; mesalamine alone (3 daily doses of 500mg), equal daily doses of mesalamine and plantago ovato seed, and plantago ovato seed alone (10g twice a day). After 12 months, it was reported that treatment failure rate was similar in all groups and that the effectiveness of each treatment was comparable. The fibre supplementation was generally well tolerated in the patients, and was found to significantly enhance faecal butyrate levels.

As known prebiotics, germinated barley foodstuffs favourably alter microbial composition, enhancing the number of bifidobacteria and lactobacillus whilst inhibiting the growth of harmful bacteria. Studies have investigated the therapeutic effects of germinated barely foodstuffs on UC symptoms, highlighting its benefits to both active [156] and quiescent [156, 157] forms of the disease.

The majority of studies investigating the oral supplementation of fibre on IBD patients are conducted in UC patients whilst in remission, with few dedicated to CD patients. However, a fairly recent small clinical trial conducted by Lindsay et al [158] reported that the daily oral supplementation of FOS (15g for 3 weeks) in patients with active CD significantly decreased disease activity and improved faecal bacterial composition by significantly enhancing bifidobacteria numbers. The expression of IL-10, an anti-inflammatory cytokine, was also increased on the lamina propria of dendritic cells following supplementation. Importantly, FOS was well tolerated by CD patients. Another recent randomised controlled trial by De Preter and colleagues [130] found that despite a reduced baseline concentration compared to healthy controls, the administration of 10g oligofructose-enriched inulin (twice daily for 4 weeks) significantly increased faecal butyrate measurements in patients with mild and inactive CD ($p=0.0011$). This change, which was associated with a reduction in disease activity as recorded using a clinical index, was not found in patients who were given the placebo.

1.4.3.3 Exclusive Enteral Nutrition

Despite the apparent therapeutic properties of dietary fibre both in a healthy GIT and in IBD patients, the exclusive of dietary fibre is known to promote remission in paediatric CD patients [122]. As an alternative to steroids, paediatric CD is often treated with a period of

exclusive enteral nutrition (EEN); a polymeric formula that provides all nutritional requirements, administered in liquid form or via a feeding tube. Although EEN is successful in terms of inducing clinical remission [159], the mechanisms by which it does so are not fully understood. However it is known that this treatment paradoxically reduces SCFA production and reduces counts of perceived beneficial bacteria [122]. This confounds existing knowledge regarding the beneficial properties of fibre and its health-promoting effect within the colon. Nevertheless, the application of EEN highlights the medicinal prospects of dietary manipulation and the role of diet in the pathogenesis of CD.

1.4.4 Current guidelines regarding intake of dietary fibre in IBD

Evidently, the relationship between dietary fibre and IBD is complicated and apparently dependant on disease activity. Current guidelines regarding fibre intake in IBD therefore take this into consideration and vary according to disease state [146]. Whilst those with active disease are advised to omit fibrous food from their diet, those in remission are encouraged to maintain the same guidelines as healthy people from the same age bracket [146]. In the United Kingdom, both male and female adults are advised to consume 30g fibre per day [160].

1.5 Measurement of colonic fermentation

The majority of studies investigating SCFA in IBD have investigated the concentration of these acids in stool. Whilst this serves as a proxy of SCFA production, most SCFAs are quickly absorbed from the colonic environment are either distributed to the systemic circulation or utilised as colonocyte fuel [80]. It is important that research attempts to investigate the actual production of SCFA in IBD, however this is easier said than done. Both *in vivo* and *in vitro* techniques have been employed to gauge SCFA production in humans.

1.5.1 *In vivo* methodology

Although the bacterial activity conducted within the human colon and its involvement in human health and disease has been of scientific interest for decades, research techniques used to study this important eco-system and its behaviour were very primitive until recently. Of course modern advancements in molecular biology have been invaluable in the profiling of bacteria; however the first issue in the study of these organisms and their metabolites is gaining initial access to their habitat.

The majority of bacterial fermentation is conducted in the proximal colon [80] , and is therefore notoriously difficult to study *in vivo*. A few studies have used intestinal cannulation techniques [161, 162]; however these have been restricted to animal studies and are deemed overly invasive and impractical for use in humans. Some human studies have used intubation of the proximal colon either via oral [163], rectal [92] or colonic [164] route, however this physical insertion into the GIT would undoubtedly cause perturbations in normal gastrointestinal function. The colonic contents of sudden death patients have been studied as an *in vivo* method of investigation, but this evidently requires strict ethical considerations and studies using this technique often have low patient numbers [165, 166]. As gut fermentation produces hydrogen and methane, breath analysis is considered a favourable, non-invasive and straightforward indication of real-time gut fermentation [80]. However this is a fairly crude technique and, as some studies have found discrepancies between expired gas and SCFA production [167], needs optimisation.

1.5.2 *In vitro* fermentation methods

Evidently, *in vivo* measurements of colonic activity are limited by the accessibility to the colon, impracticalities and ethical restraints. Although animal models, particularly those in rats [168-170] and pigs [80, 171, 172], are used as indictors of human gut activity, it is not clear how reliably these findings can be extrapolated to humans and thus *in vitro* methods have been instrumental in this field. Although they employ different fermenter models (either continuous, semi-continuous or batch-culture), the main *in-vitro* techniques are all based on the provision of appropriate growth media, the inoculation of human gut bacteria (often obtained via faeces) and conditions that mimic those within the human colon (i.e. neutral pH, body temperature and gentle shaking simulating peristalsis) [173]. Continuous cultures involve a series of vessels which aim to simulate the different regions of the GIT, and allow the continual removal of toxic materials whilst replenishing substrates, processes which naturally occur in the colon [173]. These systems are good for long-term fermentations but need constant attention. One the other hand, the batch culture fermentation model is a closed system that does not allow the addition or removal of material once the vessels have been anaerobically sealed. This system is particularly useful when studying the production of metabolites such as SCFA [173]. Substrates of choice can be fermented alongside the faecal inocula and growth media, and incubated for any chosen time period (normally no more than 48 hours). Many studies choose to take aliquots from the fermentation vessels at specific time-points using a sterile needle for further analysis of metabolites and their rate of production [174-176]. Batch culture systems are easy to

implement, don't require expensive equipment and are not as time-consuming as continuous fermenters [173]. For these reasons, and as SCFA is a primary outcome of the studies within this thesis, batch culture system were the method of choice within this thesis.

1.6 General Conclusions and the purpose of this thesis

It is clear that the aetiology of IBD is poorly understood on the whole; yet there are strong veins of cumulative research investigating the independent factors which are thought to contribute to these organic diseases. IBD is clearly multifactorial and therefore these factors are probably integrated in its causation and/or development. Therefore, research in this field should now be focused on the relationship between factors implicated in the onset of IBD using modern technology. As aforementioned, there is a strong implication that the gut microbiota plays a strong role in IBD. Whether this is a result of microbial dysbiosis and/or a reduction in functional capacity is debatable and warrants further investigation. Furthermore, prior studies have mainly investigated both bacterial and SCFA concentration in stool samples and although this offers an insight into the colonic environment, only generates a snapshot of information that must be interpreted with caution.

Thus, the aim of this thesis is to explore the interplay between dietary fibres, human gut microbiota in IBD using an established *in vitro* fermentation model. Adult IBD patients, paediatric CD patients and healthy controls will be recruited to donate a faecal sample which will give a representation of their gut microbiota. Of prime interest will be the functional capacity of the bacteria in terms of their ability to produce SCFA in response to various fibrous stimuli, of which is reported in Chapter 3. It is hypothesised that there will be variation in the capacity to produce these metabolites between IBD and healthy controls and it is likely that these changes will include a reduction in butyrate production in patients. Furthermore, it is of interest to investigate whether the composition of the gut microbiota of IBD differs from matched healthy controls, and to see if these bacteria respond differently to dietary fibre and whether incubation of the same amount and type of fibre will shift microbial communities towards the same profile (see Chapter 5).

An additional aspect of this thesis is to gain further understanding of the *in vitro* fermentation method used and thus Chapter 3 reports on the effect of increasing fibre availability during such experiments.

Ultimately the purpose of the investigations undertaken as part of this thesis is to utilise *in vitro* methodology to investigate the microbial composition and functional capacity of the gut microbiota in IBD patients and compared with healthy controls. Using

these techniques it will be possible to identify whether an initial difference in bacterial composition in IBD patients can be rectified with the addition of dietary fibre. Results of this study may shed light on both the pathogenesis of IBD and potential management strategies, contributing research to a field which desperately needs information in order to overcome the idiopathic nature of these diseases.

Chapter 2 : Subjects and Methods

2.1 Chapter outline

This chapter will outline the study design, describing participant recruitment and research methodology, as well as data and statistical analysis.

2.2 Study Design

The primary aim of this cross-sectional study was to compare the fibre fermentation capacity of the gut microbiota of IBD patients compared with healthy controls. To investigate this, *in vitro* batch culture fermentations were conducted using faecal samples obtained from CD and UC patients recruited from the local gastroenterology clinics, as well as healthy controls. Using these faecal specimens, various fibres were fermented to explore any differences in the production of SCFA between patients and healthy controls. Aliquots were extracted from the fermentation vessels before and after a 48 hour incubation period for the analysis of DNA and SCFA production.

In a mechanistic study the ability of the gut microbiota to produce SCFA during *in vitro* batch culture fermentation with increasing substrate availability was investigated. This was done using faecal samples from healthy participants. Although the standard *in vitro* methods as outlined in Section 2.4.2 was used, minor changes to this protocol are described in Chapter 3 (see section 3.3).

2.3 Recruitment

2.3.1 Ethically approval

This study gained ethical approval from the NHS, West of Scotland Research Ethics Committee 4 with respect to patient participation, and the University of Glasgow, College of Medical, Veterinary and Life Sciences (MVLS) Ethics Committee with respect to healthy participants.

2.3.2 Study Participants

Six groups were recruited for the purposes of this study:

i) *Adult CD patients in remission:* Patients (≥18years) were identified by the consultant gastroenterologist as being in clinical remission. These patients were recruited during their routine appointments at the gastroenterology outpatient clinic of Glasgow Royal Infirmary.

ii) *Adult UC patients in remission:* Patients (≥18years) were identified by the consultant gastroenterologist as being in clinical remission. These patients were recruited during their routine appointments at the gastroenterology outpatient clinic of Glasgow Royal Infirmary.

iii) *Healthy adults with no history of IBD:* Adults (≥18years) who had no personal or familial history of gastrointestinal disorders were recruited to match the patient cohorts in terms of age, gender and BMI.

iv) *Paediatric patients with CD with active disease:* These were children (≤17 years) with newly diagnosed, treatment naïve, active CD who were recruited prior to the commencement of any medical treatment.

v) *Paediatric CD patients on contemporary treatment:* These children (≤17 years) were recruited following their return to habitual diet, between 2 to 12 months following the course of EEN treatment.

vi) *Healthy children with no history of IBD;* Children (≤17 years) who had no personal or familial history of gastrointestinal disorders were recruited to match the paediatric patient cohorts in terms of age, gender and BMI.

2.3.3 Exclusion Criteria

Participants who reported a regular intake of pro- or prebiotics and/or the use of antibiotics within the 3 months prior to recruitment were not asked to partake in this study.

2.3.4 Participant recruitment

2.3.4.1 Recruitment of adult UC and CD patients in remission

Both UC and CD adult patients were recruited in gastroenterology clinics at the Glasgow Royal Infirmary between August and October 2015. The patients were originally identified by the consultant gastroenterologist during their routine appointment and if the patient obliged they were approached by the researcher who thoroughly explained the study to them. A participant information sheet (see Appendix 1) was provided to all patients and they were encouraged to ask any questions regarding the study. If the patient chose to participate in the study, they were provided with three consent forms (see Appendix 2); one for the researcher, one for hospital notes and one for the patient.

2.3.4.2 Recruitment of paediatric CD patients

The recruitment of paediatric CD patients with both active and quiescent disease was conducted in clinics at the Royal Hospital for Sick Children Yorkhill, Glasgow, between April and November 2015, as part of a larger study assessing the impact of EEN on faecal metabolites and gut microbiota in paediatric CD. The original study recruited patients prior to the commencement of EEN, tracking them throughout the duration of this treatment for 8 weeks and up to 12 months following return to habitual diet. Potential participants were identified by the consultant gastroenterologist and then approached by the PhD student who both verbally explained the study and supplied the children and their parents/guardian with a relevant information sheet. Participants were encouraged to ask any questions regarding their participation in the study and if they agreed to partake, consent and assent forms were issued in triplicate (one for the patient, researcher, and for the hospital records).

2.3.4.3 Recruitment of adult and child healthy controls

Healthy participants were recruited via online advertisements and through leaflets distributed throughout the hospital and university campus. Potential participants were encouraged to contact the researcher with the provided phone number or email address. Upon contact, responders were supplied with an information sheet (see Appendix 3) via email. If they were happy to participate, the researcher arranged to meet the participant at a place of their convenience to supply them with the stool collection kit and consent forms

(see Appendix 4). Two identical consent forms were supplied; one for the participant and the other for the researcher. Healthy controls were matched to patients in terms of age, BMI and ethnicity and gender. Healthy participants who completed the study were offered a £10 shopping voucher.

2.3.5 Collection of participant characteristics and information regarding disease activity

Patient height and weight was recorded by nurses during the appointment in which they were recruited as part of routine assessment. These measurements were passed on to the researcher and were used to calculate body mass index (BMI) (kg/m^2). Adult CD and UC patients were asked to complete a Clinical Report Form, which enquired about their age at diagnosis alongside current and previous medication. This information was verified using medical records, providing the patient had consented to these being accessed by the researcher. Information regarding disease characteristics, namely behaviour and location, was also obtained from patients' notes. For both UC and CD patients, this information was used to assess Montreal Classification [177].

All healthy controls were presented with a short health questionnaire regarding their own and familial gastrointestinal well-being. This included use of antibiotics or pre/probiotics in the 3 months previous to recruitment. Height and weight were recorded via a Stadiometer model (SECA 213, Leicester, UK) and scales (TANITA TBF-310, Cranela, UK), respectively.

2.4 Laboratory Methods

2.4.1 *In vitro* batch culture fermentations

The batch-culture fermentations chosen for use in this thesis are based on the method quantified by Edwards et al (1996) [178], who felt that there were too many discrepancies between the batch culture method used in different laboratories. This research team standardised the method in terms of media used, amount of substrate provided, inoculum injected and shaking rate, and hence validated the proposed method in 8 laboratories. 40 different faecal samples were fermented alongside various resistant starches and a non-fibrous control, and results showed that SCFA production was comparable between most of the participating laboratories. Thus, this method has been implemented in many studies investigating the fermentability of different fibres by human gut bacteria in the last two decades. For these reasons, it was deemed appropriate to implement this method in the

experiments in this thesis, which aim to characterise both the bacteria associated with IBD and the ability of these bacteria to utilise dietary fibre to produce SCFA.

Faecal samples of participants were collected promptly after defecation, after which they were added to a phosphate buffer solution to produce a slurry. This slurry was inoculated into previously sterilised fermentation vessels containing specific growth media and either one of the chosen fibres, a mixture of these fibres or no fibre at all. The fermentation vessels were incubated for 48 hours under anaerobic conditions in a shaking water bath at 37˚C. Before and after 48 hours, aliquots were taken from each vessel for future analysis of SCFA production, DNA and immune response. A flow chart of the *in vitro* process can be viewed in Figure 2.1 whilst the process for taking aliquots is displayed in Figure 2.3.

2.4.1.1 Fibre Substrates

Seven different fibres were investigated in this study: maize starch (HI-MAIZE®, National Starch and Chemical Ltd., batch no. KKI0283), apple pectin (Sigma-Aldrich Company Ltd, 76282), raftilose (Orafti® P95, BENEO, batch no. PECBR0CBR0), wheat bran (Infinity Foods Co-operative Ltd.), α-cellulose (Sigma-Aldrich Company Ltd., lot no. 100H0747), and butyrate and propionate esters (supplied by Dr Douglas Morrison, University of Glasgow, SUERC, East Kilbride). Additionally, 0.1g of maize starch, apple pectin, raftilose, wheat bran and α-cellulose were combined to provide 0.5g of mixed fibres to be included in the batch-culture fermentations. The fibres were weighed prior to sample arrival and placed in sterile 100mL McConkey bottles. The fibre utilised in this study were chosen offer a wide range of fermentability. Apple pectin, hi maize and raftilose are fermentable, whilst wheat bran is not readily fermented. Cellulose is insoluble and offers almost no fermentable properties. The range of fibres was also chosen to investigate whether the gut microbiota of IBD patients and healthy controls differed in their ability to fermenting various fibres. A non-substrate control (NSC) was conducted for each participant.

2.4.1.2 Preparation of culture media

Fermentation medium((per litre) 2.25g tryptone, 450mL distilled water, 225mL bicarbonate solution, (2g NH_4HCO_3, 17.5g $NaHCO_3$, 500mL ddH_2O) 225mL macromineral solution (2.85g Na_2HPO_4, 3.1g KH_2PO_4, 0.3g $MgSO_4.7H_2O$, 500mL ddH_2-O) 1.125mL micromineral solution(13.2g $CaCl_2.2H_20$, 10g$MnCl_2.4H_2O$, 1g $CoCl_2.6H_2O$,

8g $FeCl_3.6H_2O$, 100mL ddH_2O), 1.125mL 0.1% resazurin) and Sorenson's phosphate buffer (pH 7) were boiled for 5 minutes prior to being purged with oxygen-free nitrogen for 30 minutes, or until they reached 37 °C. A reducing solution (0.3125g cysteine hydrochloride, 0.3125g $Na_2S.9H_2O$, 2mL NaOH, 50 mL distilled water) was prepared during this period; 2 mL of which was added to each McConkey bottles containing 0.5 g of pre-weighed fibres.

Following purging and cooling, the pH of the fermentation medium was readjusted to 7 using 6M hydrochloric acid. 42mL of the fermentation medium was added to the fibre and reducing solution in the McConkey bottles, which were then sealed with a self-sealing silicon crimp top. The bottles were again purged with oxygen-free nitrogen for 1 minute to ensure anaerobic conditions.

2.4.1.3 Sample Collection

Participants were issued a sample collection kit with written instructions on how to use it. Participants were requested to collect an entire bowel movement in the provided pre-weighed plastic container, immediately replacing the perforated lid and placing it into a bag containing an anaerobic sachet (Anaerocult® A, Merck Millipore, Darmstedt, Germany). After tightly tying the bag closed, the container was placed in an insulated bag with a freezer block, after which participants were required to notify the researcher immediately to arrange prompt collection. These conditions maintained microbial viability by hindering bacterial metabolism until samples were delivered to the laboratory and processed. The majority of samples were processed within 4 hours of defaecation, with a mean processing time of 3.15 ± 0.13 hours.

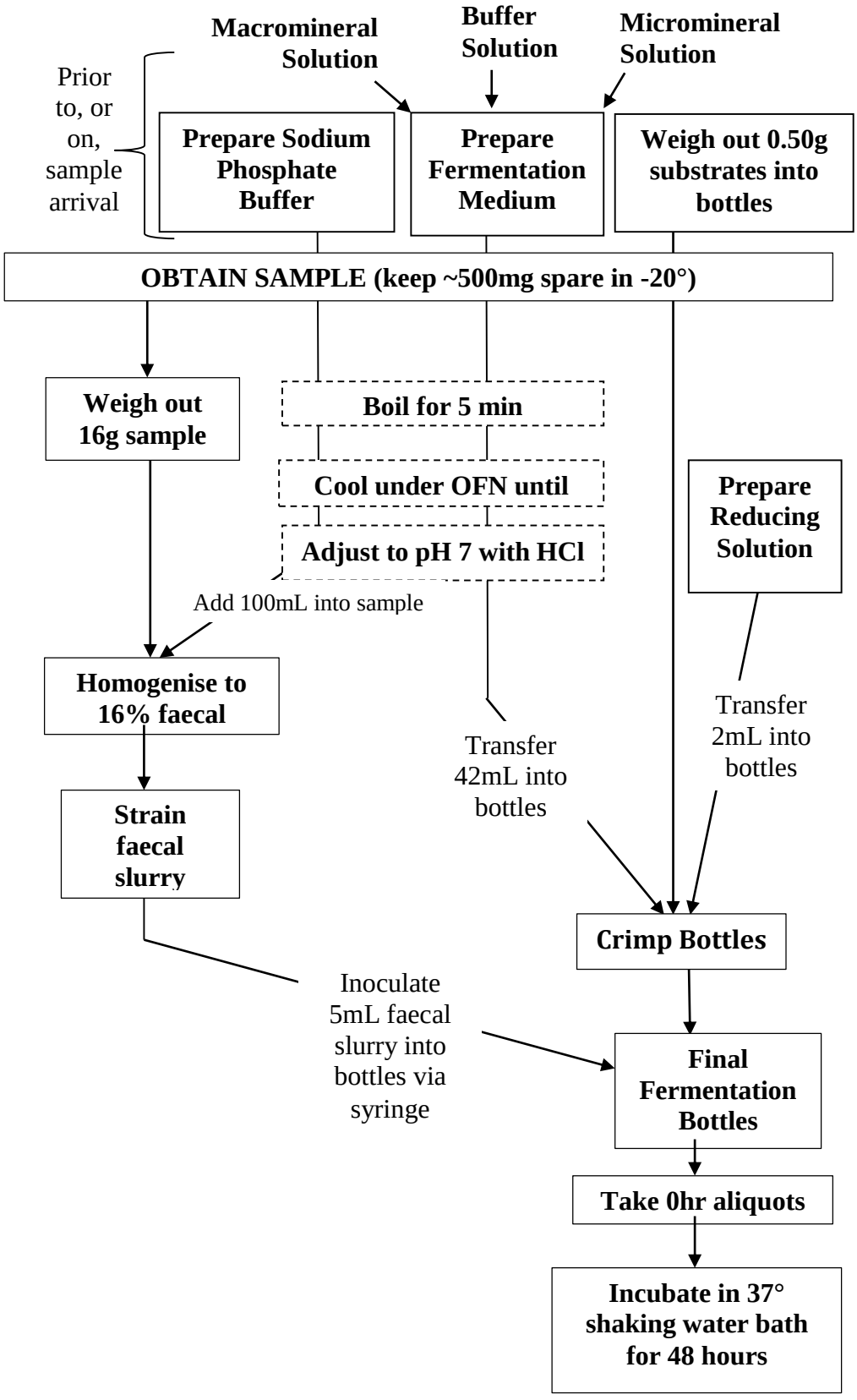
2.4.1.4 Sample processing and incubation

Sample processing was initiated with the homogenisation via mechanical kneading. Approximately 5g of stool was placed in a bijoux and stored at-20°C prior to the addition of 100mL of Sorenson's phosphate buffer to 16g of stool to create a 16% slurry using a blender (BraunTM, Kronberg, Germany). The slurry was then strained through a nylon mesh to remove large particles after which 5mL slurry was injected into each McConkey bottle using a 10mL syringe and a sterile 19-gauge needle. The bottles were once again purged with oxygen-free nitrogen for 1 minute before being manually shaken to homogenise the contents prior to 0 hr aliquots being taken. Following this, the bottles were incubated in a shaking water batch (60strokes/minute, 37°C) for 48 hours.

2.4.1.5 In vitro measurements of pH and gas production and extraction of slurry for SCFA and DNA analysis

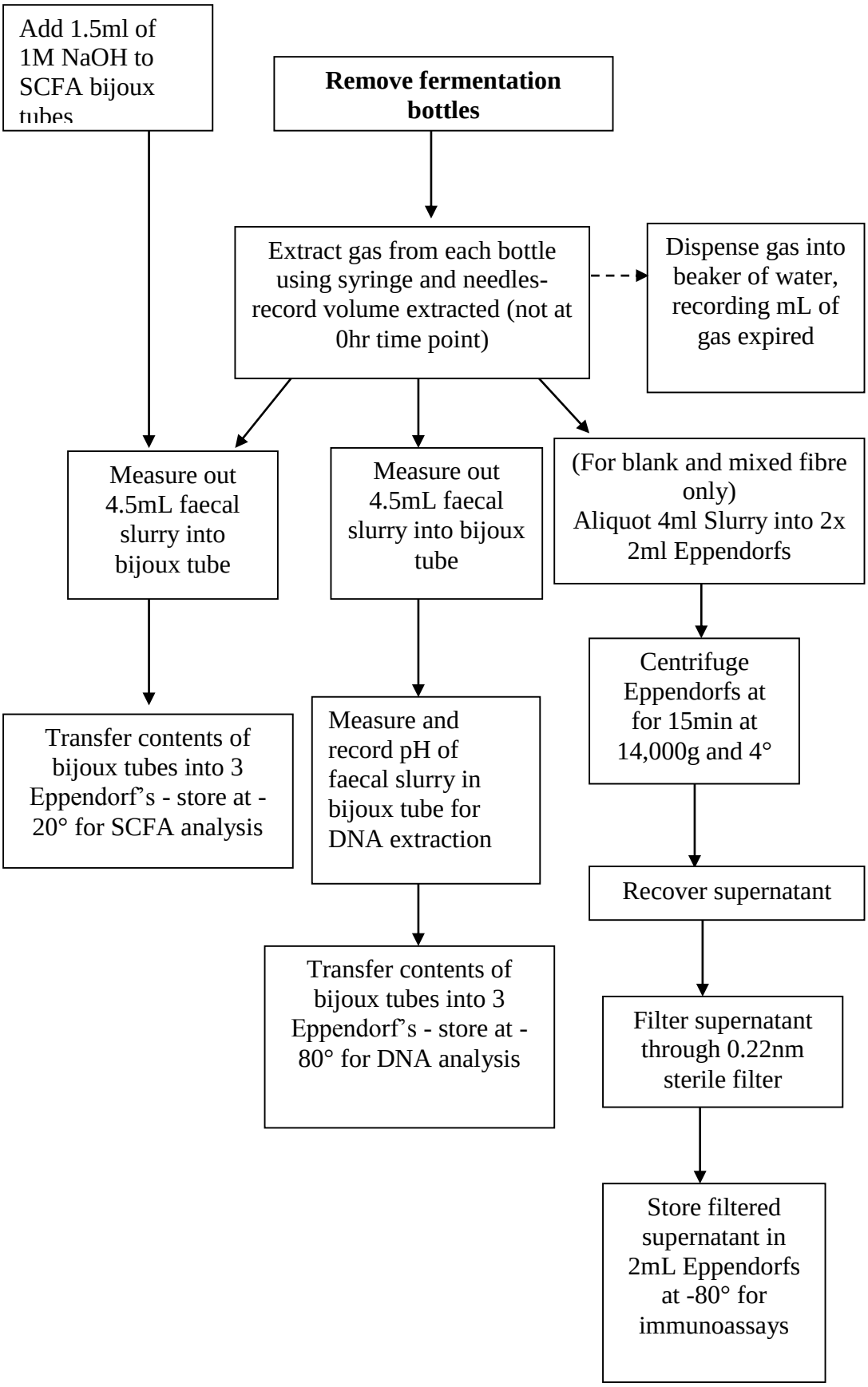
Aliquots were taken at 0 and after 48 hours of fermentation. At the 48 hour time point, the volume of gas production was measured by piercing the silicon caps with a sterile 19-gauge needle attached to a 10mL syringe. At both time points, 4.5mL aliquots were extracted from the bottles and added to 1.5mL 1M sodium hydroxide (NaOH) for SCFA analysis. Upon mixing, the slurry and NaOH mixture was separated into three 2mL eppendorfs and stored at -20°C until further analysis. A further 4.5mL extracted from each bottle and distributed into 3 eppendorfs of 1.5mL slurry. The pH of the slurry was determined from these aliquots using a calibrated pH meter (Hanna® Instruments, Rhode Island, USA). Once pH was recorded, these eppendorfs were stored at -80°C for future analysis of bacterial DNA.

Figure 2.1 Flow Chart displaying the in-vitro fermentation protocol



OFN: oxygen-free nitrogen

Figure 2.2 Flow chart displaying the procedure of taking aliquots for future immunoassays and SCFA and DNA analysis. Aliquots were taking prior to and following 48h of in vitro fermentatio



2.4.2 *In vitro* fermentation *sans* the introduction of human gut microbiota

This research was conducted using the exact *in vitro* fermentation protocol described in Section 2.4.1 with the exception that no faecal slurry was introduced to the cultures in order to allow the observation of any activity occurring in the absence of human gut bacteria. Essentially, this was conducted as a control experiment for the studies within the thesis. Thus, the cultures contained only the fermentation media alongside one of 8 different fibres, one mixed fibre and a non-fibrous control as outlined. Aliquots were taken as previously described.

2.4.3 Gas chromatographic analysis of SCFA

Gas liquid chromatography was used to identify and quantify SCFA and BCFA production. Although other detectors are available, a flame ionisation detector (FID) was employed during this study.

2.4.3.1 Principles of gas chromatography

Gas chromatography is used for the separation of volatile materials that can include gases, liquids and dissolved solids based on the differing boiling points of organic compounds. The system is dependent on a gas supply, known as the carrier gas, which is necessary for the transport of the compound through a column within an oven of a set temperature. The carrier gas (often helium, argon or nitrogen depending on the column type) is inert and highly purified, and its flow rate is monitored closely. The sample is exposed to the gas following injection into the column via a sample port, which has a temperature of at least 50˚C higher than the boiling point of the least volatile compound within the sample. This ensures that there is no loss of organic compounds prior to entry to the column. The carrier gas then transports the sample through the column where its components are separated at different speeds. Thus, these compounds reach the end of the column at different times, where they encounter the FID. At this point the compound is mixed with hydrogen and air and passed through a flame, causing pyrolysis of the compound which produces ions and electrons. These particles generate an electric current proportional to the number of reduced carbon atoms detected.

2.4.3.2 Production and analysis of a chromatogram

The electrical signals produced within the FID are transmitted to software which records these signals as peaks on a graph known as a chromatogram. The peaks are presented on the graph in the order that the corresponding electrical signals are generated, and thus the order of the peaks represents the order in which the organic compounds reached the FID. The "retention time" refers to the time taken between sample injection to the peak generation. The area produced under the peak, known as the "area under the curve" is proportional to concentration of the SCFA present.

2.4.3.3 Preparation of external and internal standards

The use of standards during gas chromatography is necessary in order for comprehension and interpretation of the gas chromatogram. The internal and external standards create a standard curve for analysis, determining the response factor. This information allows the area under the curve of each peak on the chromatogram to be calculated into a corresponding concentration of the associated SCFA/BCFA.

Internal standard was produced using 73.68mM 2-ethyl butyric acid. 1.71452g 2-ethyl butyric acid was weighed and rinsed with 2M NaOH before being added to volumetric flask containing 100mL of 2M NaOH. 2M NaOH was added to this flask until a volume of 200mL volume.

External standards were prepared by combining SCFA and BCFA of known molarities containing between 2 to 11 carbon atoms, as shown in Table 2.1. 100mL of 2M NaOH was added to a volumetric flask. Specific weights of each acid (summarised in Table 2.1) were rinsed in a small volume of 2M NaOH before being added to the volumetric flask. Once all acids had been added, 2M NaOH was added to the flask until a final volume of 200mL was obtained. The flask was shaken well to mix contents. Both internal and external standards were aliquoted into 2mL eppendorfs and stored at 4°C.

Six standards containing known concentrations of the external and internal standard were prepared each day of SCFA analysis and ran at the start of each set. 10µL, 25µL, 50µL, 100µL, 200µL and 300µL external standard were added to respectively to 790µL, 775µL, 750µL, 700µL, 600µL and 500µL distilled water respectively. 100µL internal standard was added to all standards alongside 100µL orthophosphoric acid, resulting in a 1000µL final volume. These standards were then prepared for gas chromatography as outlined in the next section.

The standard containing 100µL of external standard was run after every twelfth sample as a quality control. Table 2.1 Concentration of the external standards used in the preparation of the standards used for gas chromatography

No of Carbon atoms	Acid Name	Molar Mass (g/L)	Standard Concentration (mM)	Weight (g)	Product company	Product Code
2	Acetic acid	60.5	183.3	2.1441	Sigma Aldrich*	695092
3	Propanoic acid	74.07	133.2	1.9930	Sigma Aldrich	94425
4	Butyric acid	88.11	104.2	1.9690	Acros Organics†	108111000
5	Valeric acid	102.13	86.3	1.8367	Sigma Aldrich	240370
6	Caproic acid	11.16	74	1.8614	Sigma Aldrich	W255900
7	Enenthic acid	130.18	64.7	1.7844	Sigma Aldrich	75190
8	Caprylic acid	144.21	57.5	1.6584	Sigma Aldrich	C2875
9	Isobutyric acid	88.11	102	1.8356	Sigma Aldrich	I1754
10	Isovalerate acid	102.13	86	1.7466	Sigma Aldrich	129542
11	Isocaproic acid	116.16	50	1.2176	Sigma Aldrich	277827

*Sigma-Aldrich Company Ltd (Dorset, UK)
† Acros Organics, Thermo Fisher Scientific (New Jersey, USA

2.4.3.4 Preparation of sample for SCFA analysis

Although samples were collected for the analysis of SCFA production in response to chemically produced butyrate and propionate esters, these samples were not analysed as part of this thesis due to methodological issues. As these compounds respectively contain both butyrate and propionate, the preparation of these samples prior to analysis via gas chromatography would break the ester linkages and release these SCFA. Therefore, it was

unclear if the results obtained from gas chromatography would truly reflect *in vitro* SCFA production. Therefore, method optimisation for the analysis of these samples is necessary, however this was unfortunately out with the time limits of this study.

As aforementioned, fermentation slurry was stored at -20°C following the addition of NaOH. SCFA and BCFA are incredibly volatile, thus the 1M NaOH was added to prevent loss of these organic compounds. After thawing, 800µL slurry was added to 100µL internal standard solution and 100µL orthophosphoric acid. 3mL diethyl ether was added to this mixture, before being homogenised by vortex at 1,500rpm for 1 minute. Following homogenisation, the upper organic layer was extracted and pooled in a clean tube. The process of diethyl ether addition, homogenisation, and extraction occurred three times before the organic extract was placed in a vial, which was then sealed with a crimp top and placed in the gas chromatographer (TRACE^{TM} 2000 GC machine, ThermoQuest Ltd, Manchester, UK). 1µL of the organic extract was automatically injected into a Zebron ZB-Wax capillary column (15m x 0.53mm id x 1µm film thickness) made of polyethylene glycol (catalogue No. 7EK-G007) and converted into gaseous form. The vaporised extract was carried through the column by the carrier gas (nitrogen) and the FID was used to determine the concentration of SCFA (C2-C5) and BCFA.

The injection needle was cleaned between each sample using ether and 100% methanol. Furthermore, a sample of ether was injected every 12 samples to monitor the occurrence of contamination.

Data regarding production of SCFA was captured and analysed by Chrom-Card 32-bit software version 2.2 (Thermo Scientific®, Milan, Italy).

2.4.3.5 Calculation of SCFA and BCFA based on chromatograms

As mentioned, the area under the curve (AUC) of the peaks generated on the chromatogram is proportional to the concentration of the respective SCFA/BCFA. In order to calculate the concentration, the area ratio of the AUC of each external standard to that of the internal standard had to be deduced by the following equation;

Area ratio of external standard = AUC of external standard / AUC of internal standard

The ratio of calibrators, which relates to the relationship between internal and each SCFA within the external standards was then calculated as follows;

Concentration of individual SCFA in 100 µL external standard/ concentration of the same SCFA in the internal standard

Using these values, calibration curves were produced, from which the equation of the line generated allowed the calculation of the SCFA concentrations of the unknown standards.

2.4.4 Extraction and amplification of microbial DNA

To characterise the microbial composition of the gut microbiota and changes during the fermentation studies genomic DNA was extracted from the faecal slurries before and after fermentation. For extraction of genomic DNA the chaotropic method was used as described before [122]As mentioned, eppendorfs containing 1.5mL slurry from fermentations were stored at -80°C until extraction. Samples were stored for 73.00 (38.00) days (median (IQR) before analysis.

2.4.4.1 Freeze Drying

Prior to DNA extraction, samples were freeze-dried to remove water content. Using a sterile needle, holes were punctured in the top of the eppendorfs containing the frozen slurry after which they were returned to -80°C for 24hours. The components of the freeze drier were cleaned with methanol, and a metal tier for which the samples would rest on was placed in -80°C for 15 minutes. At this point the freeze drier was turned on and once the set temperature was reached, the samples and metal tier were retrieved from the -80°C freezer and placed in the freeze drier where they remained for 36hours. Following freeze drying, the eppendorf lids were replaced with a non-punctured lid and the freeze-dried weight of the sample was recorded. Para-film was used to seal the lids before returning them to -80°C until DNA extraction.

2.4.4.2 Chaotropic method of DNA extraction

The DNA of 11 samples were extracted in each set of extractions conducted using a protocol which straddled two days. A negative control sample containing 50µL sterile water was ran alongside each set, undergoing each of the steps outlined. Unless otherwise stated, the chemicals and reagents used in this protocol were prepared in advance. A list of these chemicals and their preparation can be found in Table 2.2.

DNA extraction was initiated with the addition of 250µL of 4M guanidine thiocyanate (Sigma Aldrich, G9277), a chaotropic agent which denatures protein and lyses

cells. Following this, 40µL of 10% N-Lauroylsarcosine (Sigma Aldrich, L9150) was added to each sample before homogenisation via vortex and centrifuging for three seconds at 4°C at 15,000g. The samples were then left to rest at room temperature for ten minutes. During this time, 20mL phosphate buffer (pH8) was added to 1g N-Lauroylsarcosine to create 5% N-Lauroylsarcosine; 500µL of which was added to each sample after the ten minute resting period. The samples were then vortexed quickly, centrifuged for three seconds at 4°C at 15,000g and then incubated in a dry bath for 1 hour (70°C, 1400rpm).

Following incubation, the samples were quickly centrifuged (15,000g, 4°C, 3 seconds). Approximately 750mg of sterile 0.1mm zirconia beads (Biospec. Products, USA) were added to each tube before vortexing shortly. The samples were then shaken in a bead beater (MP FastPrep-24) for 2x30 seconds at 6m/s, with a 15 second break in between shaking. The samples were then placed on ice for 5 minutes before undergoing a further 2 x 30 second shaking (6m/s). The combination of the addition of zirconia beads followed by extremely fast shaking via the bead beater lyses the bacterial cells, exposing the DNA.

After a further 5 minutes on ice, 15mg of polyvinylpyrrolidone (PVPP) was added to each sample. PVPP absorbs and removes polyphenols which are common in many plant tissues and can deactivate proteins which may inhibit many downstream reactions like PCR. The samples were vortexed upside down to ensure full exposure to the PVPP by dissolving the pellet. The samples were then shaken for 5 minutes at room temperature using an orbital shaker (1000rpm).

The samples were centrifuged (15,000g, 4°C, 3 mins), after which the supernatant was carefully extracted and recovered in a 2mL safe-lock Eppendorf. The pellets were washed with 500 µL TENP buffer, vortexed upside-down and again centrifuged (15,000g, 4°C, 3 mins). The supernatant was again recovered in the same 2mL eppendorf. The process of washing the pellet with TENP, centrifuging and recovering the supernatant occurred four times in total.

Following this process, the supernatant of about 2mL was centrifuged for 10 minutes at 15,000g and 4°C. It was then separated equally into two DNA/RNA free eppendorfs before adding equal volume of isopropanol (1:1, v/v) to precipitate the nucleic acids. The samples were gently mixed by hand before incubating for ten minutes at room temperature.

Samples were then centrifuged for 5 minutes (15,000g, 4°C) before discarding the supernatant carefully to leave the pellet. The eppendorfs were tapped onto paper to ensure complete dryness within the tubes before being left for 20 minutes with the lids open at room temperature. 225µL of phosphate buffer (pH 8) and 25µL potassium acetate was then

added to each sample before shaking them in the orbital shaker at room temperature for 5 minutes (1,000rpm). The addition of potassium acetate at this stage precipitates dodecyl sulphate (DS) and DS-bound proteins in the sample, allowing the removal of proteins from DNA. It is also used as a salt for the ethanol precipitation of DNA. The samples were then kept at 4°C overnight.

Table 2.2 Chemicals used in the chaotropic method of DNA extraction and their method of preparation

Chemical/Reagent	Concentration	Preparation
Guanidine Thiocyanate	4M	13.5mL water +2.6 mL of Tris Cl 1M (pH=7.5)+12.37g Guanidine Thiocyanate (molecular weight =118.16g/mol). Filter and keep in covered in foil at 4°C
Phosphate Buffer (pH 8)	0.1M	9.32mL Na_2HPO_4 (1M) + 0.68mL NaH_2PO_4 + 90mL s water. Autoclave and adjust to pH 8 using 37% HCl
N-Lauroylsarcosine	5%	1g N-Lauroylsarcosine, + 20mL phosphate buffer (pH8) Prepared on day of extraction
N-Lauroylsarcosine	10%	1.1g N-Lauroylsarcosine + 11mL water. Stored at 4°C
Potassium Acetate	5M	4.9075g potassium acetate (molecular weight 98.15 g/mol) + 10mL water
Sodium Acetate	3M	2.4609g sodium acetate (molecular weight 82.03 g/mol) + 10mL water
Tris-Cl (pH 7.5 and pH 8)	1M	12.11g Trizma base (molecular weight 121.1g/mol) + 100mL water. Autoclave, split into two tubes and adjust pH to 7.5 and 8 with 37% HCl
TENP Buffer	n/a	1mL Tris-Cl 1M (pH 8) + 0.8mL

		EDTA (0.5M) + 0.4 NaCl (5M) Add 0.2g PVPP immediately before use
RNAase	10mg/mL	RNAase 10mg, + 10µL Tris-Cl (1M, pH 7.5) + 3µL NaCl (5M) + 1mL water. Store at -20°C
Sodium Dodecyl Sulphate (SDS)	10%	10mg SDS, 90mL water

The second day of the DNA extraction was initiated by shaking the samples at room temperature on the orbital shaker for ten minutes (1000rpm) before combining the duplicate samples into one of the 2mL eppendorfs. Following 30 minutes of centrifuging (15,000g, 4°C) the supernatants were then recovered in a new 2mL tube. 5 µL of ribonuclease (RNAase) was added to each supernatant to catalyse the degradation of any RNA. Samples were vortexed shortly and centrifuged (15,000g, 4°C, 3 secs), to homogenise the contents before incubation in a dry bath of 37°C at 700rpm.

After 45 minutes of incubation, 25 µL of 10% sodium dodecyl sulphate (SDS) and 12 µL of Proteinase K were added to each tube. SDS and proteinase K are respectively responsible for the lysing of proteins via the disruption of non-covalent bonds, and their subsequent digestion. Proteinase K is active in the presence of SDS. Samples were vortexed for a few seconds prior to being placed in the dry bath for 2 hours (45°C, 500rpm).

The samples were centrifuged for 3 seconds following the 2 hour incubation, after which 54µL of 3M sodium acetate was added to further precipitate the DNA. One millilitre of 100% ethanol kept at -20°C was added to the samples, before gently inverting the tubes and placing them in -20°C for 1 hour.

The ice-cold samples were then shaken at 1,000 rpm for 10 minutes at room temperature using the orbital shaker, and then centrifuged for a further 10 minutes at 15,000g and 4°C, after which the supernatant was discarded. In order to dissolve the pellet, 240 µL DNA/RNA free water was added to the tubes and the pellet was crushed with a DNA/RNA free pipette tip. 100% ethanol (-20°C) was then added to the tubes (560 µL). The samples were again shaken for 10 minutes (1,000rpm, room temperature) and centrifuged for 10 minutes (15,000g, 4°C). The supernatant was then discarded before repeating the addition of DNA/RNA free water, pellet crushing and the addition of100%

ethanol. The process of washing and crushing the pellet, shaking, centrifuging and discarding the supernatant was repeated 3 times in total.

Following the final disposal of the supernatant, the tubes were tapped dry on paper before being left open in a biological unit for 1 hour. The pellet was then resuspended in 160µL TE buffer and finally aliquoted into 4x40µL 0.2mL PCR tubes, which were stored at -20°C for further analysis.

Measuring the concentration and purity of extracted DNA using spectrophotometry

Following the extraction of the DNA from the faecal slurry, the quantity and purity of the DNA was evaluated in order to ensure optimal performance of downstream reactions, such as polymerase chain reaction (PCR). The DNA extracted in this study was analysed using the Nanodrop 1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc, USA). Primarily, 2µL of TE buffer was loaded on to the sample pedestal and analysed, providing a "blank" measurement. Following this 2µL of extracted DNA was loaded and measured in the above fashion. Nanodrop generates 3 values of interest; the concentration of DNA, the 260/230 absorbance ratio and the 260/280 absorbance ratio. These ratios are important indicators of the purity of DNA, and their values generate an absorbance spectrum which further highlights purity. It is generally accepted that a 260/280 ratio of 1.8 indicates pure DNA, whilst a higher 260/230 ratio (2.0-2.2) is desirable. Deviations from these values imply contamination. In specific, a low a 260/230 ratio is indicative of carbohydrate carryover or phenol contamination, as these both have absorbance at 230nm. Samples with low 260/280 ratios, low concentrations and/or poor absorbance spectrums were re-extracted.

2.4.4.3 Preparing amplicon pools for sequencing

Measuring extracted DNA concentration using fluorometry

It was important to ensure that all DNA samples were amplified for 16S rRNA gene sequencing using the same starting concentration. Although the Nanodrop measurement gives an indication of the purity and concentration of the DNA present, it is not very specific and the values may be affected by protein or RNA residue. Thus, the Qubit 2.0 fluorometer (life technologies, USA) was used to specifically measure double stranded DNA. The principle of fluorometry is similar to that of spectrophotometry in that it is based on absorption of light. In this case, the intensity and wavelength distribution of fluorescent light is directly proportional to DNA concentration; the more DNA, the more

fluorescent light emitted. The Qubit assay uses specific dyes which, despite having low fluorescence independently, become intensely fluorescent when bound to DNA. The Qubit assay can be performed using broad-range (BR) or high-sensitivity (HS) reagents depending on the concentrations of the samples being measured. Prior to PCR, the DNA was measured using BR Qubit Assay (life technologies, USA). In a 500 µL thin-walled polypropylene Qubit assay tube, 1µL of BR reagent was added to 199 µL of BR buffer. These components were well mixed before removing 2 µL to allow the addition of 2 µL of DNA sample, ensuring a final volume of 200 µL. It was important that the DNA extraction was fully thawed prior to Qubit analysis. This mixture was vortexed before being placed in the Qubit for measurement. Two standards were prepared in a similar manner with the exception that 190 µL of the buffer-reagent was used alongside 10 µL of the provided BR standards. These standards were measured in the prescribed order prior to the extracted samples with the dual purpose of calibrating the Qubit and calibration curve. The DNA concentration of the subsequent samples was calculated using the equation of the line generated from the standards.

Optimising the concentration of DNA used in PCR amplification

Other studies within this laboratory working with faecal samples have successfully amplified DNA using a template concentration of 5.0 ng/µL in PCR. However, these samples did not come from fermentation studies and rather were derived from stool samples. Under these conditions, PCR was not successful in the amplification of the fermented samples used in this study (as assessed by lack of bands on the gel and extremely low final HS Qubit concentrations) despite being run alongside a positive control which was successfully amplified. It was assumed that there was a degree of inhibition occurring during the PCR reaction due to one or more of the components or chemicals used in the fermentation process. Thus, amplification was attempted in 3 samples using 4 serial dilutions (undiluted 1:10, 1:100, 1:1000) of the DNA concentration measured by BR Qubit assay. The results of this method optimisation showed that following PCR, the band on the gel and Qubit measurements were strongest at a concentration of 2.5ng/ µL. Therefore, all DNA samples extracted in this study were standardised to a concentration 2.5ng/µL in a final volume of 30 µL PCR prior to amplification.

Amplification of DNA using KAPA© HiFi PCR Kit

Each DNA extraction underwent PCR amplification in triplicate, and for each set of the same extraction a negative control was conducted in which no DNA template was added. The purpose of this negative control was to assess any contamination occurring during the preparation of the PCR. Thus, 4 x 0.2mL PCR tubes were labelled for each extraction, one of which was clearly identified as the negative control. The labelled PCR tubes were left open under UV light in a biological unit which had been cleaned thoroughly with 100% ethanol. Pipettes, DNA/RNase free filtered pipette tips and a 2mL PCR-clean eppendorf were also left under the UV light for approximately 15 minutes in order to ensure a sterile work environment. During this time, the reagents and Golay bar-coded primers necessary for the PCR were removed from the -20ºC in order to allow them to thaw.

As 10 samples were amplified in triplicate at any one time alongside the negative control, a Master Mix of PCR reagents was prepared to save time and reduce contamination risk. All reagents were obtained from the Kapa HiFi PCR Kit (Kapa Biosystems, USA) unless otherwise stated. Master Mix enough for 11 samples (10 samples + 1 spare) was prepared prior to each PCR. Primarily, 200 µL HiFi buffer was added to 605.44µL nuclease-free water in the 2mL PCR-clean Eppendorf, followed by 33 µL dNTPs mixture. 22 µL Hotstart DNA polymerase, 55 µL dimethyl sulfoxide (DMSO) (Sigma, USA) and 38.28 µL forward primer (Eurofins Genomics, Luxembourg) was also added to the Master Mix, giving a final volume of 953.72 µL. 88.52 µL of Master Mix was aliquoted into the negative controls, each assigned to specific samples. Each sample had a designated reverse primer with a unique barcode. It is imperative for bioinformatics analysis that these bar codes were different for each sample. 3.48 µL of the allocated reverse primer was carefully pipetted into the designated 0.2 mL PCR tube containing the PCR Master Mix. This tube was then vortexed before aliquoting 23 µL into each of the 3 remaining PCR tubes. Whilst 2 µL of nuclease-free water was added into the negative control tube, 2 µL of well-vortexed 2.5ng/mL extracted DNA sample was added into each of the other PCR tubes. Thus, the final volume for all PCR tubes was 25 µL. All tubes were thoroughly vortexed and centrifuged quickly before being placed in the PCR machine (Veriti™ Thermal Cycler, Applied Biosystems™, USA). Each sample was amplified using the thermal stages as follows;

a) Initial denaturation stage: 95˚C for 5 minutes
b) Denaturation stage: 98˚C for 20 seconds
c) Annealing stage; 60˚C for 15 seconds
d) Extension stage: 72˚C for 40 seconds

e) Repeat stages b) to d) for 25 cycles
f) Final extension stage; 72˚C for 1 minute

On completion of all steps, the PCR machine was cooled to 4˚C until the samples were removed.

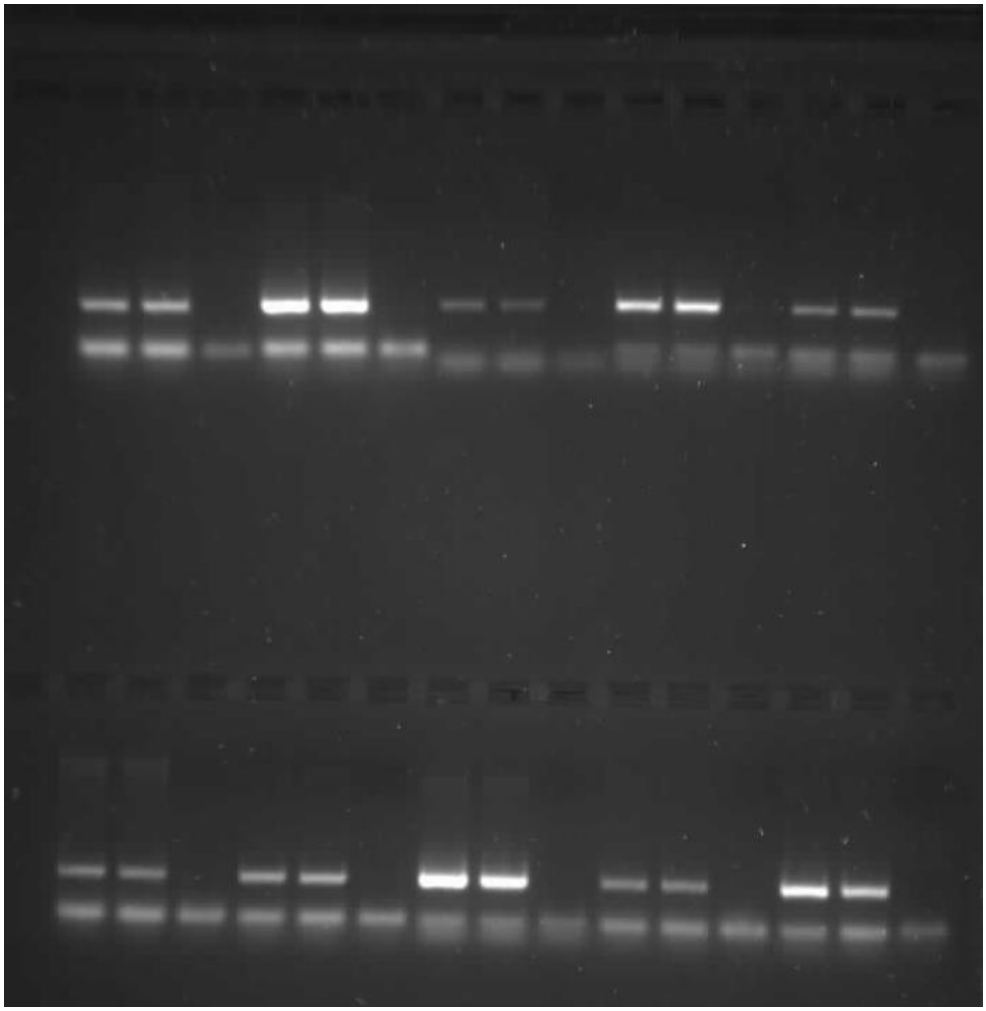
Evaluation of PCR yield and performance via agarose gel electrophoresis

Briefly, agarose gel was prepared for the purification of the PCR products. 2g agarose was added to 200 mL of 1X TAE buffer before heating in the microwave until the solution was completely clear. The solution was then left to cool to 55˚C before adding 2 µL SYBR safe DNA gel stain (Invitrogen, USA), which was thoroughly dispensed throughout the agarose-TAE solution before being poured into the gel casting base with combs inserted. The gel was left to cast for approximately 50 minutes.

On completion of the PCR, the samples were removed from the machine. Each DNA sample had been amplified in 3 PCR tubes alongside a negative control. One of the PCR tubes containing amplified DNA was split equally between the 2 remaining tubes with amplified DNA, resulting in a volume of 37.5 µL in each of the latter tubes. 3 µL 6X blue/orange loading dye (3 ml 100 % glycerol (Sigma-Aldrich, G5516), 250 µl of bromophenol blue (2% w/v) (Sigma-Aldrich, B0126), 6.5mL nuclease-free water) was added to both these tubes and the negative control, giving a final volume of 40.5 µL and 28 µL respectively. These samples were carefully loaded into the wells of the previously prepared agarose gel, ensuring to take note of the sample order. The PCR products were then separated by electrophoresis for 45 minutes at 125 volts.

Following electrophoresis, the gel was removed from the tank and viewed under UV light. Had the PCR been successful, all samples including the negative controls should have a distal band containing the primers and primer dimers, whilst the DNA samples should have an additional proximal band, which contained the amplified products. An example of a successful band is shown in Figure 2.3. If the negative control had an additional band this indicated contamination during preparation of the PCR, in which case the samples would be re-amplified. Samples we also re-amplified if there was no visible proximal band where one was expected.

Figure 2.4 Example of resultant bands following successful amplification



Distal bands represent primer dimers
Proximal bands represent amplified samples. Negative controls are those that have a distal band yet no proximal band

Purification of amplicons

Successful amplicons were carefully excised from the gel using a sterile scalpel and tweezers and placed in a DNA/RNA free 2 mL Eppendorf. As each sample was amplified twice, the two bands generated from electrophoresis were removed simultaneously and placed in the same Eppendorf. Extra care was taken to avoid contamination from other samples and/or primers. The excised bands were weighed before adding three times this weight (3x w/v) of agarose-dissolving buffer (Zymo Research, USA). The samples plus buffer were then incubated in a shaking dry bath at 50˚C (500rpm) for ten minutes to ensure that the gel had completely dissolved.

Following incubation, the samples were carefully pipetted into individual spin columns (Zymo Research, USA) which were placed in 2 mL collection tubes (Zymo Research, USA). These were centrifuged for 1 minute at 10,000g after which the flow-through was discarded. As only 700 µL could be spun at once, this process was repeated until all of the samples had been passed through the spin column. 200 mL of wash buffer (Zymo Research, USA) was then added to each spin column and spun for 30 seconds at 10,000g. This was repeated one time, discarding the flow-through after each spin.

Finally, a new collection tube was placed under each spin column. 12 µL of elution buffer (Zymo research, USA) was carefully added to the membrane of each spin column before a final 1 minute centrifuge (10,000g). The eluted DNA was recovered from the collection tube and stored in a labelled PCR tube at -20˚C until pooling.

Measuring extracted DNA concentration using fluorometry

Prior to pooling all amplified DNA samples, it was important to ensure that all samples had a minimum DNA concentration of 2.5 ng/ µL following the amplification process. This was done using the Qubit as previously described; however in this instance the High Sensitivity buffer, reagent and standards were used. Samples containing less than 2.5 ng/ µL DNA were re-amplified.

Standardising the concentration of amplicons and preparing the amplicon pool

All amplified DNA samples were to be pooled together in one 2mL Eppendorf for subsequent sequencing, thus it was necessary to ensure that all samples were of the same concentration (1.5 ng/ µL). Therefore on the day of pooling, all samples were measured using the high sensitivity Qubit. Using the aforementioned equation ($C_1 \times V_1 = C_2 \times V_2$), nuclease-free water was used to dilute samples to the desired concentration. The concentration of all samples was then checked again by Qubit to ensure they were 1.5 ng/

µL before carefully pipetting 5 µL into the final 2 mL DNA/RNA free Eppendorf containing all samples. This Eppendorf was securely wrapped in parafilm and packaged in dry ice to be transported to the sequencing centre in Birmingham in which sequencing took place.

2.4.5 Measurement of faecal calprotectin

Faecal calprotectin is a widely used marker of gut inflammation and is routinely employed to identify and monitor IBD patients.

2.4.5.1 Principles of Enzyme Linked Immunosorbent Assay (ELISA)

The kit worked on the principles of the "two-site sandwich" ELISA technique, in which two selected antibodies are utilised, each of which bind to different epitopes of the chosen antigen. Primarily, the capture antibody is bound to each well of a microtiter plate. The chosen samples and standards are then incubated with the antibody. Following incubation and washing, the antigen of interest is bound to the plate at which point the second antibody, which is specific to the chosen antigen, is added. The plate is again shortly incubated, forming an antibody-antigen-antibody "sandwich". After washing, enzyme-linked secondary antibodies used in the detection of the immunocomplex are added to the plate. The activity of the immunocomplex bound to the wall of the microtiter plate, which can be measured using spectrophotometry, is directionally proportional to the amount of antigen in the sample.

2.4.5.2 Measurement of calprotectin using Quantitative Faecal Calprotectin ELISA Kit (Epitope Diagnostics, Inc, USA)

Calprotectin was measured using a quantitative commercial ELISA kit (Epitope Diagnostics, Inc, USA) according to the manufacturer's provided protocol. Primarily, approximately 100 mg of thawed faeces was weighed out into a 15mL corning tube alongside an inoculation loop which had been broken in half so that the tube could be closed. Extraction buffer was added to each sample (w/v 1:25), which was then vortexed for 5 minutes until the stool sample had dissolved. The sample was then left vertically at room temperature for 30 minutes before being centrifuged for 5 minutes (3000g) to allow sedimentation. 150 µL of clear supernatant from each sample was then transferred into a 1.5 mL Eppendorf containing 1.2 mL of Extraction Buffer and gently vortexed.

The seven assay standards and three quality controls provided by the manufacturer were reconstituted by adding 500 µL of distilled water to each. These were left to sit undisturbed for 5 minutes before gently vortexing.

50µL of provided Assay Buffer was pipetted into the wells of the calprotectin antibody coated microtiter plate, followed by 50 µL of standards, controls and samples, each in duplicate. The plate was covered in foil, placed securely on an ELISA plate shaker, and incubated for 1 hour (425 rpm). Prior to the end of this incubation period, the Tracer Antibody working solution was prepared by adding the Calprotectin Tracer Antibody to the Tracer Antibody Diluent in a 1:21 fold dilution.

Following incubation, the wells were washed five times by dispensing 350 µL of working wash solution in to each well, before complete aspiration. 100 µL of Tracer Antibody working solution was then added to each well, which were again covered with foil, placed on the ELISA plate shaker, and incubated for 45 minutes (425 rpm). After incubation, the wells were again washed in the same fashion as previously described before 100 µL of ELISA horse-radish peroxidase (HRP) substrate was pipetted into each well. The plate was covered in foil and incubated stationary at room temperature for 12 minutes.

Following the last incubation period, the plate was quickly shaken to homogenise contents before 100 µL of ELISA Stop Solution was then quickly added to all wells and mixed gently. Finally, absorbance was read using a spectrometer (Thermo Scientific Multiskan® Spectrum) at 450nm with reference filter at 620nm.

According the manufacturer's guidelines, values of faecal calprotectin measurements over 43.2 µg/g were deemed as abnormally high.

2.5 Statistical analysis

All data was analysed using Minitab Version 16.2.2 statistical software (Pennsylvania State University, Pennsylvania, USA). The Anderson-Darling test was used to assess the distribution of data in order to determine the use of subsequent statistical tests. When assessing differences between groups of three of more, the Kruskal-Wallis test was used when data was not normally distributed whilst 1-way ANOVA was used when data was considered normal. When comparing two groups, Mann-Whitney test and 2 sample t-test were used for non-normally and normally distributed data respectively. Statistical significance was considered at $p \leq 0.05$.

Chapter 3 : Methodology Chapter: The Influence of Fibre Type and Amount on *in vitro* Fermentation Production of SCFA by Human Gut Microbiota

3.1 Chapter Outline

This thesis adopted an in-house established batch culture method which has been previously evaluated for its efficacy [93]. This technique and others like it have been developed in an attempt to mimic physiological bacterial metabolism within the human GIT. However, previous studies [179, 180] have suggested that substrate availability during these fermentation studies can influence subsequent metabolite production. This methodological chapter aims to further investigate this area by quantifying the production of SCFA in response to varying amounts of substrate in batch culture fermentations.

3.2 Introduction

With increasing advancements in molecular techniques aimed at characterising the complex bacteria that reside within the human GIT [70, 71], interest in this extensive community and its impact on human health has substantially increased in the last decade. With research linking specific microbiota to diseases such as obesity [59, 60], IBD [181], cardiovascular disease[51], and depression [182], focus on this ecosystem has now expanded and it is a topic which has attracted much media coverage and public interest. Many food and supplement brands are now available which supposedly restore bacterial homeostasis in the GIT, favouring the growth of healthy bacteria whilst inhibiting the growth of pathogenic strains [183]. These products include probiotics which are live beneficial microorganisms, usually lactobacilli, enterococci, streptococci and bifidobacteria, or fermentable foods (dietary fibre and prebiotics). Dietary fibre evades digestion in the upper GIT, presenting itself in the colon as an energy source for the resident bacteria, promoting a symbiotic relationship with their host. Therefore, the composition and number of these bacterial species can be altered by modulating such fuel sources presented in the colon.

The health-promoting effects of the colonic microflora are largely mediated by the production of short chain fatty acids (SCFA) via the anaerobic fermentation of non-digestible carbohydrates. The main benefits of the principal SCFA (acetate, propionate and butyrate) are outlined in Chapter 1. It is reported that SCFA production has an indirect

relationship with various diseases [184] hence optimising their production via diet is desirable.

Bacterial species prefer using specific fibre as fuel depending on the CAZymes they possess (described in Chapter 1), which in turn influences the production of both total and individual SCFA. Therefore, fibres which are more readily fermented than others tend to be more efficient in SCFA production. This variability is well documented in the literature. For example, pectin is highly fermented and is known to produce a high total SCFA concentration, particularly acetate [185], whilst psyllium is more propiogenic than most substrates [186, 187]. Butyrate, which is particularly beneficial to colonic health [188], is associated with resistant starch [80] and other prebiotics [189].

Many epidemiological [98-100] and intervention [106, 190, 191]studies indicate that both acute and chronic alteration of dietary fibre intake can modulate bacterial composition and activity [109], subsequently impacting SCFA production[103, 104]. It has been reported that both *Faecalibacterium prausnitzii* and *Roseburia spp* are significantly reduced following a fibre-free diet in healthy individuals [103], whilst decreasing carbohydrate intake from 399g per day to 24g per day reduces SCFA production by 50% in obese patients [105]. Total bacterial production and, in particular, production of *Clostridium Cluster XIVA* and the *Roseburia* genus, both major butyrate producers, were increased during *in vitro* fermentation of faecal samples supplemented with 14 grams per day of wheat dextrin [108]. A further *in vitro* study by Connelly and colleagues (2010) [104] investigated the impact of exposing more substrate to human bacteria. They found that larger sized, whole oat grain flakes produced more SCFA than smaller flakes, and at a quicker rate. The larger flakes also had a significantly higher total bacteria population after 24 hours fermentations, with a specific increase in Bifidobacterium genus - changes that did not reach significance with the smaller sized oats. It was also noted that whilst the smaller flakes produced a propionate-rich SCFA profile, the larger size significantly enhanced butyrate after 24 hour fermentation.

It is apparent that the amount and type of non-digestible carbohydrate consumed within in the diet influences the complex ecosystem within the GIT. We may expect that increasing intake of specific fibres will continually improve SCFA concentrations; however there is evidence to suggest that the enzymes that catalyse fermentations become inhibited with increasing substrate availability [179, 180, 192]. Khan et al [179]explored the *in vitro* potential of increasing the amount of lactulose available during batch-culture fermentations with human faecal inocula. It was reported that although net total SCFA production was steadily increased with substrate amount, these variables were not directly

proportional. In fact, the results indicated that the lowest amount of lactulose produced proportionately the most total SCFA. Similar results were reported by Stevenson et al (1996), who conducted their *in vitro* studies using rat caecal contents incubated with pectin, ispaghula and corn starch [180]. However, these results cannot be extrapolated to all batch culture studies due to issues with study design. As mentioned, Khan et al [179] only investigated one fibre and whilst Stevenson and colleagues included more substrates, it is not clear how much can be inferred from rat studies to humans. Furthermore, Khan et al did not neutralise the pH of the growth medium prior to the introduction of human bacteria, instead leaving it alkaline which may have influenced bacterial activity [193].

As *in vivo* research in this field is limited (as detailed in Chapter 1) it is important to identify the effects of increasing substrate availability in terms of SCFA profile in the presence of different fibres during *in vitro* investigations. This mechanistic study will investigate the effects of increasing the availability of various different fibres during *in vitro* batch-culture fermentations on SCFA production, hypothesising an inhibitory effect with increasing substrate availability. I performed this experiment in order to select the appropriate amount of fibre for the major study presented in this thesis.

3.3 Subjects and Methods

A single stool sample was collected from free-living, healthy adults recruited by word of mouth advertisement on the University of Glasgow campus. Participants had not been on antibiotics and/or pre/probiotics during the 3 months prior to donating a stool sample.

The *in vitro* fermentation protocol discussed in Chapter 2.4 was employed during this methodological study. The only deviation from the described method is the introduction of multiple fibre weights. During fermentations of all faecal samples, each fibre was fermented in 3 weights; 0.25g, 0.50g and 1.00g. Five different fibres were investigated in this study: maize starch, apple pectin, raftilose, wheat bran and α-cellulose. Additionally, a mixture of all 5 fibres equating to the 3 weights, were combined in equal amounts for fermentation. Thus, 0.1g, 0.50g and 1.00g of the fibre mixture contained 0.02g, 0.1g and 0.2g of each fibre respectively. A non-substrate control (NSC) fermentation was also conducted for each participant. In total, each stool sample was fermented in 19 different variations.

As described in Chapter 2, aliquots were taken before and after the 48 hour fermentation period for future analysis of SCFA production, which were stored at -20˚C. Gas chromatography was employed to assess SCFA concentrations (see Chapter 2.4.3).

This study was ethically approved by the University of Glasgow, College of Medical, Veterinary and Life Sciences (MVLS) Ethics Committee

3.3.1 Statistical Analysis

Minitab Version 16.2.2 statistical software (Pennsylvania State University, Pennsylvania, USA) and Microsoft Excel (2010). The Anderson-Darling test was used to assess the distribution of data and for those that were normally distributed, one way ANOVA was used to assess differences between groups whilst the Kruskal-Wallis test was used for non-normally distributed data. Statistical significance was considered at $p \leq 0.05$. Net SCFA production at 48 hours was calculated for each substrate by subtracting total SCFA produced in the NSC fermentation at baseline from total SCFA at 48 hours. In order to assess inhibition with increasing fibre weight, net SCFA production for 0.25g and 0.50g was extrapolated to 1.00g (0.25g x4, 0.50g x 2) in order to reflect what would be achieved if no inhibition occurred. Assuming no inhibition, total SCFA concentration for 1.00g and 0.50g substrate would be exactly four and two times that at 0.25g, respectively. Thus, net total SCFA concentration measured for each substrate was multiplied by 2 and 4 to give predicted concentration for 0.50g and 1.00g respectively compared with the 0.25 fermentations. Percentage inhibition was calculated by dividing actual total SCFA concentration by the predicted values.

3.4 Results

3.4.1 pH of faecal slurry pre- and post-48 hours fermentation with different types and amounts of substrates

There were no statistical differences between the pH recorded at baseline between neither the different substrates nor the different weights ($p<0.05$) with the median (IQR) pH at this timepoint being 7.19 (0.26) (Figure 3.1).

48 hour fermentation

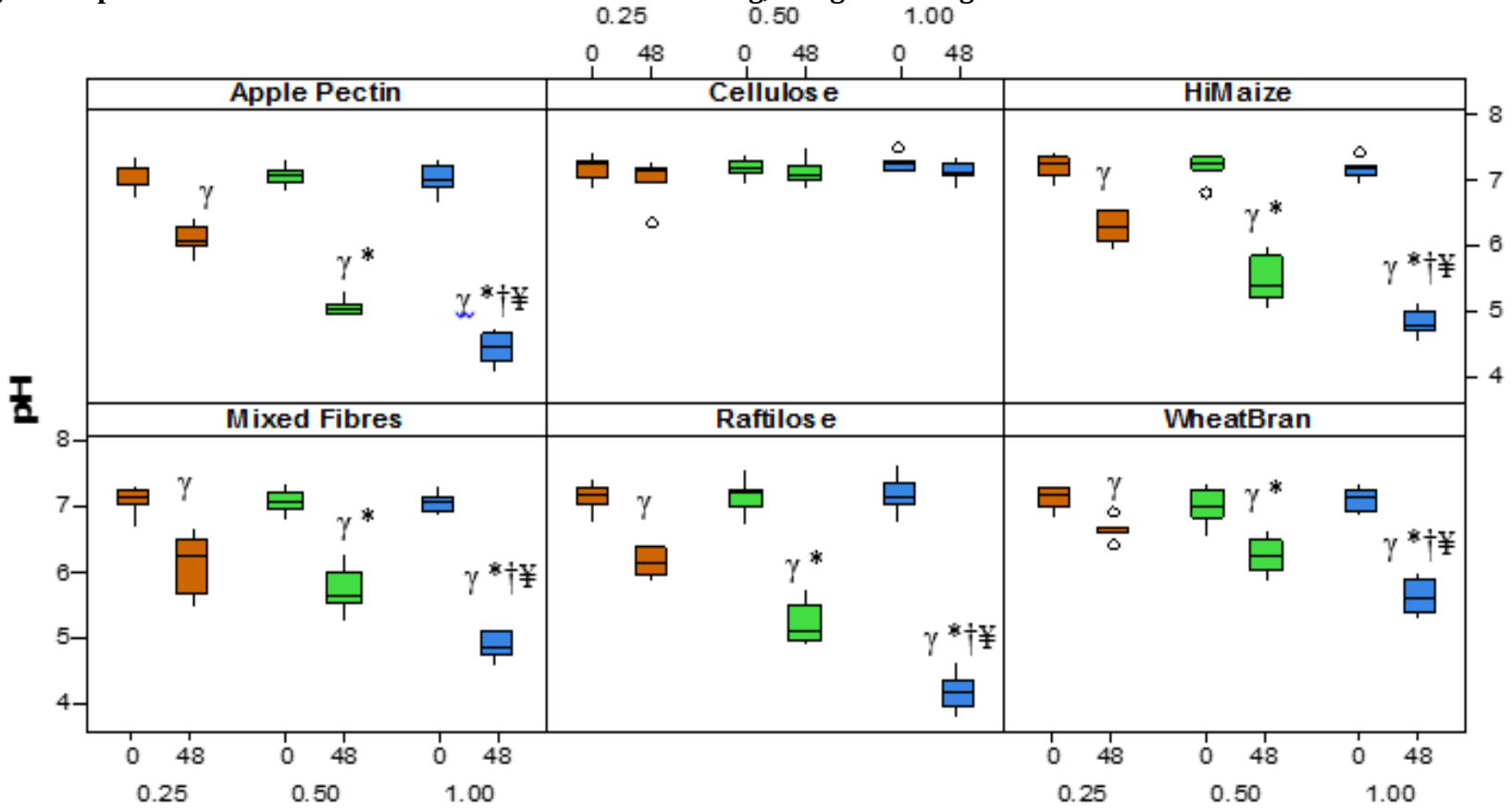
Figure 3.1 illustrates the changes in pH following 48 hour fermentation compared to baseline measurements. Furthermore, it allows comparison between both different fibres and the 3 weights used within this study. The pH of the NSC fermentation is not graphically represented, however statistical analysis did not reveal a difference between

values recorded pre- and post-fermentation (median (IQR) 0hr; 7.32 (0.32), 48hr; 7.22 (0.08) $p>0.05$). On the other hand, for all fibres except for cellulose, slurry pH was significantly lower following 48 hours fermentation compared to baseline; this was the case for all fibre weights ($p<0.05$). Not only was there no difference between pH pre-and post- fermentation with cellulose, there were also no differences found between the different weight groups (median (IQR) 0.25g; 7.16 (0.23), 0.50g; 7.10 (0.23), 1.00g; 7.14 (0.20) $p>0.05$). For all other substrates, pH was significantly decreased as fibre weight was increased (1.00g<0.50g<0.25g, $p<0.05$, Figure 3.1).

3.4.2 Volume of expired gas following 48 hours fermentation with different types and amounts of substrates

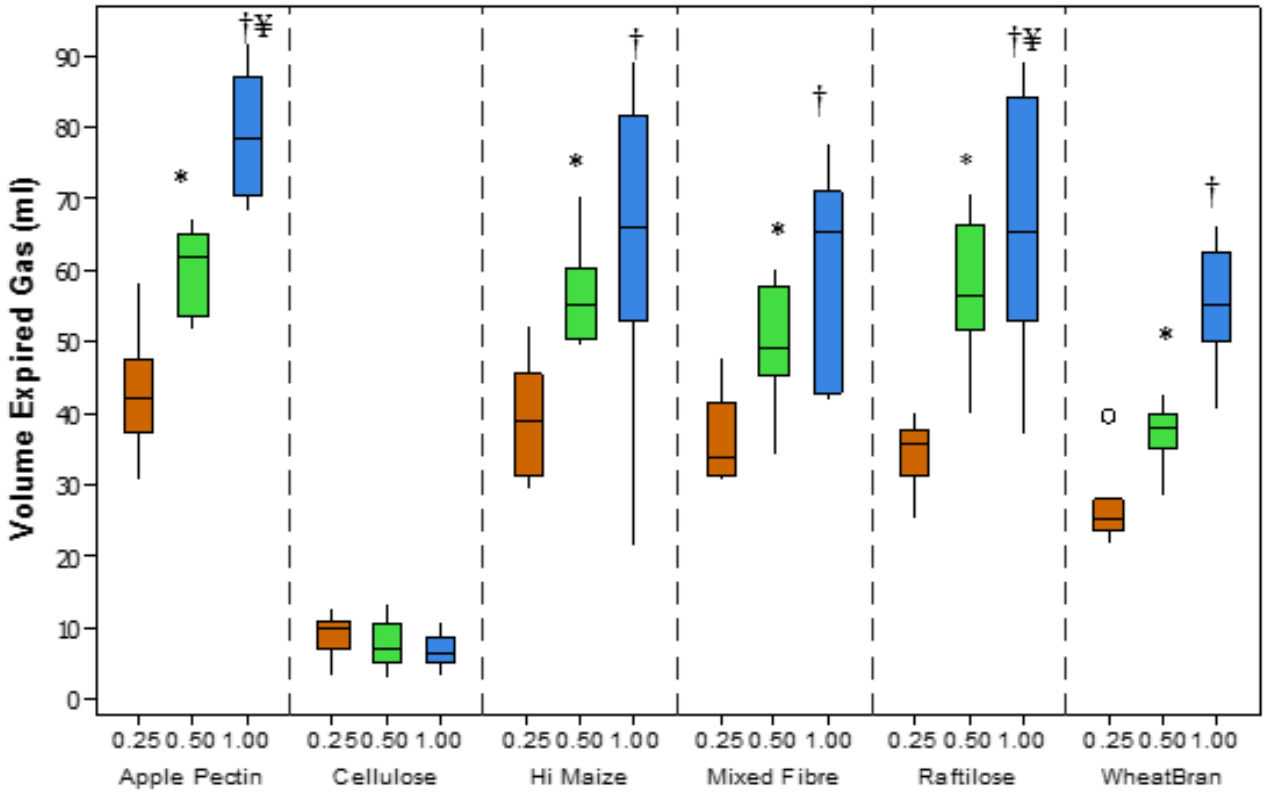
Figure 3.2 displays the volume of gas expired after 48-hour fermentation according to substrate type and weight. The fermentation with no substrate is not shown in Figure 3.2, but was found to have low expired gas (mL) (median (IQR) 6.25 (4.25)). With the exception of cellulose, the volume of expired gas appeared to increase with increasing substrate weight. In the case of wheat-bran and apple pectin, 1.00g of substrate produced significantly more gas than both 0.50g and 0.25g, whilst 0.50g produced significantly more gas than 0.25g ($p<0.05$, Figure 3.2). Whilst 0.25g of hi-maize, raftilose and mixed fibre produced significantly less gas than 0.50g and 1.00g of the same substrates, there were no differences between the latter weights.

Figure 3.1 pH before and after 48 hour fermentation with 0.25g, 0.50g and 1.00g of different fibrous substrates



o indicates outliers
* Indicates statistical difference between 0.10g and 0.50g ($p<0.05$)
† Indicates statistical difference between 0.10g and 1.00g ($p<0.05$)
¥ Indicates statistical difference between 1.00g and 0.50g ($p<0.05$)
γ Indicates statistical difference between 0 and 48 hr ($p<0.05$)

Figure 3.2 Volume of gas expired following 48 hour fermentation with 0.25g, 0.50g and 1.00g of different fibrous sut



o indicates outliers
* Indicates statistical difference between 0.10g and 0.50g ($p<0.05$)
† Indicates statistical difference between 0.10g and 1.00g ($p<0.05$)
¥ Indicates statistical difference between 1.00g and 0.50g ($p<0.05$)
γ Indicates statistical difference between 0 and 48 hr ($p<0.05$)

3.4.3 Production of the major SCFA before and after 48 hours fermentation with different types and weights of fibre

Prior to the fermentation period, there were no significant differences between neither individual SFCA concentration nor total SCFA concentration between fibres or substrate amount ($p>0.05$) (Figure 3.3). Furthermore, there were no differences between the relative contribution of acetate, propionate and butyrate to total SCFA concentration between these variables at this time point ($p>0.05$) (Figure 3.4).

48 hour fermantion

For all substrates, including the fermentation without any substrate, individual and total SCFA concentrations were signficantly higher following 48 hour fermentation for all substrate weights ($p<0.05$) (Figure 3.3). The NSC fermentation and that with cellulose had significantly lower concentrations of acetate, propionate, butyrate and total SCFA compared to all other substrates ($p<0.05$); There were no differences in the concentrations produced between cellulose and the NSC fermentation. Amongst the remaining fibres, although there was no difference in total SCFA concentration when 0.25g and 0.50g of substrate were used, 1.00g apple pectin delivered a significantly higher total SCFA concentration than the equivilent weight of all other substrates ($p<0.05$).

Figure 3.3 displays a clear tendency for increased total and individual SCFA concentration with increasing fibre weight; however this was not always significant. In the case of apple pectin, hi-maize, raftilose and wheat- bran, total SCFA concentration was significantly enhanced with each increasing weight ($p<0.05$). Although 1.00g mixed fibre produced significantly more total SCFA than 0.50g and 0.25g, there was no difference in the production between the two lesser weights ($p>0.05$). In terms of individual SCFA production, both acetate and butyrate concentration were significantly higher with increasing apple pectin weight whilst propionate was not affected by the weight of this substrate. 1.00g of hi-maize and wheat-bran produced significantly more butyrate than 0.50g and 0.25g of these substrates, with 0.25g producing the least of this SCFA ($p>0.05$). Although 0.25g of raftilose produced significantly less butyrate than 0.50g and 1.00g of this substrate, there was no significance between the latter two weights. 1.00g of mixed fibre produced significantly more of all major SCFA compared to 0.25g, but not 0.50g. Neither total nor individual SCFA was affected by cellulose weight ($p<0.05$).

Relative contribution of individual SFCA to total concentration is displayed in Table 3.2 and Figure 3.4. For all substrates the percentage contribution of propionate

tended to be reduced with increasing substrate availability, whilst that of acetate stayed relatively constant during fermentation of all fibres and all weights. Butyrate was the only SCFA to show significant differences in its percentage contribution at different weights. Fermentation with 1.00g wheat-bran, mixed fibres, pectin and hi maize produced a higher butyrate percentage than both 0.50g ($p= 0.07$, 0.046, 0.0019 and 0.04 respectively), and 0.25g ($p= 0.003$, 0.003, 0.0009 and 0.002, respectively) of the same substrate. Both raftilose ($p= 0.0009$) and apple pectin had a higher percentage contribution of butyrate with 0.50g compared to 0.25g ($p = 0.0009$). The percentage contribution of butyrate was not affected by the availability of cellulose in any case.

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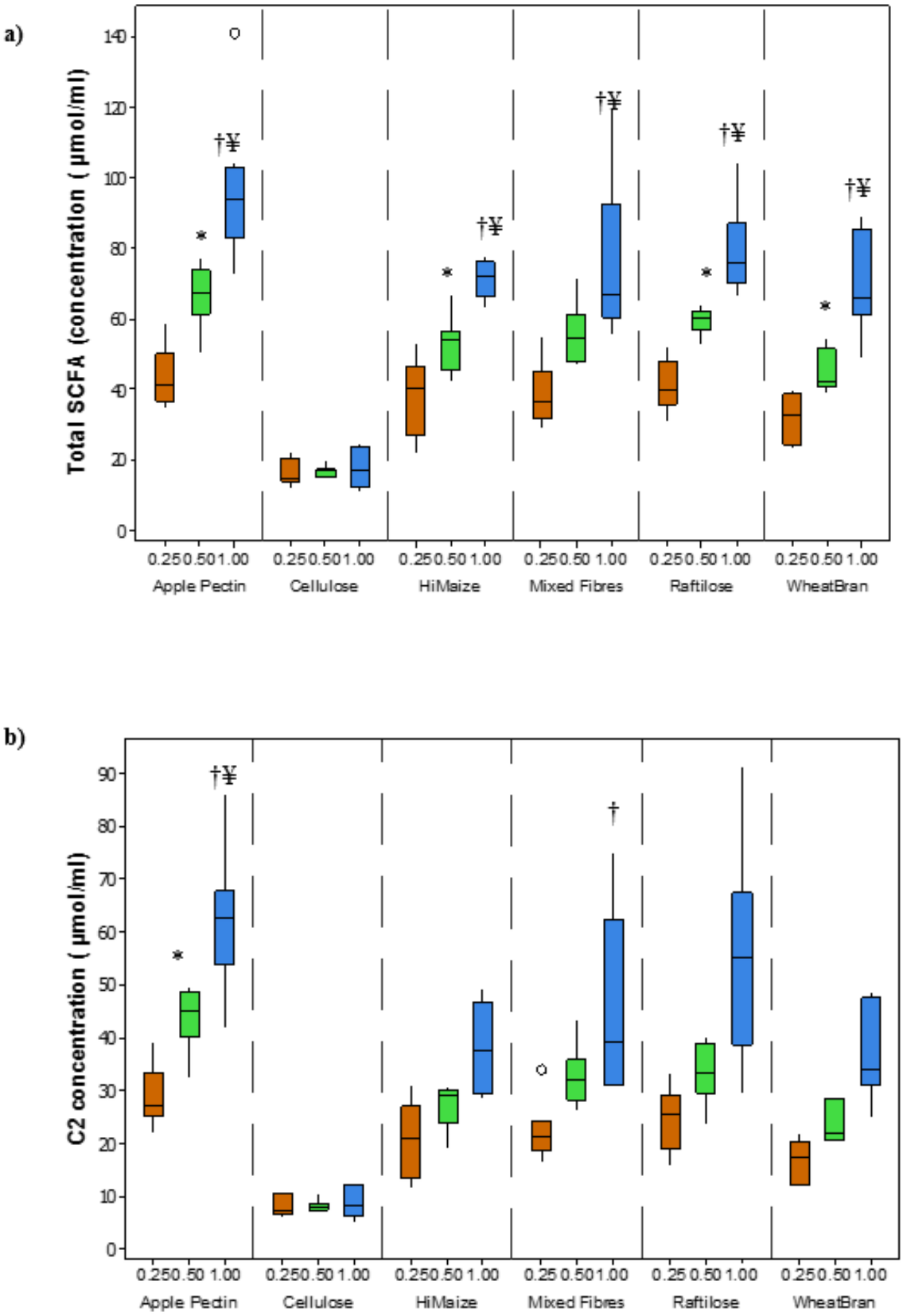
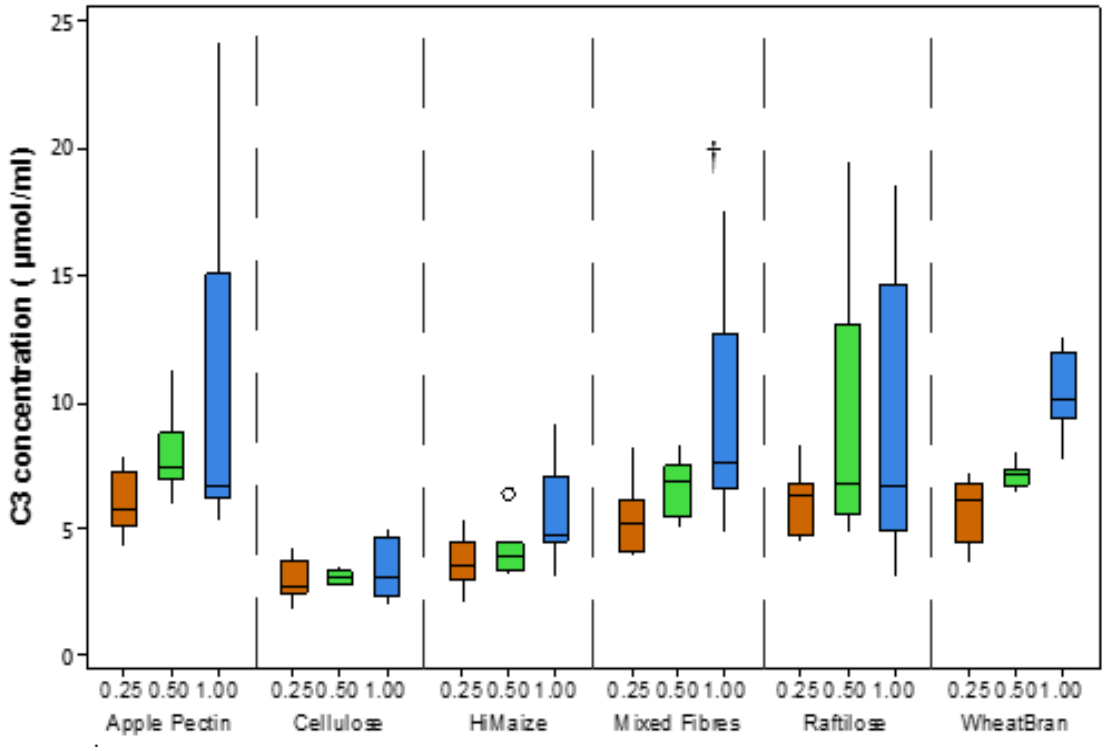
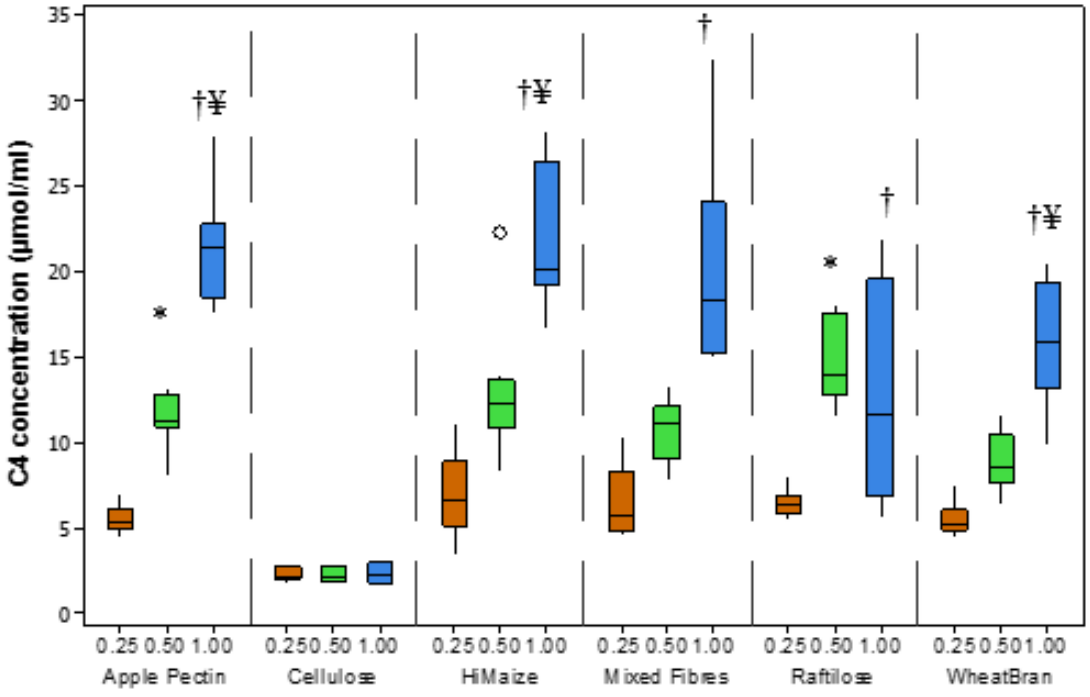
Figure 3.3 Concentration of a) total SCFA, b) acetate, c) propionate, and d) butyrate following 48 hours fermentation with different types and weights of fibrous substrates



c)

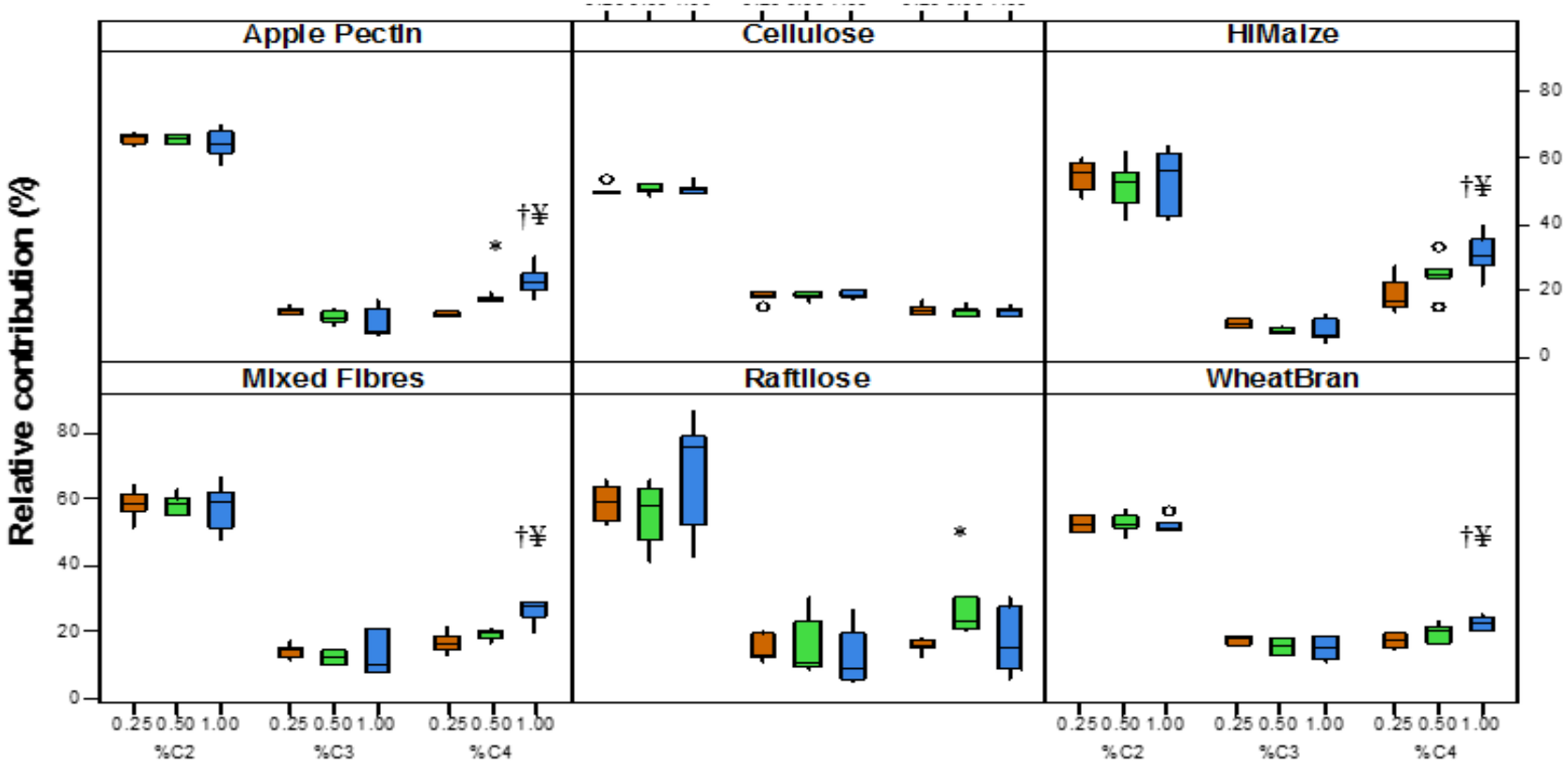


d)



C2; acetate, C3; propionate, C4; butyrate
O indicates outliers
* Indicates statistical difference between 0.10g and 0.50g ($p<0.05$)
† Indicates statistical difference between 0.10g and 1.00g ($p<0.05$)
¥ Indicates statistical difference between 1.00g and 0.50g ($p<0.05$)

Figure 3.4 Relative contribution (%) of the major SCFA to total SCFA concentration after 48 hours fermentation with different types and weights of fibrous substrates



Fibre weights presented as grams. C2; acetate, C3; propionate, C4; butyrate. O indicates outliers
* Indicates statistical difference between 0.10g and 0.50g ($p<0.05$)
† Indicates statistical difference between 0.10g and 1.00g ($p<0.05$)
¥ Indicates statistical difference between 1.00g and 0.50g ($p<0.05$)

3.4.4 Production of the major SCFA after 48 hours fermentation with different types and weights of fibre in relation to predicted production

Table 3.1 displays actual versus predicted net total and individual SCFA concentrations for each substrate, where predicted values are based on proportional extrapolations from concentrations measured with 0.25g. For all fibres, actual net total SCFA concentration produced with 1.00g was significantly lower than predicted values ($p<0.05$). This was also the case for 0.50g substrate for all fibres except hi-maize, which despite being lower was not significantly so. With this weight of substrate, inhibition ranged from 16.70% to 40.00% depending on the fibre. These values were slightly higher when 1.00g substrate was fermented (41.60% -69.53%). Similar results were found for both acetate and propionate, with significantly less measured concentrations compared to predicted values for most fibres at both 0.50g and 1.00g. This relationship was less clear with regards to actual and predicted butyrate values (Table 3.1). Cellulose was the only fibre to have significantly less butyrate production than expected with 0.50g. Wheat bran and hi maize showed slightly less butyrate concentrations than predicted, but not significantly so. On the other hand, apple pectin, mixed fibre and raftilose all showed higher actual concentrations than those predicted with 0.50g substrate. This was only significant in the case of raftilose ($p<0.05$). However, there was 53.70% inhibition in butyrate concentration when 1.00g raftilose was fermented. At this weight, wheat bran and cellulose also displayed significantly less actual butyrate concentrations than those predicted, whilst there was no significant difference for the other substrates.

Table 3.1 Actual versus predicted net total SCFA concentration (µmol/mL) following 48 hour fermentation with different fibre weights and substrates and percentage inhibition of SCFA production at 0.50g and 1.00g substrate

	0.25g (n=8)		0.50g (n=8)					1.00g (n=8)				
	Actual		Predicted		Actual		%	Predicted		Actual		%
	Median	IQR	Median	IQR	Median	IQR	inhibition	Median	IQR	Median	IQR	inhibition
Apple Pectin												
Total SCFA	39.3	13.3	78.50	26.6	**65.4***	91.0	16.70	157.10	53.2	**91.8***	20.0	41.60
C2	25.6	8.30	51.10	16.7	**43.7***	8.50	14.50	102.20	33.30	**61.3***	14.00	40.00
C3	5.5	2.10	10.90	4.20	**7.1***	1.80	34.90	21.80	8.50	**6.4***	8.90	70.60
C4	5.1	1.10	10.20	2.20	11.10	1.90	-8.80	20.40	4.50	21.10	4.40	-3.40
Cellulose												
Total SCFA	12.2	6.50	24.50	13.1	**14.7***	2.70	40.00	48.90	26.1	**14.9***	11.1	69.53
C2	5.8	4.10	11.60	8.10	**6.7***	1.50	42.24	23.10	16.2	**7.1***	5.70	69.26
C3	2.4	1.30	4.80	2.50	**2.7***	0.50	43.75	9.70	5.00	**2.8***	2.40	71.13
C4	1.8	0.70	3.70	1.50	**1.8***	0.70	51.35	7.40	2.90	**1.9***	1.20	74.32
HiMaize												
Total SCFA	38	19.6	76.00	39.1	51.80	10.8	31.84	152.00	78.2	**70.2***	9.90	53.82

C2	19.8	13.5	39.60	26.0	27.80	6.60	29.80	79.20	53.8	**36.3***	17.2	54.17
C3	3.3	1.60	6.60	3.20	**3.60**	1.10	45.45	13.10	6.30	**4.5***	2.60	65.65
C4	6.4	3.80	12.70	7.60	12.10	2.80	4.72	25.40	15.1	20.00	7.30	21.26
Mixed Fibres												
Total SCFA	34.8	13.3	69.60	26.6	**52.4***	13.1	24.71	139.60	63.0	**64.8***	32.2	53.58
C2	20.1	5.40	40.30	10.7	**30.7***	7.50	23.82	83.00	22.6	**38***	31.1	54.22
C3	4.9	2.00	9.70	4.10	**6.6***	2.00	31.96	21.50	6.60	**7.3***	6.20	66.05
C4	5.4	3.40	10.80	6.80	10.90	3.10	-0.93	19.60	16.6	18.00	9.00	8.16
Raftilose												
Total SCFA	37.8	12.6	75.60	25.1	**58.4***	4.70	22.75	151.30	50.3	**73.9***	17.1	51.16
C2	24.3	10.0	48.50	20.1	**32.1***	9.20	33.81	97.00	40.2	**53.9***	28.8	44.43
C3	6	2.10	11.90	4.20	6.50	7.50	45.38	23.90	8.40	**6.3***	9.80	73.64
C4	6.2	1.10	12.40	2.10	**13.8***	4.80	-11.29	24.70	4.20	**11.4***	12.7	53.85
Wheat Bran												
Total SCFA	30.8	14.0	61.60	28.7	**39.9***	11.1	35.23	123.20	57.3	**63.8***	23.5	48.21
C2	16.2	8.10	32.50	16.3	**20.7***	7.70	36.31	64.90	32.5	**32.6***	16.2	49.77
C3	5.8	2.40	11.60	4.80	**6.8***	0.60	41.38	23.20	9.70	**9.8***	2.60	57.76
C4	5	1.20	10.00	2.40	8.30	2.80	17.00	20.00	4.80	**15.7***	6.20	21.50

Predicted net SCFA concentrations calculated by multiplying net SCFA concentration for 0.25g of each substrate by 2 and 4 to give predicated values for 0.50g and 1.00 g, respectively

Percentage inhibition calculated by dividing difference between mean actual and mean predicted values by mean predicted values and multiplying by 100.
* indicates significant difference between actual and predicted concentrations ($p<0.05$)

Table 3.2 Relative contribution (%) of the major SCFA to total SCFA production during *in vitro* fermentation with 0.25g, 0.50g and 1.00g of various fibrous substrates

	0.25g (n=8)		0.50g (n=8)		1.00g (n=8)		
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**p-value**
Apple Pectin							
% C2	66.39	2.91	65.75	3.29	64.2	6.13	0.57
% C3	13.7	1.91	11.58	3.25	7.72	7.52	0.06
% C4	12.8	1.63	17.06	1.83	22.82	4.94	**<0.001 *†¥**
Cellulose							
% C2	47.16	0.79	47.9	3.01	48.06	4.2	0.52
% C3	18.81	1.36	19.35	2.26	19.08	1.67	0.68
% C4	13.65	2.83	13.59	2.26	13.75	2.21	0.73
HiMaize							
% C2	55.27	8.48	52.49	9.29	55.69	19.81	0.48
% C3	9.65	2.5	7.39	1.85	6.19	5.27	0.06
% C4	17.04	7.79	24.99	3.22	31.04	8.36	**<0.001 †¥**
Mixed Fibres							
% C2	58.18	5	58.32	5.62	59.22	11.91	0.96
% C3	9.65	2.5	7.39	1.85	6.19	5.27	0.16
% C4	16.51	4.44	19.75	2.54	27.91	3.95	**<0.001 †¥**
Raftilose							
% C2	59.5	10.44	57.99	15.40	76.4	27.96	0.08
% C3	13.12	7.46	10.97	14.62	8.24	14.2	0.30
% C4	16.33	2.34	23.42	9.60	15.39	19.65	**<0.001 ***
Wheat Bran							
% C2	51.59	6.15	51.99	3.38	51.31	2.57	0.58
% C3	18.19	3.14	16.01	4.79	14.96	7.41	0.40
% C4	17.32	4.11	20.5	5.36	23.00	4.16	**<0.001 †¥**

C2; acetate, C3; propionate, C4; butyrate

* indicates a statistical difference between 0.25g and 0.50g ($p<0.001$)

† indicates a statistical difference between 0.25g and 1.00g ($p<0.05$)

¥ indicates a statistical difference between 050g and 1.00g ($p<0.05$)

3.5 Discussion

Batch culture fermentations, as used in this study, are fairly cheap and easy techniques to use which are particularly useful in the evaluation of SCFA profile in response to incubation with different substrates using the same faecal inoculum [173]. It is also a useful method to allow comparison between participant groups or interventions under well-controlled conditions. However, previous studies have indicated that results of such studies should be interpreted with caution, as there is evidence of enzyme inhibition occurring with increasing the amounts of fermentable substrate [174, 180]. Thus, this study aimed to further elucidate the production of SCFA in response to increasing substrate availability, enhancing both the number of human participants and fibres researched compared to previous studies [174, 180].

The results provide further evidence that batch culture SCFA production is inhibited with increasing fibre availability. Although net total SCFA concentration is increased with increasing substrate amount, this is not a direct linear relationship, with measured concentrations being lower than predictions based on linearity. Compared with total SCFA production with 0.25g substrate, all fibres exhibited a significant inhibition at 0.50g and 1.00g substrate. The only exception was 0.50g hi maize, which did not differ significantly from predicted values. This inhibition was greater when 1.00g substrate was fermented compared to 0.50g for all substrates which suggests the more the amount of fermentable fibre the higher the inhibition is expected to be. Cellulose showed the greatest percentage inhibition however, as an insoluble, non-fermentable fibre, increasing SCFA production with cellulose was not expected hence this result was anticipated and has very little importance. Apple pectin, hi maize and raftilose on the other hand are highly fermentable fibres and yet still displayed 41.6%, 53.82% and 51.16% inhibition of total SCFA production with 1.00g substrate respectively. Although similar, it is hard to directly compare the results of this study to that of Khan *et al*' [174] as lactulose, the only fibre investigated by these authors, was fermented in much smaller quantities (25mg, 50mg, 75mg and 100mg). Furthermore, this study included multiple time points over a fermentation period of 24 hours. It was noted that after 8 hours of fermentation, 100mg of substrate produced approximately 60% less SCFA than predicted. However, after 24 hours fermentation, inhibition had been reduced to 42%. This partial recovery of total SCFA production compared to predicted values may have been due to the removal SCFA during previous time points. As shown in all *in vitro* studies within this thesis and others, pH of batch culture fermentations decreases when incubated with fibres due to the production of SCFA. Thus, the pH of the vessels becomes increasingly acidic, which may impact optimal

performance of the bacterial enzymes involved in the hydrolysis of fibres and bacterial cell death in the culture and therefore inhibit further breakdown.

This paradoxical production of butyrate is perhaps surprising given the responses of acetate, propionate and total SCFA with increasing substrate. However, a study conducted in the 1980s reported that despite extracellular pH dropping to 3.5, intracellular pH of butryogenic bacterial species such as *Bifidobactera* and *Lactobacilli* was maintained at 6.5 [194]. Therefore, these species may have the ability to continue hydrolysing fibre despite increasing acidity within in the batch culture vessels, explaining the favoured production of butyrate. As a form of resistant starch, hi-maize would be expected to increase butyrate and is therefore associated with the aforementioned bacteria. When 0.50g of hi-maize was fermented, only propionate production was significantly inhibited. Although with 1.00g hi-maize total SCFA, acetate and propionate production was inhibited by 53.82%, 54.17% and 65.65% respectively, butyrate production was only inhibited by 21.26% which was not statistically significant. As the fibre that suffered the least inhibition with increasing availability, it is probable that the bacteria responsible for hi-maize's breakdown were unaffected by extracellular pH. Furthermore, butyrate contribution to overall SCFA profile was significantly enhanced when 1.00g of all substrates was fermented compared to 0.25g, again indicating favoured butyrate production at a lesser pH.

It was hoped that the bacterial profile attributed to each fibre and their differing weights would be analysed. Unfortunately, time constraints did not allow the amplification and sequencing of the extracted DNA and therefore such results cannot be discussed within this thesis. However, there is strong evidence within scientific literature to suggest that butyrogenic bacteria thrive at a lower pH [194-198]. A study using a continuous flow fermenter system with human faecal inocula reported that at pH 5.5, butyrogenic bacteria related to *Eubacterium rectale* accounted for 50% of identified bacteria, whilst at pH 6.5, 86% of bacteria belonged to the Bacteroides group [196], which favour the production of acetate and propionate. Similar results were reported by Walker et al [195]. A very recent study by Chung et al (2016) [197] assessed the impact of pH on SCFA and bacterial profiles following incubation with pectin and inulin using continuous flow fermenters. They reported that at a lower pH *Faecalibacterium prausnitzii* replaced *Bacteriodes spp* as the dominant species, which was correlated with a static butyrate production, despite a decreased total SCFA, acetate and propionate production, reflecting the results of our current study. Butyrate-producing bacteria compete with other species at a higher pH, yet thrive at a lower pH when these competing bacteria become inhibited. This explains the reduction in the other major SCFA and total SCFA despite an increase in butyrate. Future

analysis of the microbial composition of the fermentations within this study would reveal any alterations in bacteria with increasing substrate availability and may provide further evidence that butyrate-producing bacteria take precedence with decreasing pH.

It is extremely ambiguous to relate the findings of this *in vitro* study to the activity within the human GIT. The inhibition of acetate, propionate and total SCFA at increasing substrate availability may also be due to build-up of enzymatic end-products which could cause feedback inhibition [199]. However, as a dynamic environment, there is little build-up of metabolites in the colon. The majority of SCFA are absorbed by the host in exchange for bicarbonate ions [83], hence the pH of the large intestine should not drop beyond unreasonable limits. Human studies have repeatedly shown that dietary fibre has a strong positive relationship with total SCFA production [98-100, 105, 122, 200], with the profile of individual SCFA being dependant on the fibre involved [98, 99]. As the batch culture fermentations do not allow removal of metabolites, it is not an exact representation of what occurs *in vivo*. In this case, continuous fermenters may provide a more pragmatic simulation of the *in vivo* human gut due to the periodic removal of metabolites. However, to the knowledge of this author, there are no studies investigating the influence of increasing fibre weights on SCFA and bacterial profile using a continuous fermentation model.

In conclusion, the aim of this study was to investigate inhibition of SCFA production during *in vitro* batch culture fermentations when exposed to increasing amounts of various fibres. The results echoed those of previous studies in that there is a degree of inhibition with increasing substrate. This study further showed that the relative contribution of butyrate to total SFCA is enhanced in spite of this inhibition, most likely due to the promotion or no inhibition of butyrogenic bacteria at a lesser pH. Whilst these results do not indicate the optimal substrate weight that should be used during *in vitro* fermentations, they do suggest that results should be interpreted with caution. Are the results of such studies a true representation of colonic activity or are they influenced by the *in vitro* environment? Studies of such design should be aware of this issue, and always strive to choose an *in vitro* design based on their chosen study outcomes. Importantly the results of this study suggest that comparison of findings between different studies requires that the *in-vitro* experimental conditions are the same to allow valid conclusions.

Chapter 4 : *In vitro* fermentation capacity of the gut microbiota of IBD patients and healthy control

4.1 Chapter Outline

This chapter will focus on the ability of the faecal microbiota from IBD patients in clinical remission and matched healthy controls to produce SCFA *in vitro* when they are exposed to the same amount and type of fibre. In brief, various dietary fibres were exposed to faecal microbiota of patients with IBD and matched healthy controls within 48-hour fermentation studies. This chapter will decipher if there is a difference in SCFA production in response to these different substrates between IBD patients and healthy controls.

4.2 Introduction

There is mounting scientific evidence to suggest that the bacterial production of SCFA (acetate, propionate and butyrate) within the lumen play an important role in health and has a therapeutic potential in both in diseases of the gut and beyond this organ [59, 60, 181, 182, 184]. Although the medicinal properties of these organic molecules are not restricted to their site of production, the colonic benefits of SCFA have been well documented since the 1980s [166]. The production of these acids lowers the pH of the intestinal environment, thereby promoting the growth of presumably healthy bacteria such as *Lactobacilli* and *Bifidobacterium* [194] whilst inhibiting the growth of pathogenic organisms [80]. As the main provider of colonocyte energy, butyrate has been strongly associated with colonic health. Furthermore, butyrate is involved in the regulation of both cell proliferation [87] and inflammation within the colonic mucosa [86] and thus its production has been linked to a decreased risk of colorectal cancer [89] and colitis [88].

As organic diseases of the GIT, it is logical to assume SCFA (or rather, a lack of) may play a role in either the onset or perpetuation of IBD. Studies have explored the relationship between IBD and SCFA production, based on evidence that there is a marked difference in faecal SCFA concentrations between CD [122, 129, 131] and UC [128, 131, 132, 166, 201] patients compared to healthy controls. A large observational study of 73 UC patients, 23 CD patients and 65 healthy controls reported that despite similar faecal concentration of acetate, IBD patients had a lesser concentration of propionate and butyrate [131]. Similar results were found in a Malaysian study of a similar cross-sectional design; however this study had a low patient pool (n=8). Kumari et al (2013) [128] conducted a

further cross-sectional study but focused specifically on UC patients with varying disease severity. They described a significant decrease in butyrate and acetate concentrations in patients with severe disease compared to healthy participants. Furthermore, patients with severe disease had significantly reduced butyrate levels compared to those in remission, suggesting that SCFA production is related to colonic inflammation. This preliminary evidence alluding to an inverse relationship between SCFA production and disease severity may explain why another study found no reduction in any of the major SCFA in UC patients in remission [202]. SCFA, particularly butyrate, have anti-inflammatory properties including the regulation the production of pro-inflammatory cytokines [203] and therefore it is likely that a diminished production would perpetuate inflammation although it is not entirely clear whether this reduction of SCFA is a cause or an effect of the inflammation experienced in IBD.

As dietary substrates which evade digestion in the upper GIT, fibre is transported to the large intestine where it is fermented by the resident gut microbiota; the end-product of which is SCFA. The diverse range of dietary fibres have varying fermentability and require a range of different enzymes to be fermented, hence differ in their ability to produce not only total SCFA, but also individual SCFA profile [90, 106, 107, 112]. Human trials have explored the impact of dietary fibre on SCFA in healthy individuals, reporting that fibre-free and low fibre diets significantly reduce total SCFA [98, 103, 105]. Certain fibres have been associated with enhanced butyrate production, namely resistant starch [80] and other prebiotics [189] whilst others, such as pectin, are associated with increased acetate [185]. Psyllium and guar gum have been previously reported as propiogenic [186].

There is suggestion that the lower luminal levels of SCFA seen in IBD patients may be overcome by increasing fibre within the diet, and animal studies have reported promising evidence of enhanced SCFA production in response to fibre supplementation [147-152]. These changes were often accompanied by improvements in inflammatory markers [147, 148] and colonic damage [150, 151] in animal models of colitis. The majority of clinical human studies investigating the influence of dietary fibre on SCFA have focused on UC patients [153, 154, 156, 157]. It can generally be concluded from these reports that although fibre is significantly better at relieving symptoms than a placebo [154], it is no better or worse than current drug therapies [155]. However, dietary fibre did increase faecal butyrate concentrations significantly more than the drugs studied [155]. A small clinical trial in CD patients reported that daily administration of fructo-oligosaccharide significantly decreased disease activity [158], although the authors did not comment on changes in SCFA production. De Preter et al [144] found that the

administration of oligofructose-enriched inulin significantly increased faecal butyrate measurements and decreased disease activity in patients with mild and inactive CD after 2 weeks, changes which were not seen with the placebo group. However, an opposing study reported that despite improvements in inflammatory markers IL-8 and Il-10, clinical response was not significantly changed by 4 weeks of fructo-oligosaccharide supplementation in patients with active CD [204]. SCFA production was not mentioned in this study.

Thus, there is currently not enough evidence to suggest that dietary fibre can relieve the symptoms of IBD. Indeed, there are some studies that fail to report discrepancies between faecal SCFA concentrations of IBD patients and in healthy controls [132, 202]. Machiels et al (2014) reported that, although total SCFA, acetate and propionate were significantly reduced in UC patients, concentrations of butyrate did not differ from healthy controls [132], and did not find correlations between disease severity and SCFA concentrations. Interestingly, this study did find a significant reduction in butyrate-producing bacteria *Roseburia hominis* and *Faecalibacterium prausnitzii* in patients, and these had a significant inverse correlation with disease severity. These confounding results are not easy to interpret, but perhaps suggest that UC patients have overcome this reduction in known beneficial bacteria by supporting other unidentified species. A further paradoxical finding regarding the relationship between SCFA and IBD is the reduction in faecal butyrate concentrations associated with clinical improvements in paediatric CD whilst on treatment on EEN [122], a successful form of treatment in paediatric CD [8]. Again, paradox to the belief that dietary fibre is beneficial to colonic health EEN, which is comparable to steroids in terms of inducing clinical remission [205], is completely fibre-free. This improvement of CD with a diet void of fibre further complicates current understanding of the relationship between dietary fibre, its fermentation and production of SCFA and colonic inflammation in IBD. Furthermore some, but not all, studies have implied that dietary fibre is not well tolerated by patients [204], and it is well known that fibre can aggravate functional symptoms such as bloating and abdominal discomfort making it difficult to differentiate between organic and functional disease [206]. Although IBD patients are not advised to avoid it, particularly when in remission, CD and UC patients often experience adverse effects on consumption of dietary fibre [145], and reports suggest that the colonic microbiota of IBD patients are not as efficient in the hydrolysis of fibre compared to healthy controls [125, 202]. James et al [202] reported that despite a reduced intake of dietary fibre, UC patients excreted 3 times more non-starch polysaccharide than matched healthy controls, suggesting an inability of the bacteria in the

utilisation of fibre. Similarly, an *in vitro* study found that compared to healthy controls, faecal samples from UC patients produced significantly less total SCFA and 10-fold less butyrate than those from healthy participants [125]. Thus, IBD patients may suffer from reduced SCFA production due to the ineptness of innate bacteria to ferment dietary fibre regardless of intake; which may in fact exacerbate inflammation.

The majority of studies investigating SCFA concentrations in faecal matter [128, 129, 132, 201] which, although offers a proxy of SCFA production, is not a complete representation of colonic production. The majority of SCFA are absorbed from the colon [80] for distribution around the body and thus only a small proportion are excreted in faeces [80]. As outlined in the introducition of this thesis, it is hard to gain an accurate representation of SCFA production *in vivo*. *In vitro* fermentation models help to study the ability of the gut bacteria to produce SCFA in response to different fibres. Thus, this study aims to characterise the functional capacity of gut bacteria obtained from adult IBD patients in remission by assessing differences in SCFA production between patients and matched healthy controls using a batch culture *in vitro* system.

This study was ethnically approved by the NHS, West of Scotland Research Ethics Committee 4 and the University of Glasgow, College of Medical, Veterinary and Life Sciences (MVLS) Ethics Committee.

4.3 Subjects and Methods

Participants of this study were recruited as outline in Chapter 2 2.3. Briefly, participants were recruited into one of the 6 following groups;

i) *Adult CD patients in remission*

ii) *Adult UC patients in remission:*

iii) *Otherwise, healthy adults with no history of IBD*

iv) *Newly diagnosed paediatric patients with CD with active disease*

v) *Paediatric patients with CD on contemporary treatment*

vi) *Healthy children with no history of IBD;*

Participants were asked to donate a single stool sample, which was processed as explained in Chapter 2 and used in *in vitro* fermentation studies alongside various fibres for 48 hours. pH was recorded pre- and post-fermentation, as was the volume of gas expelled after 48 hours. Aliquots were taken for future SCFA analysis and stored at -20˚C alongside 1M NaOH. Each stool sample was fermented alongside 8 different fibres, one mixed fibre and a non-substrate control (NSC). SCFA were analysed using gas chromatography. Thus 9 aliquots were taken both before and after the fermentation period, giving a total of 18 potential SCFA extractions per participant. The extraction and analysis of all SCFA was not possible due to time restraints thus SCFA were analysed from only the non-fibrous control at baseline and all fibres after 48 hours (including the mixed fibre and the non-fibrous control). However, it has been shown in many studies within this laboratory that baseline SCFA concentrations are negligible regardless of disease state or fibre. Furthermore, although desirable, it was not feasible to do the SCFA extractions in duplicate again due to lack of time and therefore each extraction was only conducted once. This gave a more reasonable total of 560 SCFA extractions. However, the results generated from gas chromatography analysis were scrutinised and any value which looked abnormally high or low was re-extracted.

Further *in vitro* fermentations were conducted using the same protocol; however in this case, no faecal slurry was introduced in order to observe fermentation in the absence of human gut microbiota (see Section 2.4.2) at both 0 and 48 hours.

4.3.1 Statistical analysis

All data was analysed using Minitab Version 16.2.2 statistical software (Pennsylvania State University, Pennsylvania, USA). Probability charts generated by the Anderson-Darling test of normality highlighted that the data collected for the age and BMI of participants was not normally distributed nor were the majority of measurements for SCFA or volume of expired gas. Thus, the Kruskal-Wallis test was used to assess the differences in these characteristics between the three groups. For measurements that were normally distributed, such as pH, 1-way ANOVA test was employed to assess differences between CD, UC patients and healthy controls. Pearson correlation was used to assess correlations between faecal calprotectin and SCFA production. Statistical significance was considered at $p \leq 0.05$.

4.4 Results

4.4.1 Participant characteristics

4.4.1.1 Demographics, anthropometric measurements and disease characteristics of adult IBD patients and healthy controls

Following the recruitment process outlined in Chapter 2, 15 CD and 17 UC adult patients were approached in the gastroenterology clinics, all of whom consented to take part in the study and met the inclusion criteria. For reasons unknown to the researcher, 2 CD and 5 UC patients did not provide a stool sample and thus results for 13 CD and 12 UC patients are presented hereafter. There was a slight male predominance in the patients who completed the study (56% males vs. 44% females). However, although 61.5% of CD patients were male, an equal male to female ratio existed in the UC patients. Likewise, there were an equal number of males and females amongst the 14 healthy adults recruited in this study. No significant differences were detected between the age or BMI of CD patients, UC patients or healthy controls ($p>0.05$), values which are displayed in Table 4.1.

Table 4.2 and Table 4.3 respectively summarise the disease characteristics and concomitant medications taken by CD and UC patients during this study. The majority of CD patients had colonic or ileal CD and most were diagnosed between the ages of 18-40 years (average 28.38 ± 0.24 years, minimum 10, maximum 46). All but 2 CD patients (15.4%) were on medication, with the majority (84.6%) taking a form of immunosuppressant. UC patients had a higher average age of diagnosis compared to CD patients (41.58 ± 0.34 years) and only 1 patient was not on medication. Pancolitis was the predominant form of UC in this study.

Table 4.1 Characteristics of adult IBD patients and healthy controls recorded at the time of recruitment.

	Males		Female		All participants	
	Median	IQR	Median	IQR	Median	IQR
All IBD Patients						
	n=14		**n=11**		**n=25**	
Age	36.50	25.63	35.00	25.00	36.00	25.00
BMI (kg/m^2)	24.88	3.19	23.92	11.62	24.82	5.55
CD Patients						
	n=8		**n=5**		**n=13**	
Age	34.5	25.21	34.00	10.50	34.00	15.92
BMI (kg/m^2)	25.05	6.46	28.12	15.41	25.95	9.07
UC Patients						
	n=6		**n=6**		**n=12**	
Age	43.00	37.98	58.00	30.25	53.50	30.73
BMI (kg/m^2)	24.88	2.34	23.90	8.46	24.70	2.50
Healthy Controls						
	n=7		**n=7**		**n=14**	
Age	37.74	28.41	37.21	27.81	37.48	27.31
BMI (kg/m^2)	25.47	5.89	25.00	17.34	25.43	0.72

All values expressed as Median (Interquartile Range(IQR)

Table 4.2 Disease characteristics according to the Montreal Classification and concomitant medications of adult UC patients

	Females n=6	**Males n=6**	**All UC patients n=12**
Age at diagnosis (median (IQR))	51.00 (28.50)	34.50 (12.5)	35.00 (19.75)
Disease location (n (%))			
E1 Ulcerative proctitis	2 (33)	0	2 (17)
E2 Left-sided UC (distal UC)	2 (33)	1 (17)	3 (25)
E3 Extensive UC (pancolitis)	2 (33)	5 (83)	7 (58)
Concomitant Medication (n)			
Aminosalicylates	4 (66)	4 (66)	8 (66)
Immunosuppresants	2 (33)	4 (66)	6 (50)
None	1 (17)	0	1 (8)

Table 4.3 Disease characteristics according to the Montreal Classification and concomitant medications of adult CD patients

	Females n=5	**Males n=8**	**All CD patients n=13**
Age at diagnosis (n (%))	**N**	**N**	**N**
A1 below 16y	1 (20)	1 (12)	2 (15)
A2 between 17 and 40y	3 (60)	7 (88)	10 (77)
A3 above 40y	1 (20)	0	1 (8)
Age at diagnosis (median (IQR))	31.50(16.75)	31.50(17.25)	31.5 (10.75)
Disease location (n (%))			
L1 ileal	3 (60)	2 (25)	5 (38)
L2 colonic	2 (40)	3 (37)	5 (38)
L3 ileocolonic	0	2 (25)	2 (15)
L4 isolated upper disease	0	1 (13)	1 (8)
Disease behaviour (n (%))			
B1 non-stricturing, non-penetrating	4 (80)	4 (50)	8 (61)
B2 stricturing	1 (20)	3 (37)	4 (31)
B3 penetrating	0	1 (13)	1 (8)
Concomitant medication (n (%))			
Aminosalicylates	0	3 (38)	3 (23)
Immunosuppresants	4 (80)	7 (88)	11 (85)
Corticosteroids	1 (20)	1 (13)	2 (15)
None	1(20)	1 (13)	2 (15)

Table 4.4 displays faecal calprotectin values for all adult IBD patients and the healthy controls. CD patients had significantly higher calprotectin compared to healthy controls ($p<0.0106$), whilst despite a tendency, there was no difference in the values measured between UC patients and healthy participants ($p<0.069$).

According to the manufacturer's cut-off value of 43.2ug/g, 46% of CD patients had high faecal calprotectin, whilst all UC and healthy controls had normal values.

There were no statistical differences in water content of faecal samples between patients and controls (Table 4.5).

Table 4.4 Faecal calprotectin of CD patients, UC patients and healthy controls

	Faecal Calprotectin (µg/g)					
	Median	**(IQR)**	**Min Value**	**Max Value**	**% patients over 43.2µg/g**	**% patients over 200µg/g**
Crohn's Disease	48.86*	(528.78)	5.00	534.00	46.00	38.50
Healthy control	2.62	(2.37)	1.33	3.70	0	0
Ulcerative Colitis	6.44	(19.70)	2.40	22.40	0	0

Faecal calprotectin values displayed as µg per gram of wet stool sample

* indicates a statistical difference between CD and HC

Table 4.5 Percentage water content of faecal samples obtained from CD patients, UC patients and healthy controls

	Water Content (%)		
	Median		**IQR**
Crohn's Disease	72.94	±	14.58
Healthy Control	76.69	±	4.67
Ulcerative Colitis	78.53	±	10.85

4.4.1.2 Anthropometric measurements and disease characteristics of paediatric Crohn's Disease patients and healthy controls

As outlined in Chapter 2, paediatric patients were recruited as part of a wider study by another PhD researcher. This researcher primarily recruited for their own study; however if the stool sample provided by the child was large enough it was used for the fermentation experiments within this study. 12 different children with CD supplied a stool sample that were included in this study; 6 of whom had active disease and were yet to start their treatment on EEN whilst the remaining 6 had were already on medication. The stool samples used in the paediatric cohort were predominately from boys, with 9 out of the 11 recruits (81.8%) being male. Out of a total of 6, only one female paediatric patient was included in the treated group for this study. Of the 5 healthy children who were recruited and who provided a stool sample for this study, 3 were male (60%). The age and BMI of the paediatric patients and the healthy controls are displayed in Table 4.6. No significan t differences were detected in these characteristics between patient groups and healthy controls.

The majority of both treatment naïve and treated paediatric CD patients had ileocolonic disease (66.67 % and 83.33% respectively, Table 4.7). All but one previously diagnosed patient was on a form of immunosuppressant; the remaining patient was treated with maintenance enteral nutrition. All of the newly diagnosed patients were treatment naïve.

Table 4.6 Characteristics of paediatric Crohn's Disease patients (pCD) and healthy controls recorded at the time of recruitment

	Male	Female	Male and Female
All pCD patients			
	n=9	**n=3**	**n=12**
Age	11.05 ± 1.05	9.53 ± 0.29	10.67 ± 0.80
BMI (kg/m^2)	17.34 ± 0.74	16.96 ± 1.93	17.25 ± 0.69
Treatment Naive pCD Patients			
	n=4	**n=2**	**n=6**
Age	12.00 ± 1.66	9.80 ± 0.20	9.84 ± 1.05
BMI (kg/m^2)	18.45 ± 0.62	17.88 ± 2.93	16.60 ± 0.94
Treated pCD Patients			
	n=5	**n=1**	**n=6**
Age	12.00 ± 1.34	9.00±n/a	11.50 ±1.20
BMI (kg/m^2)	18.45 ±1.03	15.11±n/a	17.89± 1.01
Healthy Controls			
	n=3	**n=2**	**n=5**
Age	10.00 ± 3.00	9.50 ± 2.50	9.80 ± 1.83
BMI (kg/m^2)	17.61 ± 2.61	17.31 ± 1.46	17.49 ± 1.50

All values expressed as Mean ± Standard Error of the Mean (SEM)

Table 4.7 Disease characteristics according to the Montreal Classification and concomitant medications of paediatric CD patients (pCD)

	Treatment Naïve pCD patients			Treated pCD patients		
	Females (n=2)	**Males (n=4)**	**Female and male (n=6)**	**Females (n=1)**	**Males (n=5)**	**Female and male (n=6)**
Age at diagnosis (median (IQR))	10 (0)	11 (3.27)	10.66 (2.58)	8	11.80 (2.38)	11.11 (2.63)
Disease location (n (%))						
L1 ileal	0	0	0	0	0	0
L2 colonic	0	0	0	0	1 (20)	1 (16.67)
L3 ileocolonic	2 (100)	2 (50)	4 (66.67)	1 (100)	4 (80)	5 (83.33)
L4 isolated upper disease	0	2 (50)	2 (33.33)	0	0	0
Concomitant medication (n (%))						
Aminosalicylates	0	0	0	0	0	0
Immunosuppressant	0	0	0	1 (100)	4 (80)	5 (100)
Corticosteroids	0	0	0	0	0	0
Maintenance Enteral Nutrition	0	0	0	0	1 (20)	0
None	2 (100)	4 (100)	6 (100)	0	0	0

Table 4.8 presents the faecal calprotectin of paediatric CD patients and healthy controls, highlighting that both treatment naive and treated CD patients had significantly higher calprotectin than healthy controls. All patients who were newly diagnosed and untreated had higher than normal calprotectin values (>43.2 µg/g) whilst 5 out of the 6 (83.33%) children on treatment had high measurements. One of the healthy children had a slightly higher than normal calprotectin measurements (45.00 µg/g).

Table 4.8 Faecal calprotectin (µg/g) of paediatric CD patients (pCD) and matched healthy children

Faecal Calprotectin (µg/g)						
	Median	**IQR**	**Min Value**	**Max Value**	**% patients over 43.2µg/g**	**% patients over 200µg/g**
Treatment Naive pCD	232.10*	218	233.3	649.9	100%	100%
Treated pCD	578.90*	873	13.00	1235	83.33%	83.33%
Healthy controls	8.27	108	4.52	53.97	20.00%	0%

Faecal calprotectin values displayed as µg per gram of wet stool sample
pCD; paediatric Crohn's Disease
* indicates a statistical difference from HC

4.4.2 pH of faecal slurry pre- and post-48 hours fermentation

4.4.2.1 Adults

As shown in Table 4.9, the difference between the pH pre- and post-incubation of the non-substrate control was not signficantly different when each subject group was studied independently; however there was a slight increase following fermentation when the 3 subject groups were combined (median (IQR) 0hr; 7.13 (0.18), 48hr; 7.29 (0.21), p= 0.0073), information not shown on Table). Similarly, when considering all participants simultaneously, the pH recorded after the fermentation period with cellulose was signficantly higher than at baseline (median (IQR) 0hr; 7.13 (0.16), 48hr; 7.23 (0.25) p=0.0337), information not shown on Table). As shown in Table 4.9, in the case of all substrates except apple pectin, there were no statistically significant differences between the pH measured in CD patients, UC patients and healthy controls pre- or post-fermentation. With regards to apple pectin, the initial pH of healthy controls' was

significantly higher than both CD and UC patients; however this difference did not remain after 48 hours fermentation (Table 4.9).

4.4.2.2 Children

Table 4.10 displays the results of the pH measurements recorded prior to and after the fermentation period in paediatric CD patients and the child healthy controls. With the exception of the substrate-free fermentation and that with cellulose, the pH of faecal slurries fermented alongside all substrates significantly decreased after 48 hours in all groups. In all subject groups, the pH was slightly higher after fermentation with cellulose and the non-fibrous control; however, this was not found to be significant. For fermentations with all substrates, there was no significant differences in the initial pH or that after 48 hours between the 3 groups.

Table 4.9 pH of faecal slurry of adult CD, UC and healthy controls before and after 48 hour fermentation with fibrous substrates

	CD (n=13)				UC (n=12)				Healthy Controls (n=14)			
	0hr		48hr		0hr		48hr		0hr		48hr	
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
NSC	7.16	0.23	7.30	0.15	7.05	0.16	7.17	0.30	7.15	0.19	7.29	0.24
Apple Pectin	7.11	0.14 †	4.84	0.38*	7.04	0.24 ‡	4.88	0.57*	7.23	0.13	4.89	0.27*
Butyrate Ester	7.14	0.15	5.70	0.38*	7.19	0.22	5.38	0.53*	7.07	0.19	5.65	0.55*
Cellulose	7.14	0.12	7.26	0.26	7.05	0.17	7.18	0.23	7.18	0.23	7.26	0.29
Hi-Maize	7.14	0.12	5.41	0.86*	7.03	0.15	5.47	0.69*	7.16	0.24	5.16	0.49*
Mixed Fibre	7.12	0.17	5.40	0.49*	7.01	0.15	5.64	1.34*	7.14	0.19	5.69	0.94*
Propionate Ester	7.14	0.17	5.76	0.63*	7.03	0.24	5.90	0.62*	7.10	0.16	5.65	0.51*
Raftilose	7.18	0.11	4.50	0.33*	7.09	0.19	4.51	0.81*	7.20	0.18	4.53	1.12*
Wheat Bran	7.10	0.13	6.05	0.24*	7.02	0.20	6.15	0.82*	7.13	0.15	6.25	0.58*

* represents a statistical difference between pH at 0hr and 48hr ($p<0.05$)
† represents a statistical difference between pH of CD and healthy controls ($p<0.05$)
‡ represents a statistical difference between pH of UC and healthy controls ($p<0.05$)
NSC: non-substrate control

Table 4.10 pH of faecal slurry of treatment naive paediatric CD patients, paediatric CD patients who have been on treatment and healthy controls before and after 48 hour fermentation with fibrous substrates

	Treatment Naive (n=6)				Treated (n=6)				Healthy Controls (n=6)			
	0hr		48hr		0hr		48hr		0hr		48hr	
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
NSC	7.16	0.50	7.20	0.49	7.06	0.18	7.18	0.24	7.24	0.31	7.33	0.49
Apple Pectin	7.09	0.47	5.05	0.45*	7.03	0.16	4.75	0.42*	7.25	0.32	5.11	0.63*
Butyrate Ester	7.17	0.32	5.57	0.69*	7.10	0.17	5.70	0.65*	7.23	0.38	5.55	0.59*
Cellulose	7.19	0.32	7.26	0.48	7.10	0.17	7.14	0.24	7.28	0.47	7.17	0.54
Hi-Maize	7.17	0.51	5.64	1.18*	7.08	0.18	5.06	0.54*	7.24	0.41	5.63	0.79*
Mixed Fibre	7.09	0.48	5.27	1.16*	7.06	0.18	5.12	0.63*	7.15	0.42	5.50	0.58*
Propionate Ester	7.18	0.56	5.78	1.45*	7.08	0.16	5.93	0.89*	7.18	0.41	5.70	0.88*
Raftilose	7.13	0.36	4.61	0.92*	7.07	0.19	4.64	0.41*	7.29	0.34	4.75	1.34*
Wheat Bran	7.13	0.45	6.53	0.64*	7.03	0.13	6.05	0.48*	7.18	0.52	5.97	0.70*

*represents statistical difference between pH at 0hr and 48hr ($p<0.05$)
NSC; non-substrate control

4.4.3 Volume of expired gas following 48 hr fermentation

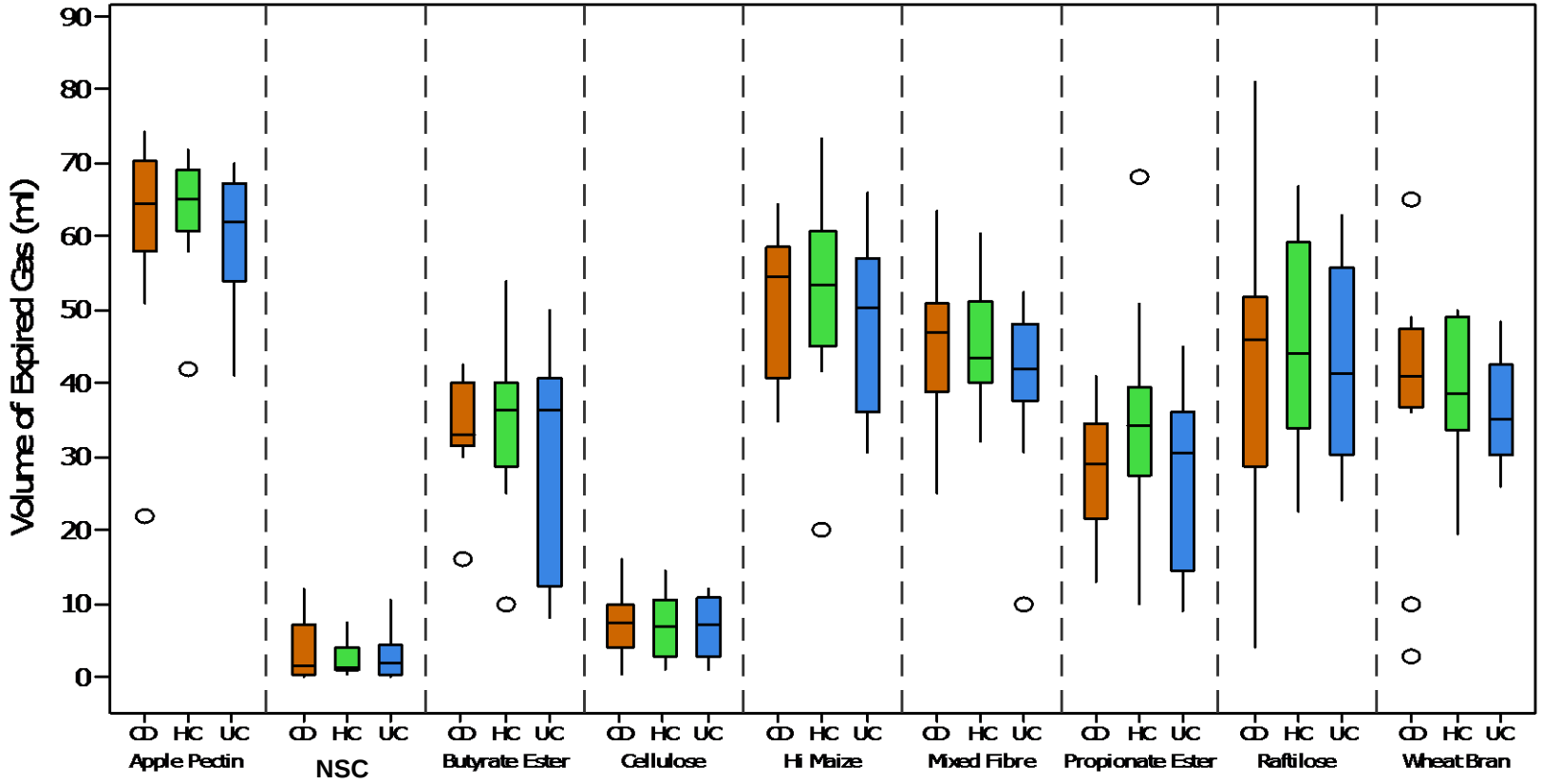
4.4.3.1 Adults

As illustrated in Figure 4.1, there were no significance noted in the differences between the volume of gas produced between CD patients, UC patients and healthy controls for all substrates and the NSC. In all participant groups very little, if any, gas was expired after 48 hours of fermentation for both cellulose and the non-fibrous control, whilst apple pectin produced the highest production of gas (median (IQR) CD; 62.25 (8.38), UC; 62.00 (13.25), healthy controls; (64.50 (12.50).

4.4.3.2 Children

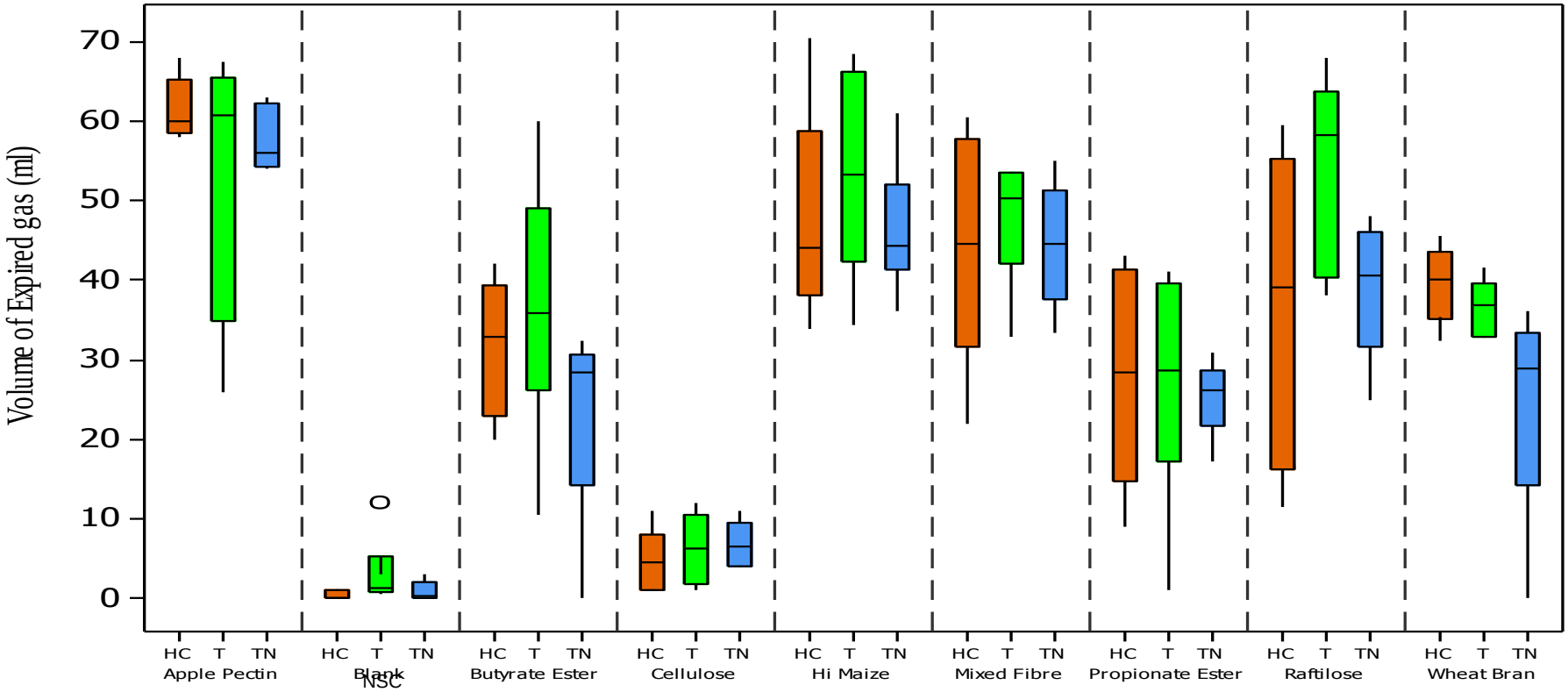
Figure 4.2 displays the volume of expired gas from all substrates after 48 hour fermentation in the two paediatric CD groups and the matched healthy children. Similar to the results found for adults, there were no signficant differences between the subject groups for any of the substrates. Apple pectin was the highest producer of gas for all participant groups (median (IQR) newly diagnosed pCD: 56.00 (7.88), treated pCD; 60.75 (30.63), healthy controls 60.00 (6.75), whilst there was minimal gas expelled after fermentation with no fibre or with cellulose.

Figure 4.1 Box-plot illustrating the volume of expired gas (ml) after the 48 hr fermentation period for Crohn's Disease (CD) patients, ulcerative colitis (UC) patients and healthy (HC) adult participants



O indicates outliers
NSC; non-substrate control

Figure 4.2 Box-plot illustrating the volume of expired gas (ml) after the 48 hr fermentation period for treatment naive CD patients (pCD), pCD patients who have received treatment, and healthy children



O indicates outliers

HC; healthy control, T; treated pCD patient, TN; treatment-naïve pCD patient, NSC; non-substrate control

4.4.4 Production of the major SCFA before and after 48 hours fermentation with different fibrous substrates

As aforementioned (Section 2.4.3.4), the *in vitro* production of SCFA in response to fermentation with butyrate and propionate esters was not investigated in this study due to methodological issues. The results of all other fibres however, are reported hereafter.

4.4.4.1 Adults

Baseline measurements of SCFA

SCFA concentrations at baseline were analysed only for the NSC fermentation. For both patient groups and the healthy controls, SCFA concentration was very low for the non-substrate control (Table 4.11). Only acetate was detected and there were no difference between groups (Table 4.11).

Table 4.11 Concentration of the major individual SCFA and their proportional contribution to total SCFA (%) prior to 48hours fermentation with faecal slurry from CD patients, UC patients and healthy controls with no fibrous substrate

	CD (n=13)		UC (n=12)		Healthy Controls (n=14)	
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Total SCFA	0.93	1.49	0.84	0.30	1.05	1.20
C2	0.91	1.33	0.81	0.29	0.92	0.76
C3	0.00	0.00	0.00	0.00	0.00	0.00
C4	0.00	0.00	0.00	0.00	0.00	0.19
C2%	100.00	16.63	100.00	0.00	100.00	16.76
C3%	0.00	6.46	0.00	0.00	0.00	0.00
C4%	0.00	0.00	0.00	0.00	0.00	15.31

CD; Crohn's Disease patient, UC; ulcerative colitis patient, HC; healthy control
C2; acetate, C3; propionate, C4; butyrate
concentrations expressed as µmol/mL

SCFA production after 48 hours

Differences in SCFA concentrations and proportional contribution to total SCFA between substrates

The median (IQR) concentration of the major SCFA and their relative contribution to the total SCFA measured after 48 hours of fermentation with all fibres and the non-fibrous control for all participant groups are presented in Table 4.11 and in Figure 4.3.

For all participant groups, apple pectin produced a significantly higher total SCFA concentration compared to all other substrates ($p<0.05$) whilst the NSC fermentation and that with cellulose produced significantly less total SCFA than all other substrates ($p<0.05$). Furthermore, the fermentation with no substrate and that with cellulose produced a signicantly lesser concentration of total SFCA compared to all other fibres ($p<0.05$). However, in no case was there any statistical differences in SCFA concentrations or their relative contributions to total SCFA between the NSC and cellulose fermentations.

The fermentation with apple pectin produced significantly more acetate than all other substrates ($p<0.05$). Apple pectin also resulted in a significantly higher concentration of propionate than hi-maize and raftilose, as did wheat bran ($p<0.05$). Butyrate concentration was signficantly increased after fermentation of apple pectin, hi-maize and mixed fibres compared to that observed with raftilose and wheat bran ($p<0.05$).

In terms of proportional contribution to total SCFA concentration, acetate was the predominant SCFA for all substrates, contibuting to an average of 58.28% (14.62) when all substrates were assessed simultaneously, whilst propionate and butyrate were respectively accounting for 14.10% (6.75) and 14.05% (9.86) of total SCFA on average (values expressed as median (IQR). The relative contributions of these SCFA for each fibre individually can be seen in Table 4.12 and Figure 4.4. The contribution of acetate to total SCFA was proportionally higher after fermentation with raftilose and apple pectin compared to wheat-bran, hi-maize and the mixed fibres ($p<0.05$), whilst wheat-bran had a significantly higher percentage of propionate than all other substrates ($p<0.05$). Relative contribution of butyrate to total SCFA production was highest after the fermentation with hi-maize compared to the other studied fibres ($p<0.05$).

Differences in SCFA concentrations and proportional contribution to total SCFA between participant groups

Although there were tendencies for increased concentration of acetate, propionate and butyrate concentration in healthy controls, statistical analysis did not reveal significant differences between patient groups and healthy controls for the majority of SCFA and substrates (Figure 4.3, Table 4.12). In terms of total SCFA concentration, there was a trend for enhanced production in healthy controls for all substrates except wheat-bran. However, this was only significantly different between healthy controls and UC patients after fermentation with hi-maize (median (IQR) HC; 58.87 (17.69) vs. UC; 44.92 (21.49) $p=0.008$) Although the median concentration was also lower in CD patients than healthy

controls, it did not reach significance. On the other hand, total SCFA concentration was statistically higher in healthy adults than in both UC and CD patients after the fermentation period with raftilose (median (IQR) HC; 51.76 (22.02) vs. CD; 41.12 (23.28) vs. UC; 41.94 (14.72) p=0.02). The proportional contribution of butyrate to total SCFA following fermentation with mixed fibre was also signficiantly higher for healthy controls compared to UC patients (median (IQR) HC; 10.76 (8.26) vs. CD; 8.75 (4.74) p=0.044) but not CD patients (p=0.260).

In no cases were there any significant differences between the SCFA concentration or relative contribution in CD or UC patients (Figure 4.4).

Table 4.12 Concentration (µmol/mL) of the major individual SCFA and their proportional contribution to total SCFA (%) after 48 hours fermentation with different fibrous substrates and faecal slurry from CD patients, UC patients and healthy controls

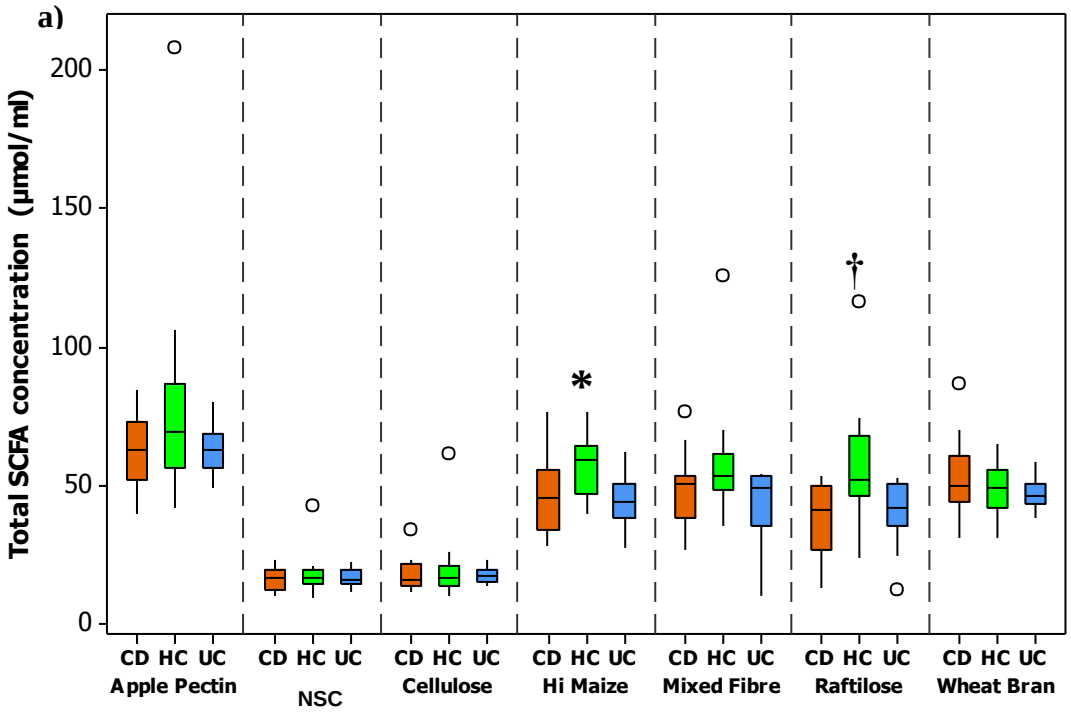
	CD (n=13)		**UC (n=12)**		**Healthy Controls (n=14)**		**p-value**
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	
NSC							
Total SCFA	16.22	7.49	15.89	5.20	15.93	5.26	0.96
C2	8.31	5.06	8.35	1.95	8.49	3.17	0.97
C3	2.47	0.73	2.38	1.08	2.16	1.47	0.74
C4	1.61	1.00	1.87	1.13	1.82	1.03	0.65
C2%	53.78	6.96	49.34	9.73	54.60	8.75	0.49
C3%	15.70	8.23	14.37	4.15	14.78	4.35	0.68
C4%	11.22	2.70	13.24	4.81	11.54	2.40	0.39
Apple Pectin							
Total SCFA	62.43	20.70	62.67	12.22	68.88	30.92	0.24
C2	41.83	11.87	43.45	8.88	49.12	21.49	0.29
C3	8.16	4.90	6.85	6.28	7.59	6.00	0.50
C4	11.47	7.03	9.11	4.21	10.49	8.20	0.33
C2%	68.86	8.36	69.90	3.62	68.22	9.14	0.52
C3%	12.92	6.01	10.96	5.17	10.77	5.79	0.66
C4%	16.05	9.51	17.53	6.53	15.14	8.44	0.51

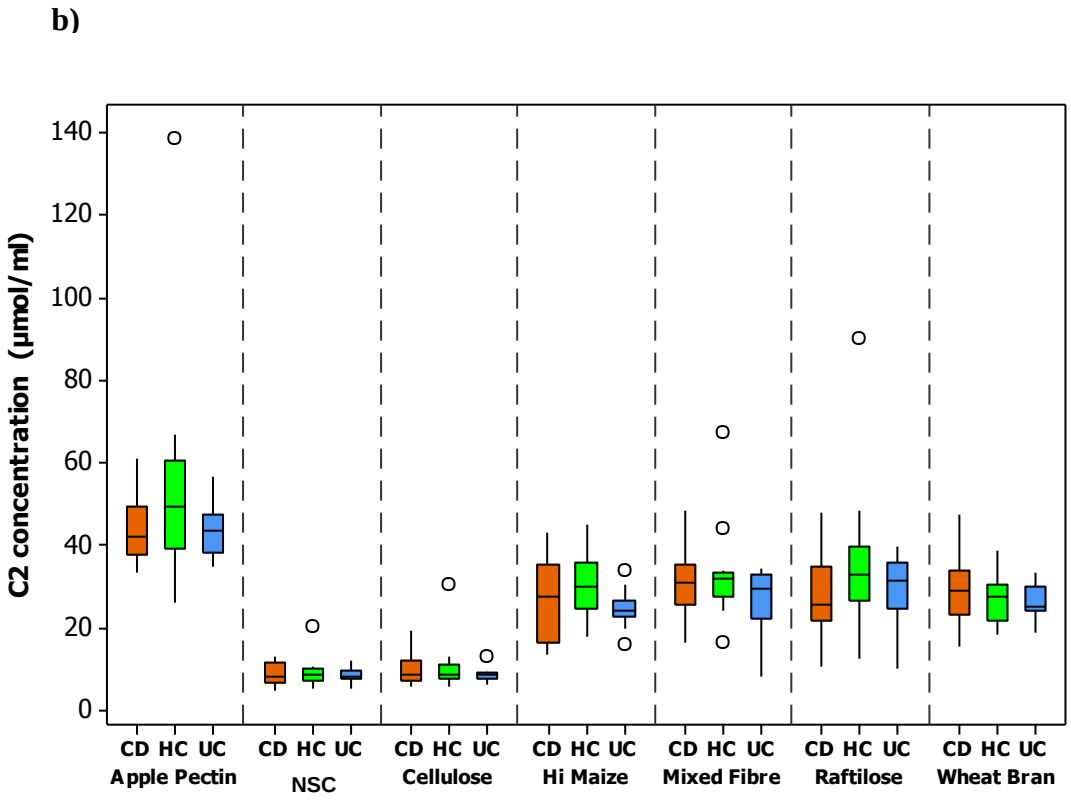
Cellulose							
Total SCFA	15.55	7.95	16.84	4.37	16.60	7.00	0.99
C2	8.70	4.93	8.74	1.20	8.40	3.39	0.10
C3	3.03	1.06	2.52	0.92	2.48	1.64	0.41
C4	1.81	0.93	2.03	0.75	1.97	0.91	0.78
C2%	52.96	9.07	50.25	8.19	54.92	7.60	0.45
C3%	17.01	7.83	15.02	4.51	16.45	4.31	0.51
C4%	10.50	3.00	12.29	3.24	11.15	2.39	0.26
Hi Maize							
Total SCFA	44.92	21.49	43.82	12.64	**58.87***	17.69	0.02
C2	27.27	18.70	24.03	4.05	29.97	11.14	0.13
C3	6.46	3.87	5.36	3.89	6.29	2.96	0.17
C4	10.29	9.61	9.67	8.05	13.99	11.58	0.20
C2%	57.17	11.43	53.13	14.39	58.46	18.80	0.87
C3%	13.42	9.40	10.87	8.25	12.47	6.65	0.34
C4%	22.70	12.86	22.48	17.18	24.39	17.19	0.47
Raftilose							
Total SCFA	41.12	23.28	41.94	14.72	**51.76†**	22.02	0.02
C2	25.47	12.82	31.32	10.97	32.81	12.88	0.27
C3	2.81	3.59	3.66	8.94	6.07	6.12	0.14
C4	3.61	9.90	3.04	7.22	8.42	12.52	0.14
C2%	73.95	20.85	78.02	30.47	61.16	31.16	0.21
C3%	9.79	9.13	11.72	17.01	10.42	12.71	0.58
C4%	10.48	19.83	8.31	14.27	12.84	22.43	0.36
Wheat Bran							
Total SCFA	49.25	16.41	45.85	7.75	48.97	14.24	0.43
C2	28.87	10.47	25.05	5.89	27.50	8.88	0.50
C3	10.27	4.97	8.07	1.13	7.76	3.88	0.11

C4	8.56	7.30	7.65	2.29	7.94	4.05	0.98
C2%	56.01	7.92	54.75	7.89	57.22	9.17	0.98
C3%	17.70	10.04	18.02	2.63	15.79	4.10	0.51
C4%	15.02	7.00	17.06	2.69	16.18	3.60	0.42
Mixed Fibre							
Total SCFA	50.26	15.32	48.80	18.17	53.36	12.44	0.12
C2	30.97	9.76	29.26	10.59	31.69	6.15	0.45
C3	7.12	5.87	5.37	3.93	7.15	4.87	0.18
C4	8.75	4.74	8.02	7.26	**10.76***	8.26	0.049
C2%	63.79	15.69	63.15	19.63	59.61	12.90	0.32
C3%	14.07	5.59	11.81	5.20	13.53	5.39	0.20
C4%	17.23	7.92	15.32	15.94	21.82	17.58	0.19

CD; Crohn's Disease patient, UC; ulcerative colitis patient, HC; healthy control C2; acetate, C3; propionate, C4; butyrate, NS; non-substrate control
concentrations expressed as µmol/mL
* indicates a statistically significant difference between healthy control and UC
†indicates a statistically significant difference between healthy control and UC/CD

Figure 4.3 Concentration of a) total SCFA, b) acetate, c) propionate, and d) butyrate following 48 hours fermentation with different fibrous substrates and faecal slurry from CD patients, UC patients and healthy controls





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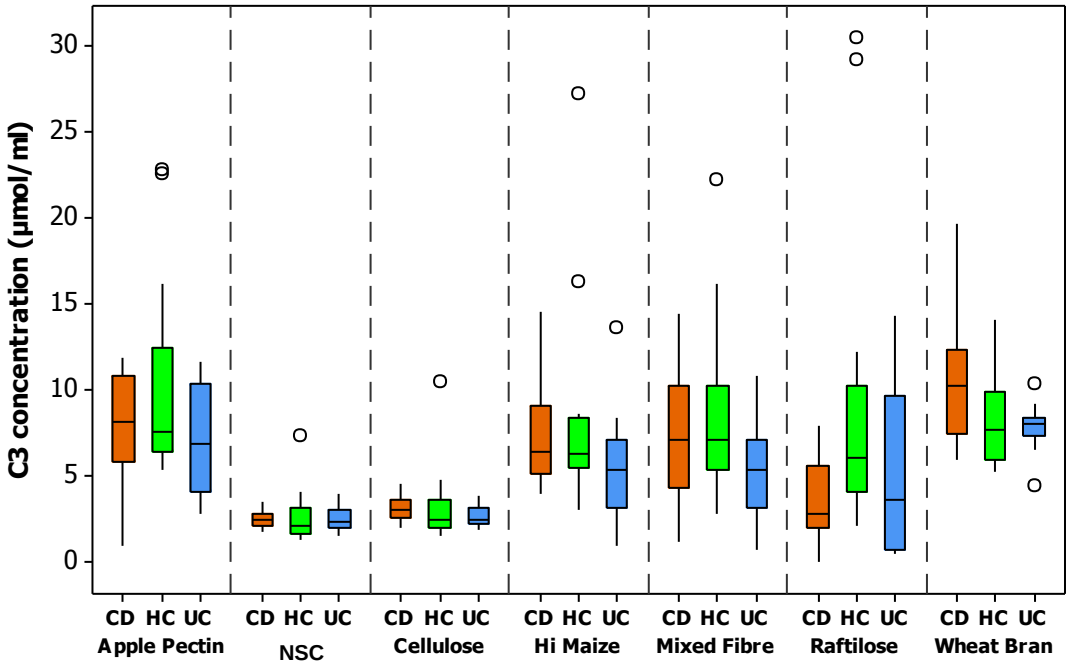

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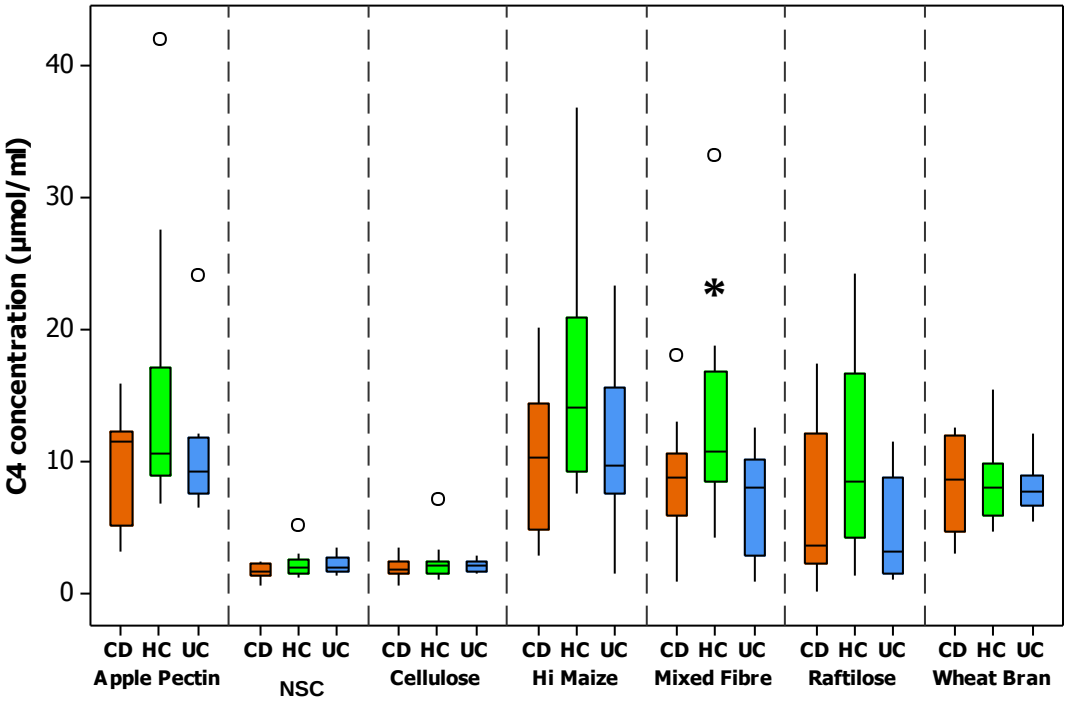
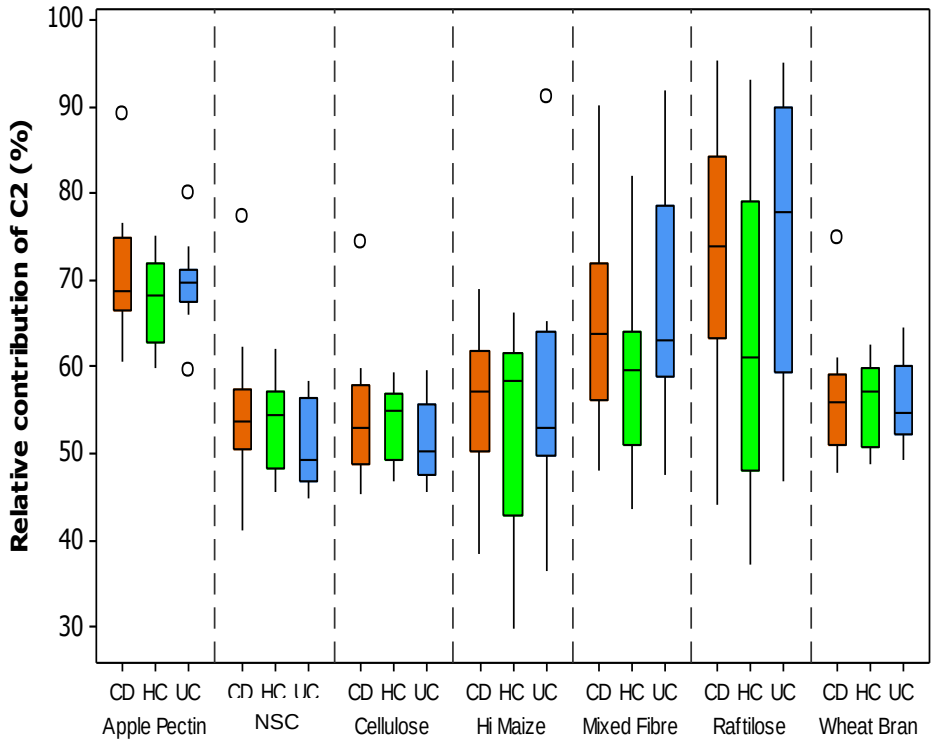


CD; Crohn's Disease patient, UC; ulcerative colitis patient, HC; healthy control, NSC;; non-substrate control
C2; acetate, C3; propionate, C4; butyrate
All concentrations expressed as µmol/mL.
O indicates outliers
* indicates significant difference between healthy control and UC
† indicates significant difference between healthy control and UC/CD

Figure 4.4 Relative contribution (%) of a) acetate, b) propionate and c) butyrate to total SCFA concentration after 48 hours fermentation with different fibres and faecal slurry from CD patients, UC patients and healthy controls

a)



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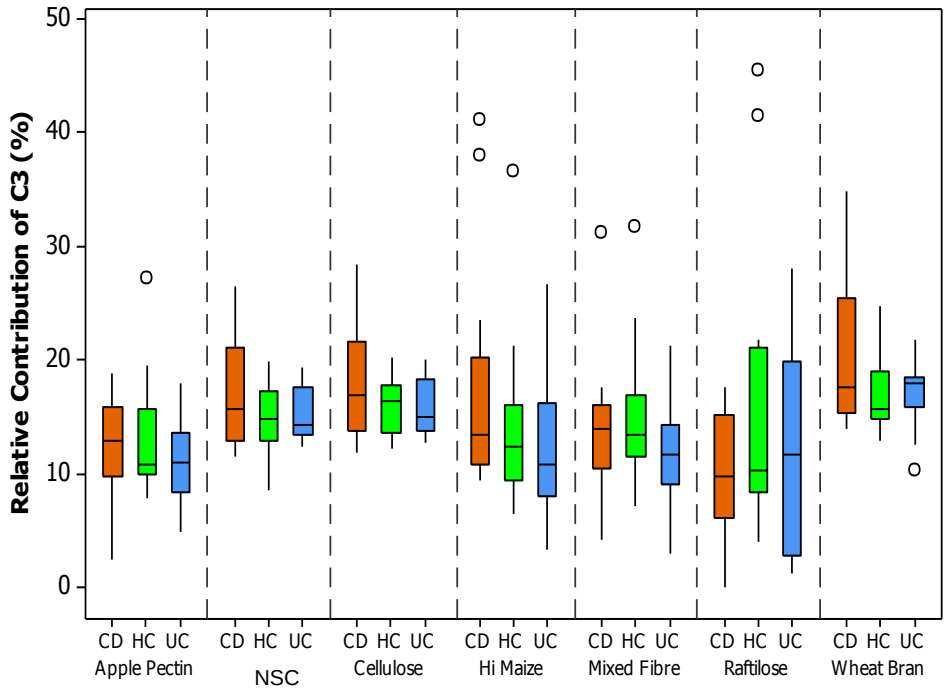
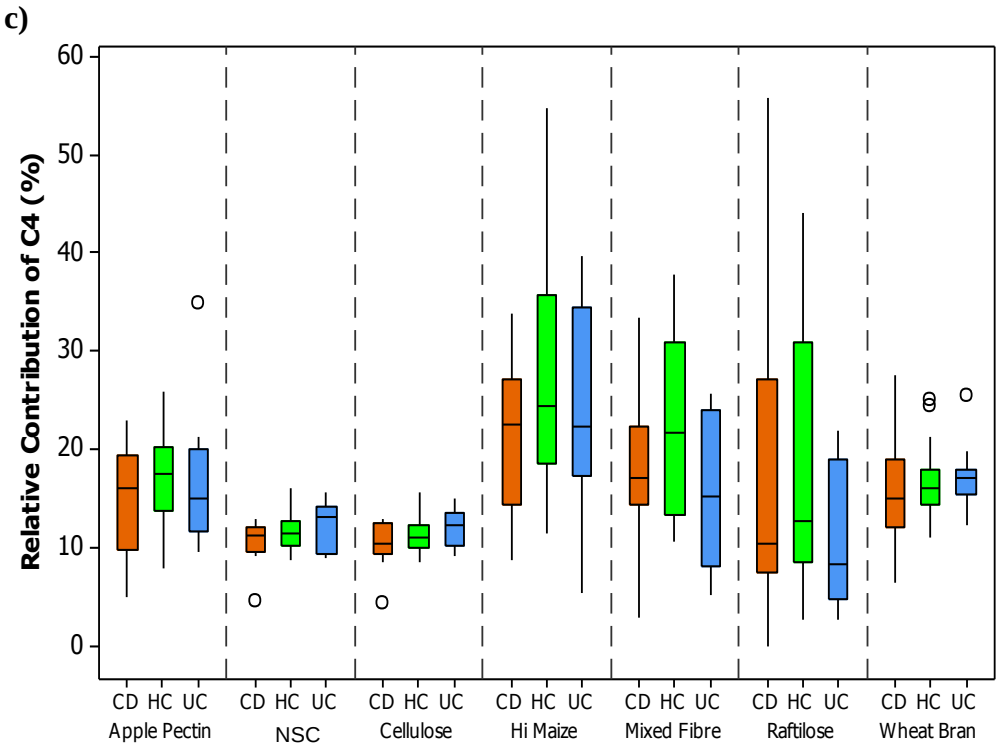




CD; Crohn's Disease patient, UC; ulcerative colitis patient, HC; healthy control, C2; acetate, C3; propionate, C4; butyrate, NSC; non-substrate control
O indicates outliers

4.4.4.2 Children

Baseline measurements of SCFA

There was both minimal total and individual SCFA production at baseline for the non-substrate control in all participant groups (Table 4.12) and there were no statistical differences between groups for these measurements ($p>0.05$). For all groups, acetate was the highest proportional contributor to total SCFA and there were no differences between groups in terms of proportional contribution of individual SCFAs to total concentration.

Table 4.13 Concentration of the major individual SCFA and their proportional contribution to total SCFA (%) prior to 48hours fermentation with faecal slurry from treatment naive CD patients (pCD), pCD patients who have received treatment, and healthy children with no fibrous substrate

	Treatment Naive pCD (n=6)		Treated pCD (n=6)		Healthy Controls (n=5)	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
NSC						
Total SCFA	1.07	± 0.20	1.25	± 0.16	1.62	± 0.55
C2	0.99	± 0.15	1.04	± 0.09	1.23	± 0.37
C3	0.03	± 0.03	0.08	± 0.08	0.17	± 0.11
C4	0.06	± 0.06	0.13	± 0.06	0.20	± 0.09
C2%	95.29	± 4.71	86.20	± 4.83	64.26	± 17.23
C3%	1.42	± 1.42	5.04	± 5.04	6.41	± 3.95
C4%	3.29	± 3.29	8.75	± 3.94	8.50	± 3.48

C2; acetate, C3; propionate, C4; butyrate, NSC; non-substrate control
Concentrations expressed as µmol/mL

SCFA production after 48 hours

Differences in SCFA concentrations and proportional contribution to total SCFA between substrates

Table 4.14 and Figure 4.5 both display individual SCFA concentrations and their proportional contribution to total SCFA concentration for all participant groups and substrates.

Similar to the adult cohort, the NSC fermentation and that with cellulose consistently produced significantly less total and individual SCFA ($p<0.05$). In no case was there a statistical difference between SCFA concentrations between the NSC fermentation and that with cellulose. Apple pectin produced both, a significantly higher total SCFA concentration and acetate concentration than all other substrates ($p<0.05$). With exception of the NSC and cellulose fermentations, no difference was noted in propionate concentration amongst different substrates. Regarding butyrate production, although apple pectin on average resulted in a higher concentration this was only significant when compared to fermentations with wheat-bran, NSC and cellulose.

When considering all fibres collectively, acetate, propionate and butyrate respectively contributed to an average of 60.65 ± 0.90%, 15.30 ± 0.69% and 17.46 ± 0.83% of total SCFA concentration. However, proportional contibution of the individual SCFA varied according to substrates (Table 4.13).

Differences in SCFA concentrations and proportional contribution to total SCFA between participant groups

There was large variation amongst the concentration of SCFAs for all child groups. Figure 4.5 illustrates a tendancy for total SCFA, acetate, propionate and butyrate concentrations to be highest in healthy children for most substrates; however these differences did not reach statistical significance ($p>0.05$). The exceptions to this was total SCFA and acetate concentration in response to fermentation of hi-maize, which was significantly higher in healthy controls compared to newly diagnosed patients (total SCFA; mean ± SEM, healthy controls; 57.99 ± 5.11 vs newly diagnosed; 38.89 ± 3.23, $p=0.01$, acetate; mean ± SEM, healthy controls; 32.93 ± 2.93 vs newly diagnosed; 21.41 ± 2.48, $p=0.02$) but not those who were on medication. Although not significant, there was in fact a trend for total SCFA concentration to be the highest in healthy children, lower in patients who had already been on treatment, and the lowest in newly diagnosed patients for all substrates. This trend was not as pronounced for the SCFA when considered individually.

In terms of proportional contribution to total SCFA, there were no significant differences between groups (Figure 4.6).

Table 4.14 Concentration of the major individual SCFA and their proportional contribution to total SCFA (%) after 48 hours fermentation with different fibrous substrates and faecal slurry from treatment naive CD patients (pCD), pCD patients who have received treatment, and healthy children

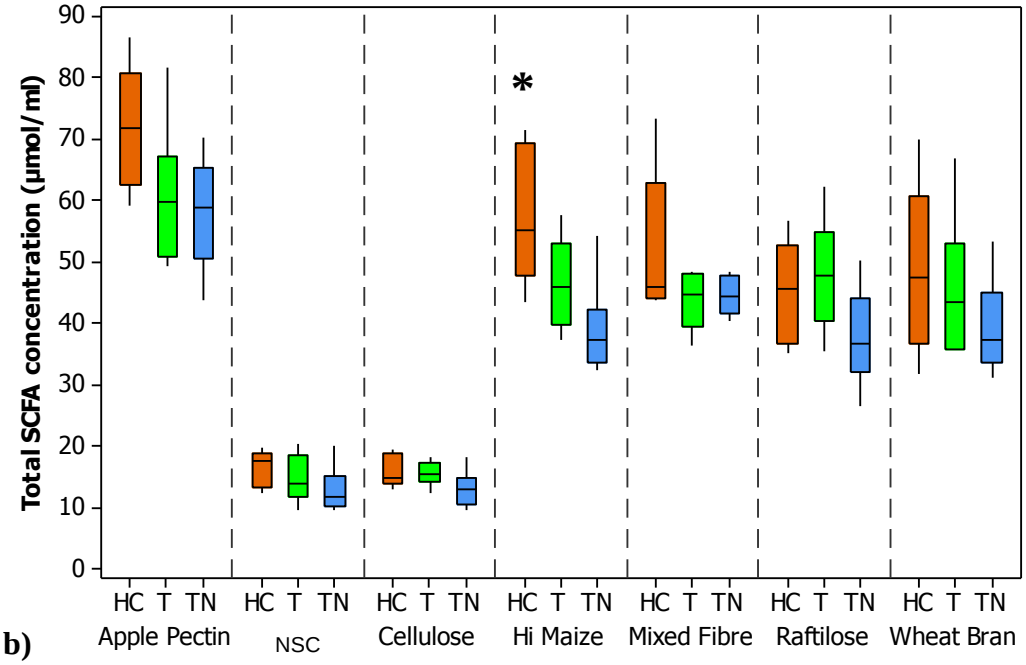
	Treatment Naive pCD (n=6)		Treated pCD (n=6)		Healthy Controls (n=5)		p-value
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	
NSC							
Total SCFA	12.85 ±	1.55	14.72 ±	1.57	16.26 ±	1.35	0.32
C2	7.33 ±	0.90	8.09 ±	0.99	9.34 ±	0.61	0.31
C3	2.00 ±	0.32	2.32 ±	0.38	2.21 ±	0.22	0.78
C4	1.59 ±	0.18	1.94 ±	0.25	2.06 ±	0.15	0.26
C2%	57.31 ±	2.83	55.58 ±	4.47	57.97 ±	2.19	0.88
C3%	15.32 ±	1.14	15.79 ±	2.50	13.64 ±	0.90	0.68
C4%	13.06 ±	1.86	13.28 ±	1.20	12.96 ±	1.33	0.99
Apple Pectin							
Total SCFA	58.07 ±	3.72	60.66 ±	4.68	71.69 ±	4.61	0.11
C2	42.62 ±	3.08	41.92 ±	3.81	47.11 ±	2.76	0.53
C3	7.27 ±	1.62	7.31 ±	1.67	11.91 ±	2.60	0.21
C4	7.35 ±	0.57	10.31 ±	1.60	11.38 ±	0.67	0.06
C2%	73.44 ±	2.56	68.91 ±	2.57	65.92 ±	1.99	0.13
C3%	12.40 ±	2.74	11.90 ±	2.79	16.01 ±	2.69	0.55
C4%	12.86 ±	1.19	17.47 ±	3.41	16.28 ±	1.83	0.38
Cellulose							
Total SCFA	12.89 ±	1.21	15.56 ±	0.83	16.08 ±	1.18	0.12
C2	7.42 ±	0.66	8.90 ±	0.61	9.34 ±	0.43	0.09
C3	2.02 ±	0.27	2.45 ±	0.39	2.36 ±	0.25	0.58
C4	1.57 ±	0.18	1.88 ±	0.20	1.91 ±	0.14	0.35
C2%	58.01 ±	2.33	57.65 ±	4.04	58.67 ±	2.42	0.98
C3%	15.37 ±	0.74	15.69 ±	2.47	14.63 ±	0.76	0.90
C4%	12.75 ±	1.89	12.28 ±	1.44	12.07 ±	1.27	0.95
Hi Maize							
Total SCFA	38.89 ±	3.23	46.42 ±	3.26	**57.99*** ±	**5.11**	0.01
C2	21.10 ±	2.48	26.50 ±	2.79	**32.93*** ±	**2.26**	0.02
C3	6.98 ±	1.57	5.83 ±	1.15	10.57 ±	3.13	0.26
C4	9.54 ±	1.49	12.61 ±	1.20	10.12 ±	1.86	0.32
C2%	53.88 ±	3.66	57.57 ±	3.78	58.41 ±	2.39	0.62
C3%	18.00 ±	4.06	11.06 ±	2.78	18.73 ±	4.56	0.40
C4%	24.92 ±	4.15	29.24 ±	3.93	14.64 ±	1.64	0.07
Raftilose							
Total SCFA	37.67 ±	3.28	47.92 ±	3.72	44.93 ±	3.83	0.14
C2	27.48 ±	5.06	27.65 ±	2.69	26.66 ±	1.79	0.28
C3	3.76 ±	1.88	6.92 ±	1.70	8.60 ±	2.65	0.98
C4	6.18 ±	2.75	13.21 ±	1.70	8.16 ±	2.84	0.14
C2%	71.02 ±	8.28	59.94 ±	3.45	60.17 ±	4.15	0.39
C3%	10.64 ±	5.13	9.95 ±	3.88	18.88 ±	5.57	0.43

C4%	17.45	±	8.22	29.91	±	3.57	17.94	±	5.87	0.43
Wheat Bran										
Total SCFA	39.28	±	3.26	45.70	±	4.71	48.48	±	6.32	0.40
C2	22.70	±	2.32	27.20	±	3.04	29.13	±	3.72	0.79
C3	8.10	±	0.72	7.03	±	1.38	9.54	±	2.00	0.47
C4	6.21	±	0.82	8.17	±	1.09	6.70	±	1.45	0.43
C2%	57.39	±	1.62	59.61	±	3.42	60.85	±	5.25	0.83
C3%	20.91	±	1.96	15.57	±	3.27	19.20	±	2.34	0.35
C4%	15.82	±	1.62	17.85	±	1.67	14.30	±	3.07	0.51
Mixed Fibre										
Total SCFA	44.53	±	1.35	43.78	±	2.03	51.98	±	5.55	0.18
C2	27.42	±	2.14	27.13	±	2.25	29.08	±	3.65	0.86
C3	7.32	±	1.73	5.69	±	1.35	9.20	±	1.99	0.38
C4	9.23	±	1.36	9.87	±	1.21	11.64	±	2.23	0.57
C2%	61.39	±	3.93	61.56	±	2.88	55.67	±	2.98	0.42
C3%	16.76	±	4.18	12.90	±	2.88	17.28	±	3.06	0.63
C4%	20.51	±	2.73	23.15	±	3.59	23.85	±	5.89	0.83

C2; acetate, C3; propionate, C4; butyrate, NSC; non-substrate control
concentrations expressed as µmol/mL
* indicates a statistically significant difference between healthy controls and newly diagnosed paediatric CD patients

Figure 4.5 Concentration of a) total SCFA, b) acetate, c) propionate, and d) butyrate following 48 hours fermentation with different fibrous substrates and faecal slurry from treatment naive CD patients (pCD), pCD patients who have received treatment, and healthy children

a)



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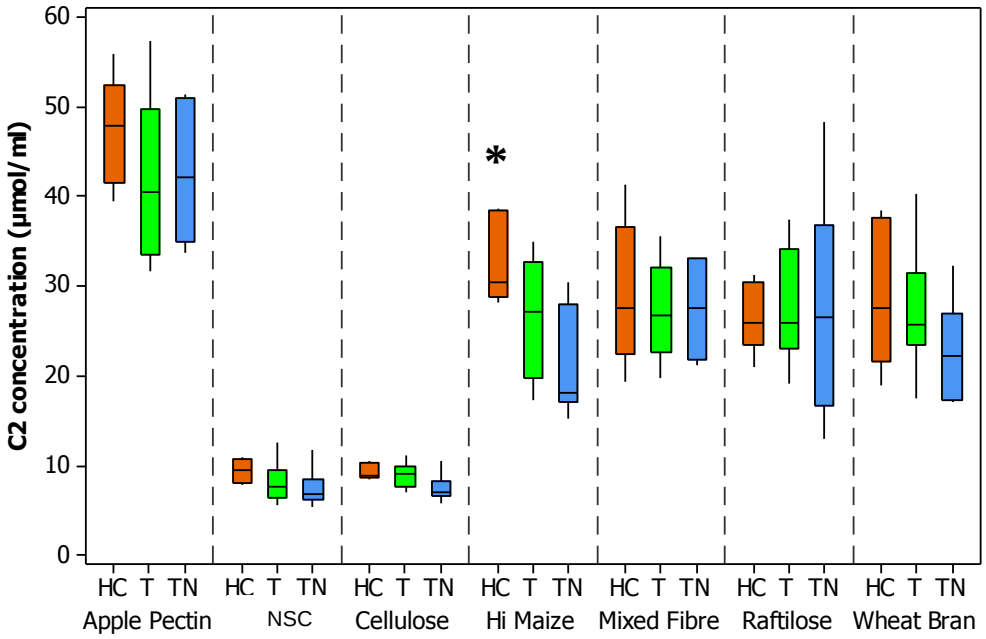

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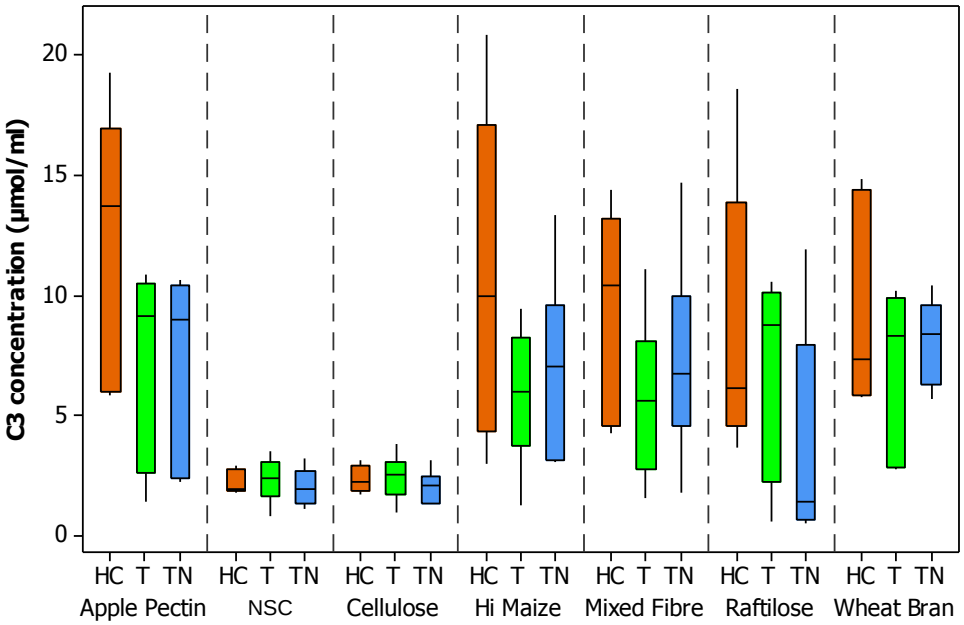

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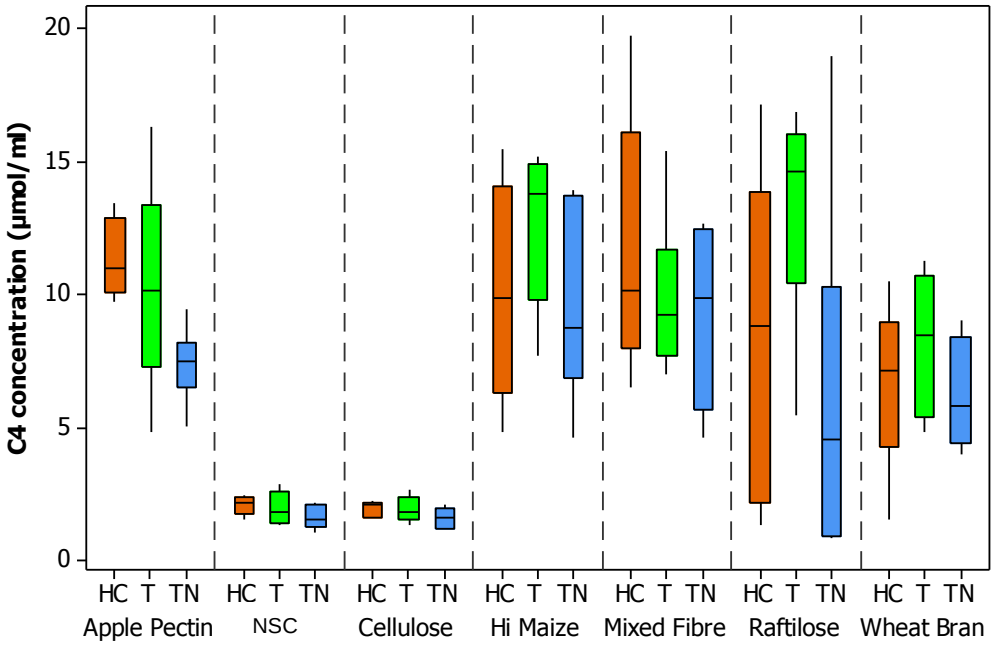
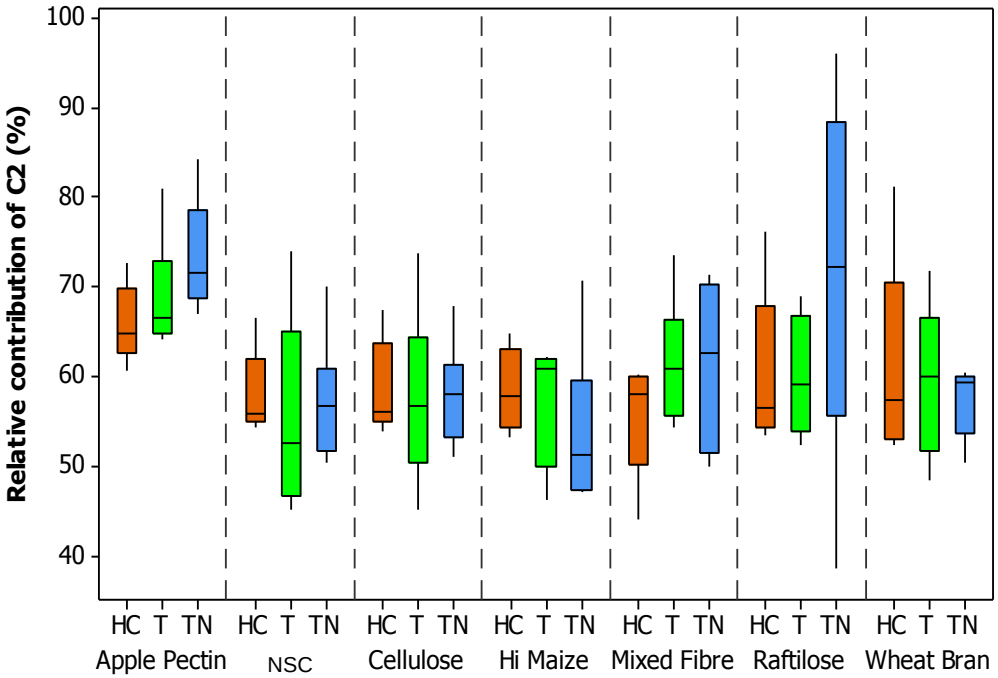


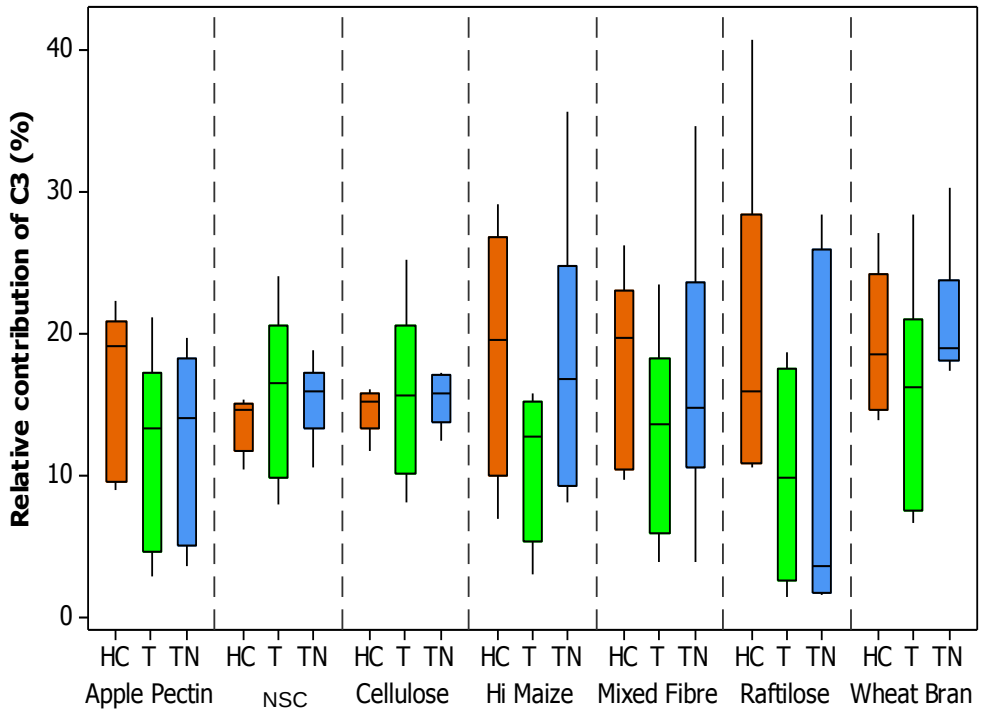
HC; healthy control, T; treated pCD patient, TN; treatment naive pCD patient, C2; acetate, C3; propionate, C4; butyrate. NSC; non-substrate control

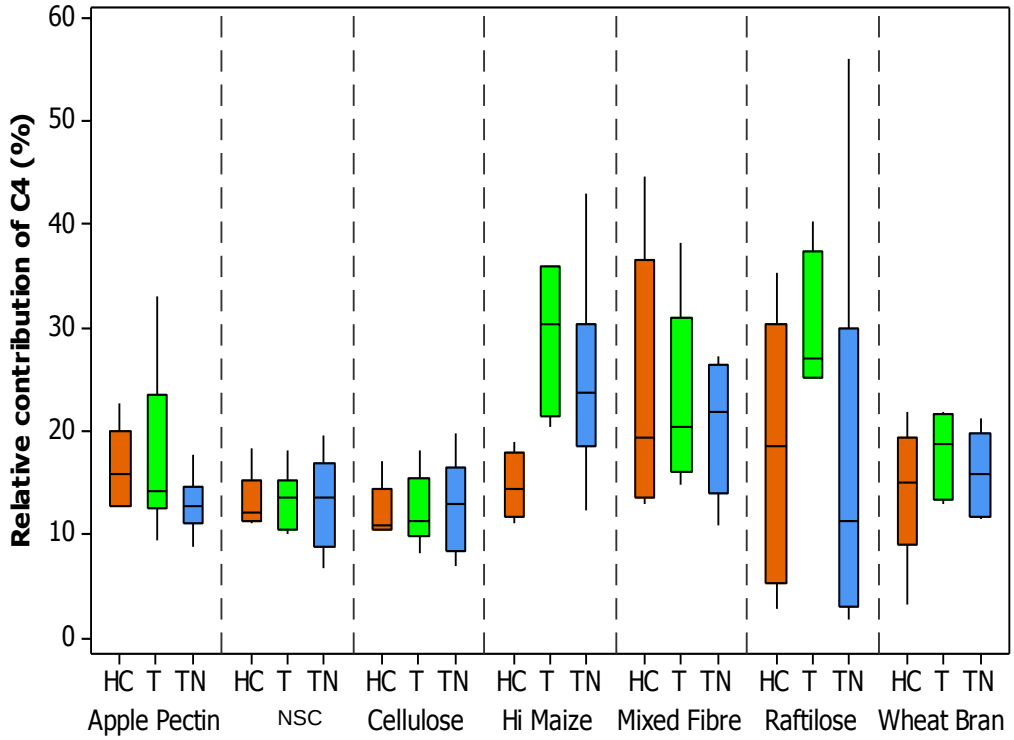
Concentrations expressed as µmol/ml

* indicates a statistically significant difference between healthy controls and newly diagnosed paediatric CD patients

Figure 4.6 Relative contribution (%) a) acetate, b) propionate and c) butyrate after 48 hours fermentation with different fibres and faecal slurry from treatment naive CD patients (TN), pCD patients who have received treatment (T), and healthy children (HC)







HC; healthy control, T; treated pCD patient, TN; treatment naive pCD patient, C2; acetate, C3; propionate, C4; butyrate. NSC; non-substrate control

4.4.5 Correlations between faecal calprotectin levels and production of SCFA after 48 hours of fermentation with different fibres

Adults

Table 4.15 and Table 4.16 display the correlations between acetate, propionate, butyrate and total SCFA production and faecal calprotectin measurements in all adult CD and UC patients respectively. No correlations were found between these measurements for any fibres for either patient group. Similarly, the proportional contribution of individual SCFA to total production was not correlated with calprotectin.

Table 4.15 Correlations of total and individual SCFA produced after 48 hours of fermentation with various fibres with faecal calprotectin measurements in adult CD patients

	All CD patients (n=13)	
	R	**p -value**
NSC		
Total SCFA	0.827	0.075
C2	0.087	0.799
C3	-0.250	0.459
C4	0.157	0.645
C2%	0.091	0.791
C3%	-0.349	0.293
C4%	0.169	0.620
Apple Pectin		
Total SCFA	-0.322	0.334
C2	-0.415	0.204
C3	-0.196	0.564
C4	-0.099	0.773
C2%	-0.136	0.690
C3%	-0.021	0.952
C4%	0.187	0.581
Cellulose		
Total SCFA	-0.097	0.778
C2	-0.056	0.870
C3	-0.415	0.205
C4	0.011	0.974
C2%	0.104	0.761
C3%	-0.402	0.220
C4%	0.164	0.630
Hi Maize		
Total SCFA	-0.389	0.237
C2	-0.347	0.296
C3	-0.235	0.487
C4	-0.273	0.417
C2%	-0.060	0.862
C3%	-0.049	0.885
C4%	0.006	0.987
Raftilose		
Total SCFA	-0.051	0.881
C2	0.173	0.611
C3	-0.236	0.485
C4	-0.366	0.268
C2%	0.398	0.226
C3%	-0.109	0.750
C4%	-0.344	0.300

Wheat Bran		
Total SCFA	-0.427	0.191
C2	-0.351	0.290
C3	-0.580	0.062
C4	-0.050	0.884
C2%	0.102	0.766
C3%	-0.300	0.370
C4%	0.236	0.485
Mixed Fibre		
Total SCFA	0.030	0.931
C2	0.030	0.931
C3	0.061	0.859
C4	0.153	0.654
C2%	-0.164	0.629
C3%	-0.019	0.955
C4%	0.290	0.387

C2; acetate, C3; propionate, C4; butyrate

R; Pearson correlation

Table 4.16 Correlations of total and individual SCFA produced after 48 hours of fermentation with various fibres with faecal calprotectin measurements in adult UC patients

	All adult UC patients (n=12)	
	R	**p -value**
NSC		
Total SCFA	0.279	0.380
C2	0.469	0.124
C3	0.046	0.887
C4	0.081	0.802
C2%	0.326	0.301
C3%	-0.293	0.355
C4%	-0.042	0.897
Apple Pectin		
Total SCFA	0.030	0.927
C2	0.092	0.775
C3	-0.363	0.246
C4	0.244	0.444
C2%	0.181	0.574
C3%	-0.442	0.150
C4%	0.194	0.546
Cellulose		
Total SCFA	0.369	0.238
C2	0.556	0.061
C3	-0.055	0.865
C4	0.224	0.483
C2%	0.371	0.235
C3%	-0.386	0.215
C4%	0.033	0.920
Hi Maize		
Total SCFA	0.475	0.119
C2	0.059	0.855
C3	-0.400	0.197
C4	-0.270	0.396
C2%	0.568	**0.054**
C3%	-0.405	0.192
C4%	0.249	0.436
Raftilose		
Total SCFA	-0.300	0.344
C2	-0.049	0.881
C3	-0.468	0.125
C4	-0.222	0.488
C2%	0.371	0.235
C3%	-0.525	0.080
C4%	-0.147	0.648

Wheat Bran		
Total SCFA	-0.079	0.807
C2	0.074	0.820
C3	-0.039	0.905
C4	-0.029	0.929
C2%	0.197	0.538
C3%	0.061	0.850
C4%	0.007	0.982
Mixed Fibre		
Total SCFA	-0.424	0.170
C2	-0.420	0.174
C3	-0.414	0.181
C4	-0.136	0.673
C2%	0.190	0.554
C3%	-0.434	0.159
C4%	0.082	0.800

C2; acetate, C3; propionate, C4; butyrate, NSC; non-substrate control
R; Pearson correlation

Children

Some significant correlations were noted between SCFA production and calprotectin in both newly diagnosed paediatric CD patients and treated paediatric CD patients. In children who were treatment naive, calprotectin showed a significant negative correlation with both total SCFA production and acetate in response to fermentation with mixed fibre (R= -0.942, p= 0.005, R= -0.864., p= 0.027, respectively). These correlations are respectively displayed in Figure 4.7 and Figure 4.9. In those who had received treatment, total SCFA production had a significant positive correlation with calprotectin measurements when following fermentation with hi maize (R= 0.860, p= 0.028, Figure 4.10). A further significant positive correlation was established between calprotectin and acetate production during the fermentation of apple pectin in treated paediatric patients (R= 0.872, p= 0.024, Figure 4.11), and with propionate production during the NSC fermentation in treatment naive children (R= 0.846, p= 0.034, Figure 4.8).

Table 4.17 Correlations of total and individual SCFA produced after 48 hours of fermentation with various fibres with faecal calprotectin measurements newly diagnosed pCD patients

	All newly diagnosed pCD patients (n= 6)	
	R	**p -value**
NSC		
Total SCFA	0.663	0.152
C2	0.432	0.392
C3	0.846	**0.034***
C4	0.223	0.671
C2%	0.610	0.199
C3%	0.173	0.742
C4%	0.353	0.492
Apple Pectin		
Total SCFA	0.771	0.073
C2	0.627	0.183
C3	0.373	0.466
C4	0.014	0.979
C2%	-0.102	0.848
C3%	0.820	0.046
C4%	-0.612	0.196
Cellulose		
Total SCFA	0.718	0.108
C2	0.462	0.356
C3	0.722	0.105
C4	0.215	0.683
C2%	0.732	0.098
C3%	0.691	0.128
C4%	-0.263	0.615
Hi Maize		
Total SCFA	0.399	0.433
C2	0.040	0.941
C3	0.810	0.051
C4	-0.137	0.796
C2%	-0.560	0.247
C3%	0.717	0.108
C4%	-0.391	0.443
Raftilose		
Total SCFA	-0.160	0.762
C2	-0.093	0.861
C3	-0.408	0.422
C4	0.226	0.667
C2%	-0.151	0.775
C3%	-0.246	0.638
C4%	0.266	0.611

Wheat Bran		
Total SCFA	-0.261	0.617
C2	-0.356	0.488
C3	0.153	0.773
C4	0.523	0.287
C2%	-0.602	0.206
C3%	0.388	0.448
C4%	0.419	0.409
Mixed Fibre		
Total SCFA	-0.942	**0.005***
C2	-0.864	**0.027***
C3	0.573	0.235
C4	-0.469	0.348
C2%	-0.594	0.214
C3%	0.632	0.178
C4%	-0.319	0.538

C2; acetate, C3; propionate, C4; butyrate, NSC; non-substrate control
R; Pearson correlation
*Indicates significant correlation

Table 4.18 Correlations of total and individual SCFA produced after 48 hours of fermentation with various fibres with faecal calprotectin measurements treated pCD patients

	All treated pCD patients (n= 6)	
	R	**p -value**
NSC		
Total SCFA	-0.410	0.419
C2	-0.435	0.388
C3	-0.110	0.836
C4	-0.295	0.570
C2%	0.110	0.835
C3%	-0.067	0.900
C4%	0.200	0.704
Apple Pectin		
Total SCFA	0.727	0.101
C2	0.872	**0.024**
C3	0.381	0.457
C4	-0.491	0.323
C2%	0.671	0.144
C3%	0.176	0.739
C4%	-7.280	0.101
Cellulose		
Total SCFA	0.535	0.274
C2	-0.149	0.778
C3	0.264	0.613
C4	0.553	0.255
C2%	0.354	0.491
C3%	-0.181	0.732
C4%	-0.136	0.797
Hi Maize		
Total SCFA	0.86	**0.028**
C2	0.715	0.11
C3	0.458	0.361
C4	0.086	0.871
C2%	0.274	0.726
C3%	0.66	0.340
C4%	-0.869	0.131
Raftilose		
Total SCFA	0.708	0.116
C2	0.751	0.085
C3	0.326	0.529
C4	0.018	0.097
C2%	3.589	0.411
C3%	0.301	0.699
C4%	-0.930	0.070
Wheat Bran		
Total SCFA	0.306	0.556

C2	0.400	0.432
C3	0.194	0.713
C4	0.074	0.889
C2%	0.291	0.576
C3%	-0.009	0.987
C4%	-0.385	0.451
Mixed Fibre		
Total SCFA	0.614	0.194
C2	0.759	0.080
C3	0.163	0.758
C4	-0.694	0.126
C2%	0.738	0.094
C3%	0.089	0.867
C4%	0.757	0.081

C2; acetate, C3; propionate, C4; butyrate, NSC; non-substrate control
R; Pearson correlation

Figure 4.7 Pearson rank correlation between faecal calprotectin and acetate production following fermentation with mixed fibre by gut microbiota of newly diagnosed pCD patients

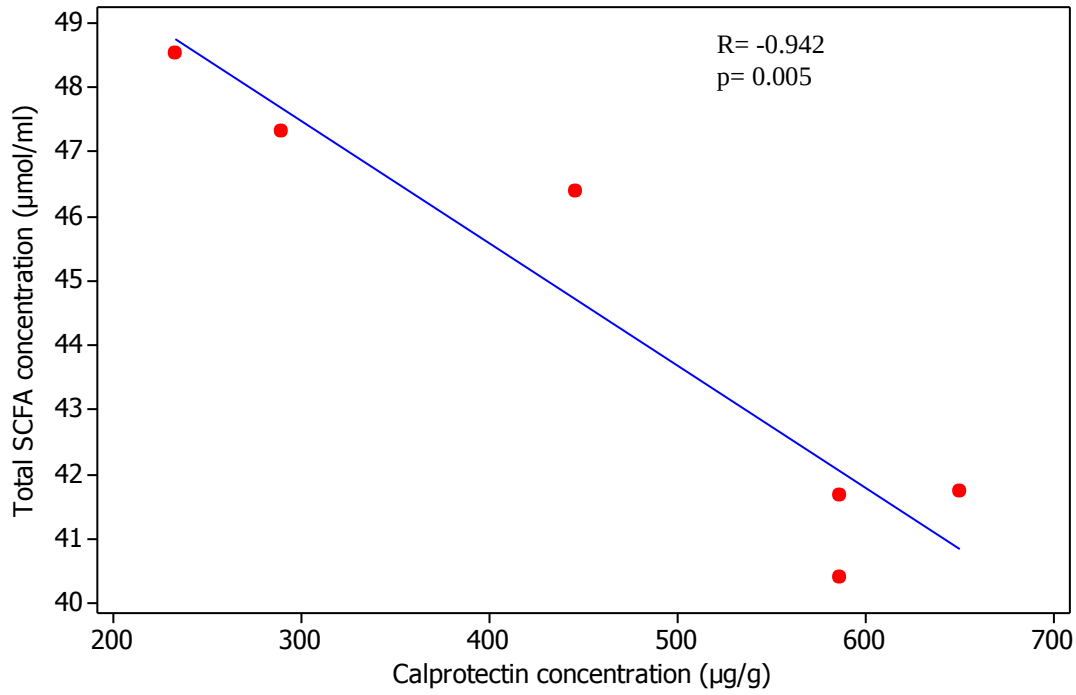


Figure 4.9 Pearson rank correlation between faecal calprotectin and acetate production following fermentation with hi maize by gut microbiota of newly diagnosed pCD patients

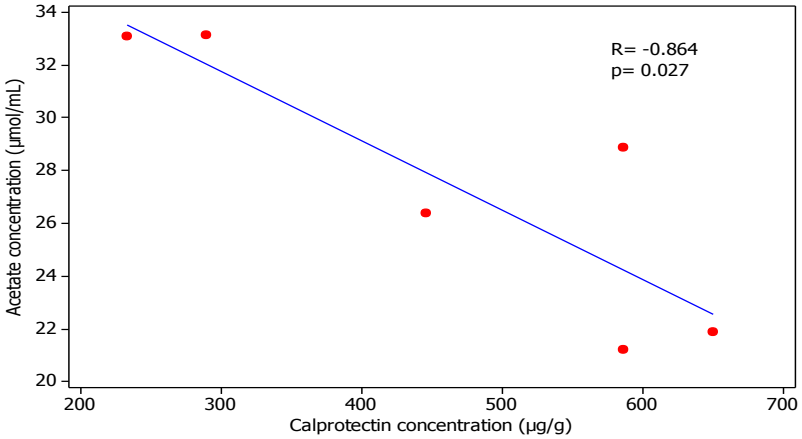


Figure 4.8 Pearson rank correlation between faecal calprotectin and propionate production following fermentation by gut microbiota of newly diagnosed pCD patients

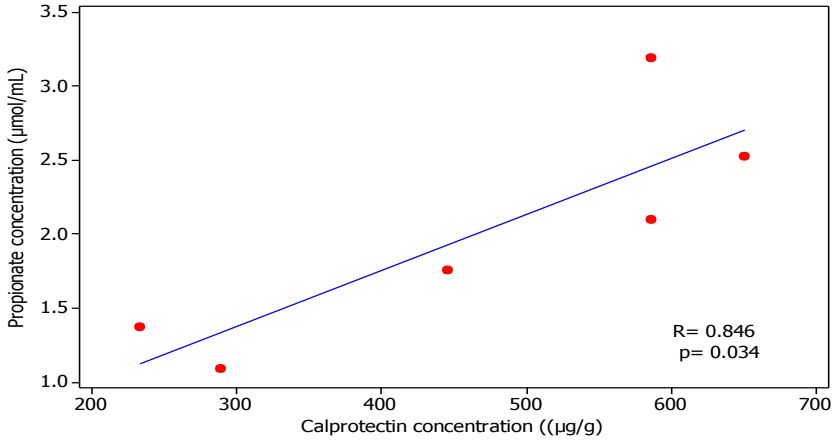


Figure 4.11 Pearson rank correlation between faecal calprotectin and acetate production following fermentation with apple pectin by gut microbiota of treated pCD patients

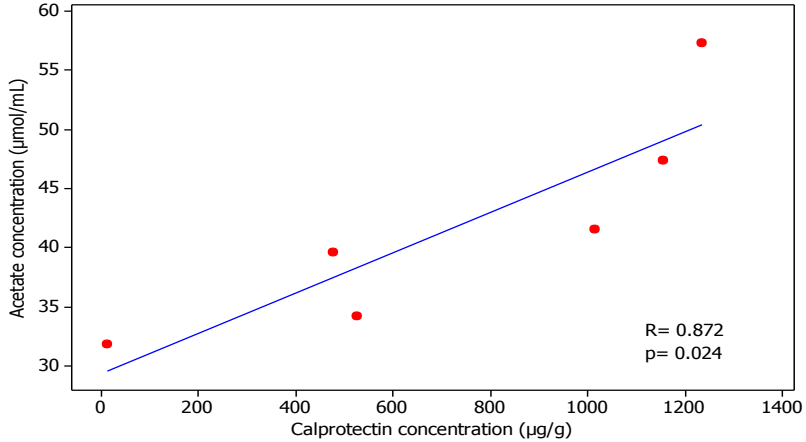
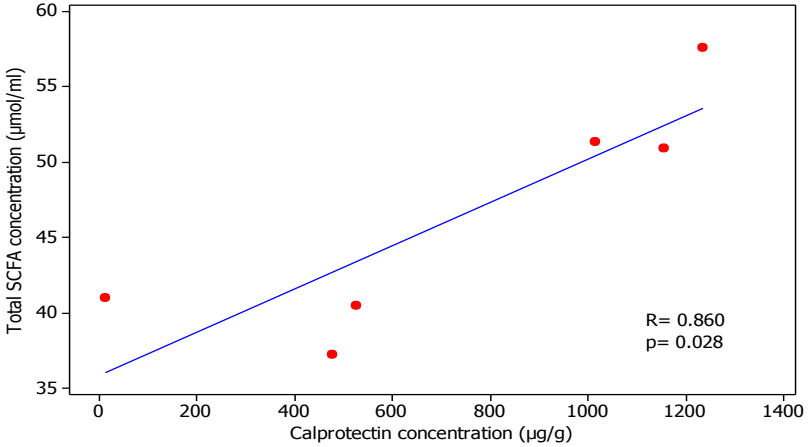


Figure 4.10 Pearson rank correlation between faecal calprotectin and total SCFA production following fermentation with mixed fibre by gut microbiota of treated pCD patients



4.4.6 *In vitro* fermentations *sans* human gut microbiota

4.4.6.1 pH of culture pre- and post-48 hours fermentation with different substrates

Four control *in vitro* fermentations were conducted. The pH of these experiments recorded pre- and post-fermentations can be viewed in Table 4.19.There were no significant differences in pH of the different substrates recorded at baseline ($p<0.05$). However at 48 hours, the fermentations with apple pectin and the mixed fibre were significantly lower than that recorded for all other substrates, whilst those with cellulose, hi-maize and no fibre were significantly higher after 48 hours compared to baseline ($p<0.05$).

4.4.6.2 Volume of expired gas following 48 hours fermentation with different substrates

As shown in Figure 4.12, the majority of fibres did not result in a high production of gas over the 48 hour time period (less than 10mL); however fermentations with apple pectin, mixed fibre and wheat-bran produced significantly more gas than all other substrates (median (IQR) apple pectin; 16.50 (6.625), mixed fibre; 23.50 (13.75), wheat-bran; 12.00 (8.00)).

4.4.6.3 Production of SCFA prior to and following 48 hours in vitro fermentation with different substrates

At baseline, no SCFA production was detected from any of the fibres investigated, including the fibre-free control.

The production of SCFA after one *in vitro* fermentation without human gut microbiota is shown in Table 4.20. As only one sample from each condition was analysed, statistical analysis was not conducted. As shown, fermentation with neither the fibre-free control (NSC) or hi maize produced any SCFA *in vitro.* Acetate was the only SCFA produced during fermentations with apple pectin (3.01 µmol/ml), raftilose (2.95 µmol/ml), cellulose (0.93 µmol/ml) and the mixed fibre (11.51 µmol/ml); however these values were negligible. The only other SCFA produced during these fermentations was butyrate in response to wheat bran (1.90 µmol/ml), which contributed 35.97% to total SCFA production, whilst acetate was responsible for 64.03% (3.39 µmol/ml).

Table 4.19 pH of ferment before and after 48 hour fermentation period with different fibre substrates

	0hr (n=4)		48hr (n=4)	
Substrate	**Median**	**IQR**	**Median**	**IQR**
NSC	7.04	0.27	**7.38***	0.25
Hi Maize	7.01	0.17	**7.27***	0.17
Apple Pectin	7.05	0.79	**6.05***	0.73
Raftilose	7.09	0.33	7.16	0.47
Wheat Bran	7.01	0.25	6.76	0.39
Cellulose	7.06	0.24	**7.24***	0.25
Mixed Fibre	7.07	0.28	6.19	1.59
Butyrate Ester	7.09	0.32	7.16	0.31
Propionate Ester	7.05	0.33	7.23	0.32

* indicates statistical difference between pH recorded at 0 and 48 hours

Figure 4.12 Volume of expired gas (ml) following 48 hour fermentation period with different substrates

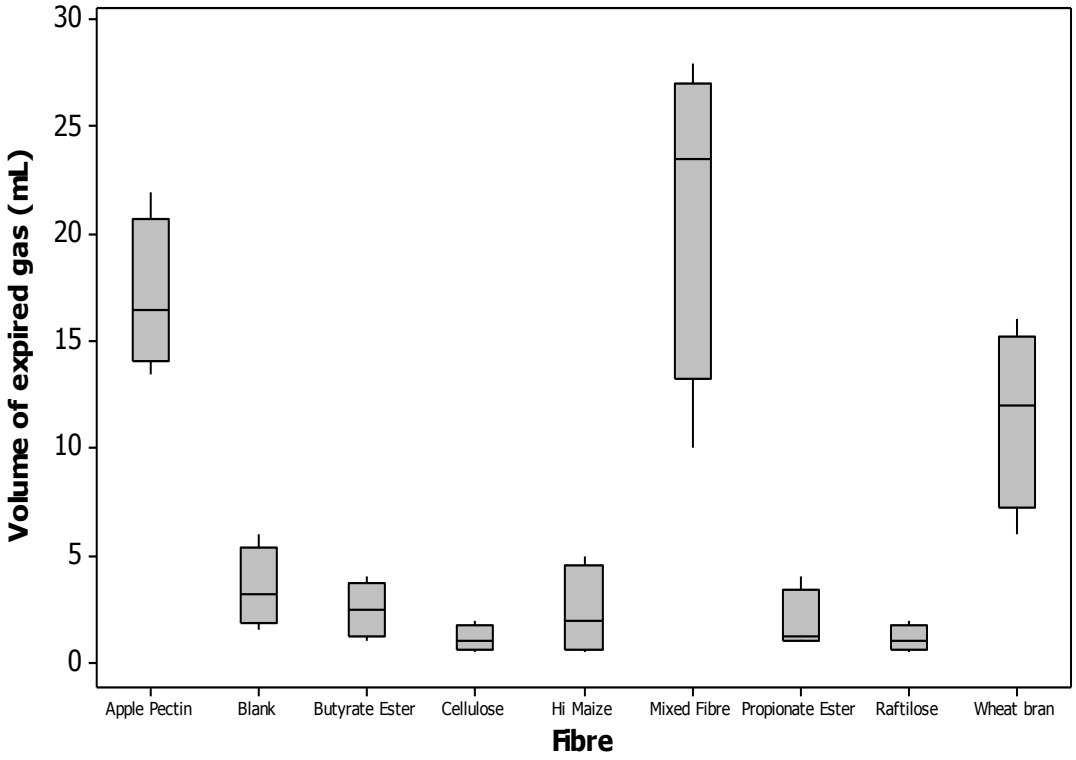


Table 4.20 Production of SCFA (µmol/mL) following 48 hours *in vitro* fermentation with different substrates but no human gut microbiota

	Total SCFA	Acetate	Propionate	Butyrate	% Acetate	% Propionate	% Butyrate
NSC	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Hi Maize	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Apple Pectin	3.01	3.01	0.00	0.00	100.00	0.00	0.00
Raftilose	2.95	2.95	0.00	0.00	100.00	0.00	0.00
Wheat Bran	5.29	3.39	0.00	1.90	64.03	0.00	35.97
Cellulose	0.93	0.93	0.00	0.00	100.00	0.00	0.00
Mixed Fibre	11.51	11.51	0.00	0.00	100.00	0.00	0.00

Concentrations expressed as µmol/ml

4.5 Discussion

The primary aim of this study was to investigate the ability of the gut bacteria of IBD patients to ferment various fibres and produce SCFA *in vitro*. The results of this study suggest that there was a tendency for reduced SCFA production, particularly total SCFA and butyrate, in adult IBD patients and paediatric CD patients.

An interesting finding within the adult cohort was the significant reduction in total SCFA concentration in UC patients and both UC and CD patients when fermented alongside hi maize and raftilose, respectively. These highly fermentable fibres are known prebiotics and hence stimulate the growth of beneficial organisms such as bifidobacteria and lactic acid bacteria [111]. This positive manipulation of the colonic flora subsequently enhances total SCFA [207] and butyrate [208] [209] production in healthy individuals. Butyrate yield was also significantly reduced in UC patients compared to healthy participants when fermented alongside mixed fibres. As the SCFA which has most relevance to colonic health, this reduction in *in vitro* butyrate production by the microbiota of UC patients may partly explain the exaggerated immune response characteristic of the disease. A reduction in these important metabolites in IBD may suggest that the microbiota of patients cannot efficiently metabolise dietary fibres, including prebiotics, and subsequently colonic cells will not fully benefit from the immunomodulatory [210], anti-inflammatory [88] and energy-rich properties [143] of SCFA. The use of prebiotics in IBD has been explored in only a few studies, most of which have reported therapeutic effects in both CD [130, 158] and UC [154] in terms of functional symptoms and inflammation markers. However, this evidence is preliminary and it is important to understand the mechanisms behind the potential medicinal properties of prebiotics. The hindered production of SCFA in response to prebiotic fermentation in IBD patients seen in this study indicates an inefficiency of the innate gut bacteria. It is likely that these bacteria therefore need extra stimulation to help enhance their reduced functional capacity, which may explain why the dietary administration of prebiotics has been known to improve disease activity. A potential issue in the dietary supplementation of fibre in IBD is the causation of excess gas as a by-product of fermentation, which can cause abdominal distension and discomfort. However, there was no difference in the production of gas in response to *in vitro* fermentation of the fibres between patients and controls, implying that these fibres are no less tolerated by patients. Therefore, the results of the current study therefore highlight the importance of fibre in the diet of adult UC and CD patients in remission. Current daily guidelines for fibre consumption in the United Kingdom is 30

grams, however this is not achieved by the majority of adults, with an average intake of 18 grams per day [211]. Although to the knowledge of this author there has been no study regarding dietary intake in IBD patients in specific, it is likely that the majority do not achieve recommended values. Therefore, the importance of dietary fibre, particularly fermentable, should be stressed not only to the general population but to IBD patients in remission to ensure that they can maximise the benefits despite the reduced capabilities of their gut bacteria.

A further aim of this study was to explore any differences in the functional capacity of the gut bacteria of newly diagnosed paediatric patients with CD who were treatment naive to both those on medication and to healthy children. Total SCFA production tended to be reduced in newly diagnosed patients compared to both healthy controls and those who were on treatment; however this was only significant in the case of hi maize, which produced higher total SCFA and acetate concentrations when fermented with faecal slurry from healthy controls than with that from treatment naive patients. This information correlates with other studies which have found a reduction in faecal SCFA in newly diagnosed paediatric CD patients compared to healthy controls [122]. It is clear that the aforementioned anti-inflammatory properties of SCFA are somewhat demoted in active IBD, and the results of this study and others suggest that this may stem from a reduced production of these beneficial anions in the diseased state. Whether this reduction is a cause or effect of the disease is not clear, however Gerasimidis et al [122] reported that EEN reduces faecal butyrate concentrations despite inducing remission, implying that diminished SCFA cannot be the sole perpetrator of the disease, and is rather a collateral impact of inflammation. If disease activity played a role in SCFA production, we would expect to see a difference between newly diagnosed paediatric patients who have active disease, and those on medication. Unfortunately, the paediatric cohort of this study was small and therefore likely to be underpowered, which may explain the lack of significant results reported. However, there was evidence of a trend of increased total SCFA and butyrate production in treated patients compared to those who were treatment naive, particularly for raftilose and wheat bran. This apparent restoration of SCFA production with medication is in agreement with other studies that imply that faecal concentrations of SCFA is inversely associated with inflammation [128]. However, patients with active disease are likely to suffer from diarrhoea, which would dilute the faecal concentrations of SCFA. Thus, the batch culture *in vitro* system was employed in this study in order to assess the ability of the gut microbiota when stimulated with fibre, mimicking colonic activity. Although this technique was still based on faecal samples, the well-developed *in vitro*

methodology described in the Chapter Two of this thesis, provides a sounder representation of the capabilities of the bacteria in a controlled environment [178]. Nevertheless, bacterial load is also compromised in diarrhoea samples, and this may contribute to the reduction in fermentation capacity seen in the newly diagnosed patients of this study. Evaluating baseline bacterial load is therefore advisable in future studies in order to assess its impact on subsequent SCFA production.

With their known medicinal benefits, the production of SCFA in IBD patients has been of scientific and clinical interest for many years. Many studies have explored differences in the concentration of SCFA between IBD patients and healthy controls, with many reporting a reduction in the diseased state [128, 131, 132, 201, 202]. However, as mentioned, alternate reports have highlighted that disease reporting that that UC patients with moderate [128] and quiescent [202] disease have similar faecal SCFA concentrations to healthy controls. Although not significant, the paediatric patients on medication in the current study tended to have an increased *in vitro* ability to produce SCFA compared to those with active disease. Although the UC and CD adult patients in the current study did display a reduced capacity to produce SCFA *in vitro* in response to fermentation with hi maize and raftilose, a more pronounced reduction may have been apparent in patients with active disease. Although a high percentage of CD patients in the current study (46%) had abnormally high calprotectin values, they had been identified as being in clinical remission based on their current symptoms. However, it is universally acknowledged by clinicians and scientists that clinical remission does not necessarily correlate to subclinical mucosal inflammation levels [212]. Furthermore, there is debate regarding what the cut-off should be when evaluating inflammation using calprotectin [213]. This study used a cut-off of 40 µg/g based on the manufacturer's guidelines, however studies have found that over 50% of patients who have achieved clinical remission still have faecal calprotectin over 150 µg/g [213-215]. It is out with the scope of this thesis to evaluate the validity of using calprotectin versus traditional disease scores in the assessing disease activity. Thus, despite high calprotectin values, the patients of this study are still considered to be in clinical remission.

As a marker of inflammation and disease severity, it could be expected that an increase in calprotectin would result in a reduced SCFA production (or perhaps vice versa) due to their contrasting relationship with inflammation. In this study, baseline calprotectin measurements were in some cases correlated with SCFA production, more so in the paediatric patients than in the adult participants. However, this was not always a negative correlation as may be expected. Similar results were published by Gerasimidis et al [122]

who documented a reduction in both calprotectin and butyrate production in paediatric CD patients throughout their course of treatment on EEN. The absence of fibre in the polymeric formula undoubtedly accounts in part for the reduction in butyrate seen. Fibre may aggravate colonic symptoms [206] via the stimulation of colonic bacteria, subsequently activating the immune system of CD patients [216], and therefore the reduction in calprotectin may also be attributed to the absence of dietary fibre whilst on EEN. Alternatively, the anaerobic fermentation of fibre may produce harmful metabolites that contribute to active disease. Therefore, although fibre is generally perceived as health-promoting, its involvement in active IBD may have detrimental consequences. As aforementioned, a more pronounced difference may have been noted between SCFA in healthy controls and adult UC and CD patients with active disease; however, fibre supplementation during flare-ups is not necessarily beneficial even if it does enhance SCFA production. Certainly, the results of Gerasmidis et al' study indicate that the therapeutic benefits of butyrate can be foregone in the treatment of paediatric disease [122]. It is therefore more likely that the supplementation of dietary fibre and subsequent SCFA production is important for the maintenance of intestinal homeostasis during remission.

A recent investigation of fibre usage in quiescent UC reported that despite having a similar dietary intake, faecal concentrations of starch and non-starch polysaccharides were significantly higher in patients compared to healthy participants [202]. Whilst this may imply a reduction in fermentation capacity, concentration of SCFA and other metabolites was not different between groups. The discrepancies within these findings could not be explained by whole gut transit time and the authors therefore credited an abnormality of fibre usage in UC patients rather than a reduction. Therefore, this study does not strongly support the notion that SCFA production is drastically reduced in IBD. There was a lack of statistical significance regarding the *in vitro* SCFA production in the study presented and whilst this may be due to insufficient power, it may also reflect that SCFA production is not impaired in IBD. It is plausible that CD and/or UC patients suffer from an inability to efficiently utilise SCFA despite being able to produce them. Indeed, studies have indicated that butyrate oxidation by the colonic cells of UC patients is significantly impaired compared to healthy cells [142-144] and this was not overcome with increasing butyrate availability [144], which may explain why some studies have found that butyrate enemas do not improve the symptoms of UC [136, 141]. Therefore, the issue of SCFA in IBD may not be their production, but in fact their usage.

Nevertheless, the results of this study imply that microbial fermentation capacity and subsequent SCFA production is compromised in both quiescent and active IBD. However the implications of this may depend on disease activity. Patients in remission would likely benefit from consuming the recommended daily intake of dietary fibre in order to maintain intestinal homeostasis via the stimulation of bacterial activity and SCFA production. However, as those with active disease benefit from the elimination of fibre from the diet, the promotion of SCFA should be secondary to treating inflammation. This, and the persistence of a reduced *in vitro* production in remission implies that although SCFA production is an issue in IBD, it is not the direct cause of disease. Further information is needed to understand why, despite its health-promoting qualities, the elimination of dietary fibre is beneficial in active disease and therefore other metabolites of anaerobic fermentation should be investigated as possible perpetuators of disease.

Chapter 5 : The relationship between gut microbial composition and IBD following *in vitro* fermentation with dietary fibre

5.1 Chapter Outline

There is growing interest in the involvement of the gut bacteria in IBD. This chapter will explore differences in the gut bacteria present in IBD patients and healthy controls both prior to and after *in vitro* fermentations with various fibres. We aim to characterise not only the bacteria associated with the diseased states, but also to identify any bacterial patterns related to the different substrates.

5.2 Introduction

It was as early as 400 BC when Hippocrates stated that "*death sits in the bowels*", yet the weight of this statement is only recently becoming understood by scientists and clinicians. With the growth of metagenomics databases and molecular techniques which are continuously improving scientific understanding of the extensive range of gastrointestinal bacteria, there has been much research in the role of this micro-community in human health. This expansive, heterogeneous and dynamic community is comprised of trillions of microbes that, in a healthy GIT, maintain a symbiotic relationship with their host. However, dysbiosis can have serious consequences and has been linked to multiple disease states [59, 72, 73, 217]. Whilst there is scientific evidence to suggest that IBD has genetic origins [18, 20], the rapid global growth of both UC and CD cannot be attributed to genetics alone [4], and the gut bacteria are now considered to play a large causative role in these conditions. Indeed, multiple studies have demonstrated that mice which have been reared in germ-free conditions are void of colonic disease, yet develop intestinal inflammation on exposure to bacteria [117, 118, 218], highlighting the importance of such organisms in disease onset.

It is not yet fully comprehended how this community interferes with host health in the onset of IBD. The human GIT burdens the responsibility of protecting both intestinal tissues and the systemic circulation from luminal pathogens, and has direct contact with the bacteria that reside within it. There is a strong scientific notion that the enteric flora of IBD patients aggravates the intestinal response resulting in exaggerated inflammation [27, 219]. Whether this is due to the presence of "bad" bacteria, an inappropriate immune response to harmless species, or the combination of both is yet to be fully elucidated. Medical

research is currently attempting to profile the gut flora of IBD patients in the hope of identifying key bacterial players involved in its pathogenesis. Although results are varied in terms of specific bacteria, there is a general agreement that the complexity of the gut bacterial profile is reduced in both CD [86, 129, 131, 220]and UC [125, 128, 131, 132, 220] patients. A study by Frank et al (2007) [220] used 16S rRNA sequencing to investigate bacterial species extracted from intestinal biopsies of UC (n=55) and CD (n=35) patients, comparing them to healthy controls (n= 34). The authors suggested that there were two main groups based on the presence of specific operational taxonomic units (OTU); "normal" and "IBD specific". The IBD specific group contained only one healthy sample yet was otherwise comprised of IBD biopsies, whilst the normal group included all other healthy samples, as well as colitis samples. These groups differed significantly, with reductions in both the Bacteriodetes and the Firmicutes phyla in the IBD specific group. Similarly, an alternative study reported that although 43 distinct Firmicutes species were associated with the faecal microbiome of healthy participants, only 12 were identified in CD patients [221]. This reduction in microbial diversity, particularly beneficial bacteria, may allow the overgrowth of pathogenic strains such as sulphate-reducing bacteria and adherent-invasive E Coli [125], both of which have been found to be more prevalent in IBD patients [125, 222].

However, the aetiology of IBD is thought to be a multifactorial and whilst gut bacteria appear to be disturbed in such patients, there is evidence to suggest that the interaction of these indigenous bacteria with external environmental factors, such as diet, is influential. A crucial role of the colonic bacteria involves the breakdown of dietary substances that are otherwise undigested by the enzymes of the GIT. These substances are usually a type of dietary fibre, a term which encompasses resistant starch and prebiotics as well as other non-starch polysaccharides. Whilst some gut bacteria are termed as "generalists" due to their ability to hydrolyse multiple different substrates, other species are more specific in terms of their fuel choice. These "specialist" bacteria have a limited resource of CAZymes which are responsible for the breakdown of fibre, whilst generalist species have a much more extensive repertoire [43]. Thus, the type of fibre that enters the colon dictates the bacterial response and in this way, the composition of our gut inhabitants can be manipulated to some extent. Both *in vivo* and *in vitro* studies have highlighted the potential to which specific fibres support the growth of particular bacteria. To this effect, prebiotics, such as fructooligosaccaride and resistant starches, have been developed with the aim of improving gut health via the stimulation of *Bifidobacteria* and *Lactobacillli* growth.

If, as research suggests, gut bacteria can be promoted or demoted in response to certain fibrous stimuli, it is plausible that the dysbiosis stigmatised with IBD patients could be rectified in response to dietary fibre. Clinical trials which have investigated this in both UC and CD patients have reported therapeutic effects on dietary fibre supplementation which coincided with improvements in microbial profile [130, 154-158]. The administration of 15g/d of FOS to 10 patients with moderately active CD reduced disease activity, enhanced faecal bifidobacterial counts and increased the production of the anti-inflammatory cytokine IL-10 in the lamina propria, indicating that the interaction between fibre and bacteria influences immune response [158]. However, an alternative study in 103 active CD patients that supplemented the same dosage of FOS for 4 weeks did not report any improvements in disease activity, nor any change in microbial composition [204].

Indeed, there is further preliminary evidence to suggest that the gut bacteria of IBD patients are less proficient in the hydrolysis of dietary fibre compared to healthy counterparts. James and colleagues (2015) recently described fibre usage in UC patients as abnormal compared to healthy counterparts in an 8-week cross-over trial [202]**.** The authors suggested that, despite a decreased habitual fibre intake, UC patients displayed an increased faecal concentration of both starch and non-starch polysaccharides and this was not corrected by increasing fibre intake. Although this study discredited the effect of gut transit time on this increased fibre output, indicating that fibre usage in UC is potentially impaired by bacterial inefficiencies, they did not report a difference in metabolite production. These paradoxal findings complicate our understanding of bacterial fermentation capacity and IBD.

The analysis of gut bacteria and their response to dietary fibre is hard to study *in vivo* due to practical issues regarding timely access to the colon. Therefore, *in vitro* systems have been developed to mimic the colonic environment in order to study this micro-community. Using this system, it is possible to investigate the bacterial response to the fermentation of various fibre, and to compare these results between participant groups. This study primarily aims to characterise the baseline microbial composition of CD patients, UC patients and matched healthy controls. Furthermore, this study aims to investigate the bacterial response to various fibrous stimuli, and to gauge whether this is either compromised in the disease state, or if in fact the provision of fibre improves the microbial profile of IBD patients.

5.3 Subjects and Methods

Participants of this study included adult CD and UC patients in remission and healthy controls matched to patients in terms of age, gender and BMI. They were recruited as described in Chapter 2. Due to time restraints and cost of sequencing, DNA analysis was only conducted on a subset of participants (8 CD patients, 8 UC patients and 8 healthy controls).

All participants were required to donate a single stool sample, which was processed as explained in Chapter 2.4. In summary, these samples were fermented for 48 hours alongside various fibres using a batch culture *in vitro* fermentation method. A NSC (fibre-free) fermentation was also conducted for each sample. Prior to the 48 hour incubation, 3 x 1.5 mL of faecal slurry was extracted from the NSC fermentation vessel using a sterile syringe and needle. This was repeated following the 48 hour time period, however at this time point aliquots were extracted from all fermentation vessels containing fibres as well as the NSC vessel. These aliquots were stored at -80˚C until further analysis.

Prior to DNA extraction, the faecal slurries were freeze dried to remove water content. Following this, the chaotropic method of DNA extraction was used to isolate and purify DNA from the samples, as outlined in Chapter 2.4.4. The quality of this DNA was assessed using spectrophotometry and fluorometry, prior to amplification using PCR. The amplified PCR products were separated by gel electrophoresis, after which the band representing the DNA amplified from the samples was extracted, washed and eluted using a commercial kit. The final barcoded amplicons of all samples were diluted to a final concentration of 1.5ng/µL before pooling 5µL of all samples together in one 2mL Eppendorf. This final pool was secured in parafilm and packaged in dry ice before being sent to an external laboratory (MicrobesNG, University of Birmingham) for 16S sequencing.

5.3.1 Statistical Analysis

The bioinformatics analysis of the sequencing was conducted by Mr Christopher Quince and Mr Asker Brejnrod at the University of Warwick who kindly constructed Shannon diversity plots and principal component analysis plots. Statistical analysis, where possible, was conducted using R. Changes in microbial diversity pre and post-fermentation were analysed using a one-sample t-test whilst changes in phyla and community structure were considered using a paired Wilcoxin test. Participant characteristics were analysed using

Minitab Version 16.2.2 statistical software (Pennsylvania State University, Pennsylvania, USA).

5.4 Results

5.4.1 Anthropometric characteristics of the IBD patients and healthy controls of whom DNA analysis was conducted

The characteristics of the patients and healthy controls of whom DNA analysis was conducted on is displayed in
Table 5.1. The gender balance was equal for all participant groups, and there were no significant differences in age or BMI between the three groups.

Table 5.1 Characteristics of adult IBD patients and healthy controls of whom DNA analysis was conducted, recorded at the time of recruitment.

	Males		**Females**		**Males and Females**	
	Median (IQR)		**Median (IQR)**		**Median (IQR)**	
CD Patients						
	n=4		**n=4**		**n=8**	
Age	31.3	(2.2)	31.3	(2.1)	31.3	(1.4)
BMI (kg/m^2)	24.0	(1.0)	25.4	(4.1)	24.7	(2.0)
UC Patients						
	n=4		**n=4**		**n=8**	
Age	35.0	(6.4)	46.0	(6.9)	40.5	(4.8)
BMI (kg/m^2)	25.9	(0.9)	23.4	(1.1)	24.6	(0.8)
Healthy Controls						
	n=4		**n=4**		**n=8**	
Age	33.9	(3.2)	41.0	(6.5)	37.4	(3.6)
BMI (kg/m^2)	25.6	(1.2)	25.0	(2.3)	25.3	(1.2)

5.4.2 Microbial diversity of healthy controls, CD and UC patients pre- and post-*in vitro* fermentation

Microbial diversity of participants was considered using the Shannon Diversity Index, which is a measure of bacterial richness and evenness. Due to the small sample size,

statistical analysis was not conducted on the differences in diversity between microbial communities of the three participant groups; however descriptive statistics can be explored. Statistical analysis was, however, conducted on pre- and post-fermentation diversity (Table 5.2 p-values representing the change in microbial diversity following 48-hour fermentation with various substrates in UC patients, UC patients and healthy controls

5.4.2.1 Baseline microbial diversity of CD patients, UC patients and healthy controls

Figure 5.1 illustrates diversity both pre- and post-fermentation. When focusing on the pre-fermentation values, it appears that healthy controls tend to have a higher diversity index (range: 1.80- 4.50) compared to both UC (range: 2.60 – 3.60) and CD (range: 1.25- 4.00) patients.

5.4.2.2 Microbial diversity of CD patients, UC patients and healthy controls following 48 hours in vitro fermentation with various fibrous substrates

Figure 5.1 (a-f) displays the Shannon diversity index of microbial communities before and after *in vitro* fermentation with different substrates for all participant groups. The p-values for these changes are outlined in Table 5.2. As shown, the Shannon diversity index appears to be reduced in the majority of participants following fermentation with hi maize (87.5% CD patients, 100% UC patients, 100% healthy controls), pectin (87.5% CD patients, 100% UC patients, 87.5% healthy controls), raftilose (87.5% CD patients, 100% UC patients, 100% healthy controls), and mixed fibre (87.5% CD patients, 100% UC patients, 100% healthy controls). These reductions were only significant for specific fibres in CD patients (hi maize ($p = 0.008$), mixed fibres ($p = 0.030$)) and healthy controls (hi maize ($p = 0.005$), mixed fibre ($p = 0.050$), apple pectin ($p = 0.033$), raftilose ($p = 0.043$)). In no case was the change in microbial diversity significantly altered in UC patients. Fermentation with wheat bran did not have a distinct effect on the microbial diversity in any group whilst there was an apparent increase in Shannon diversity in the non-fibre control (NSC) fermentation for 87.5% healthy controls, 75% of CD patients and 87.5% UC patients; however this was not significant (healthy controls; $p = 0.731$, CD patients; $p = 0.990$, UC patients; $p = 0.089$).

As previously discussed, the baseline diversity of IBD patients appeared lower than that of healthy controls (Figure 5.1). Following fermentation with raftilose ((range) CD: 2.0-3.5, UC: 2.3-3.35, healthy control: 2.5-3.6), apple pectin, ((range) CD: 1.7-2.8, UC: 1.7-3.3, healthy control: 2.2-3.4), hi maize ((range) CD: 2.4-3.4, UC: 1.4-3.3, healthy

controls: 1.75-3.75) and mixed fibres ((range) CD: 1.6-3.4, UC:1.4-3.3, healthy control: 1.8 – 3.9) the diversity of the three participant groups became less distinct (Figure 5.1).

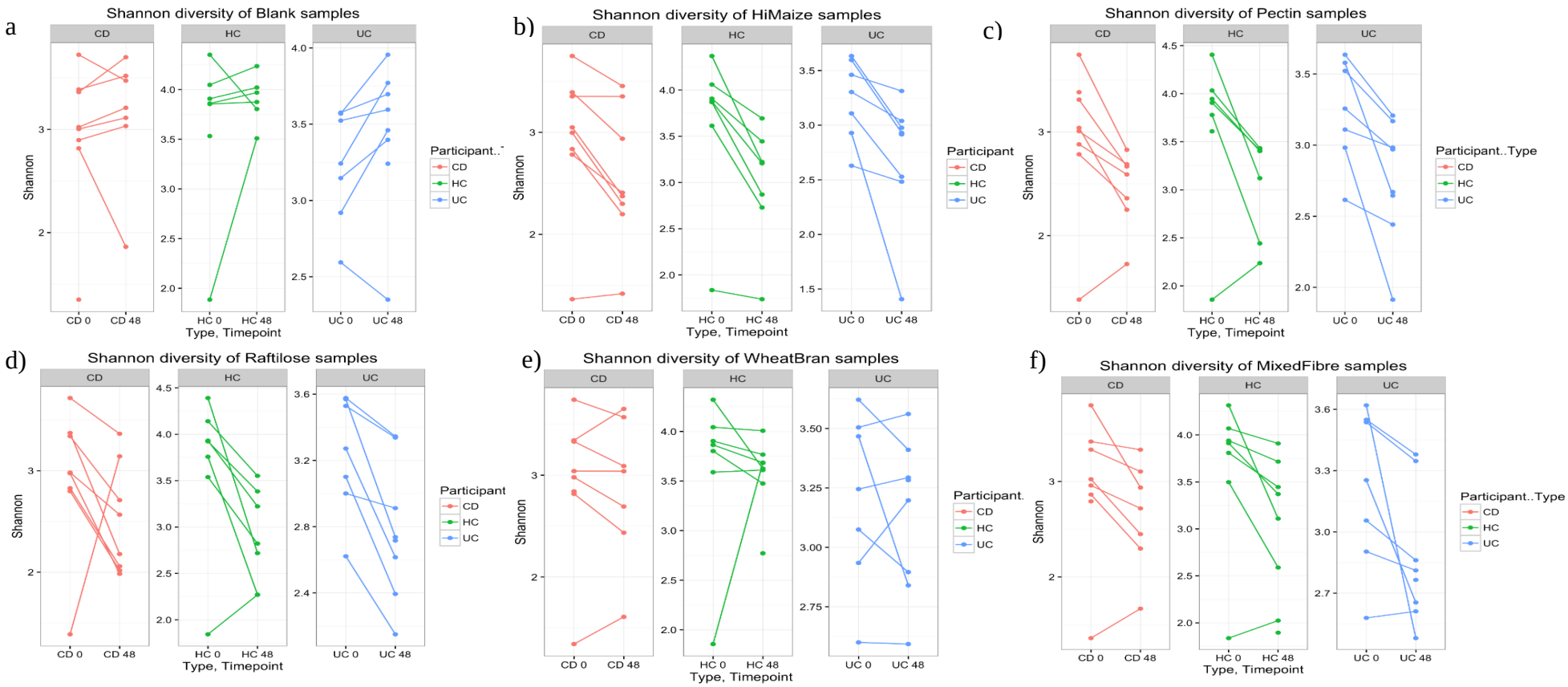
Table 5.2 p-values representing the change in microbial diversity following 48-hour fermentation with various substrates in UC patients, UC patients and healthy controls

	UC (n=8)	**CD (n=8)**	**Healthy Control (n=8)**
NSC	0.089	0.990	0.731
Hi Maize	0.327	**0.008***	**0.005***
Apple Pectin	0.236	0.084	**0.033***
Raftilose	0.245	0.228	**0.043***
Wheat Bran	0.656	0.268	0.803
Mixed Fibre	0.482	**0.030***	**0.050***

UC: Ulcerative Colitis, CD: Crohn's Disease, NSC; non-substrate control

* Indicates statistical significance between pre-and post-fermentation diversity , considered when $p<0.05$

Figure 5.1 Microbial diversity of CD patients, UC patients and healthy controls pre- and post *in vitro* 48hr fermentation with a)non-substrate control (blank), b) hi maize, c) apple pectin, d) raftilose, e) wheat bran, and f) mixed fibres



CD; Crohn's Disease patient, UC; Ulcerative Colitis patient, HC: healthy control

5.4.3 Microbial community of CD patients, UC patients and healthy controls at phylum level

Taxa plots were used to display the relative abundance of bacterial phyla present in the DNA of each participant at baseline and post-fermentation. Each individual participant is represented individually but according to their participant group. Statistical analysis was conducted on the OTUs present prior to and after fermentation, but no formal testing was conducted between groups to reduce the rate of Type I and Type II errors.

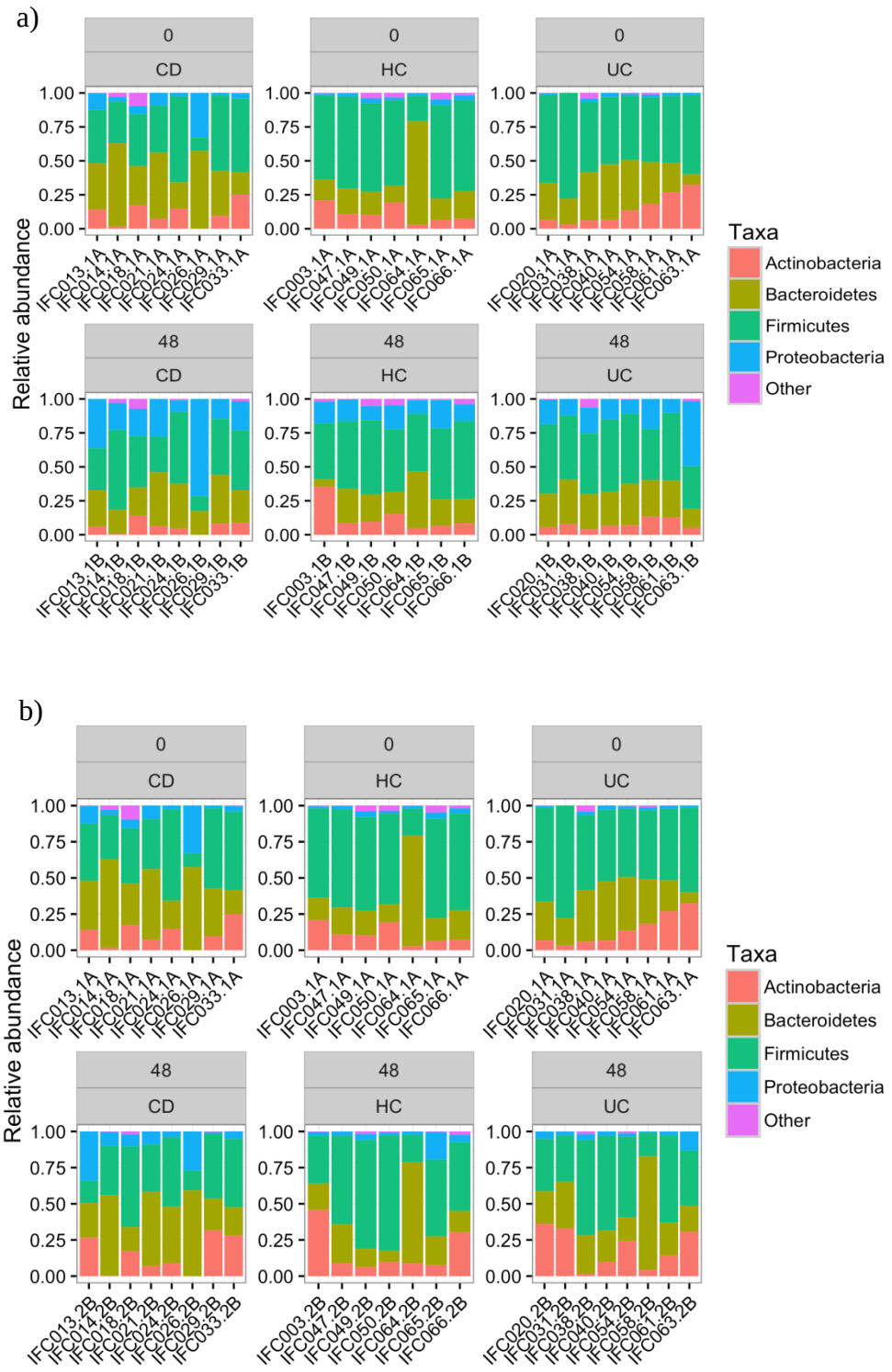
5.4.3.1 Baseline microbial community of CD patients, UC patients and healthy controls

Figure 5.2 highlights the microbial composition of each individual participant prior to fermentation. It is apparent that CD patients have increased representation of species within the Proteobacteria and other unidentified phylum, in lieu of Firmicutes compared to both healthy controls and UC patients. No obvious differences can be seen between UC patients and healthy controls.

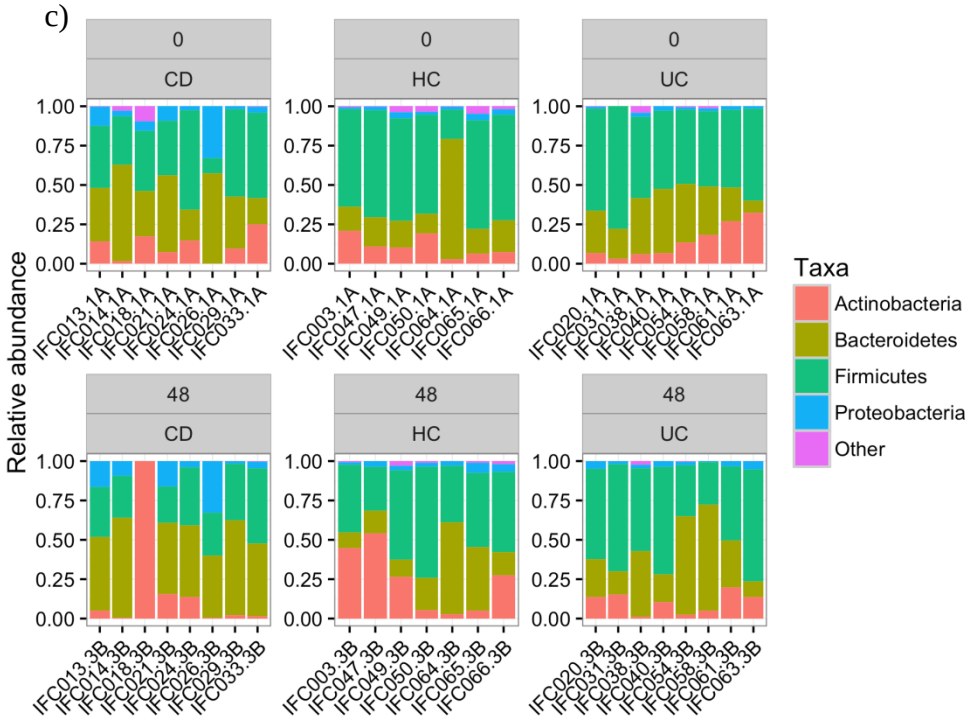
5.4.3.2 Changes in the microbial community of CD patients, UC patients and healthy controls following 48-hour in vitro fermentation with various fibrous substrates at phylum level

The taxa plots displayed in Figure 5.2 show the changes in bacterial phylum in response to fermentation with the chosen fibres for each individual participant according to their group. Following 48 hours fermentation with no substrate, there appears to be an increase in Proteobacteria for both IBD patient groups and healthy controls. In all groups, Actinobacteria appears to thrive in response to fermentation with raftilose, largely at the expense of Firmicutes. However, in regards to the remaining fibres, there is no clear pattern of change at phylum level following fermentation, and there is no obvious distinction between the microbial response of CD patients, UC patients or healthy controls at this level. As shown in Appendix 5, the adjusted p-values representing the change in OTUs before and after fermentation are not significant($p=1.00$).

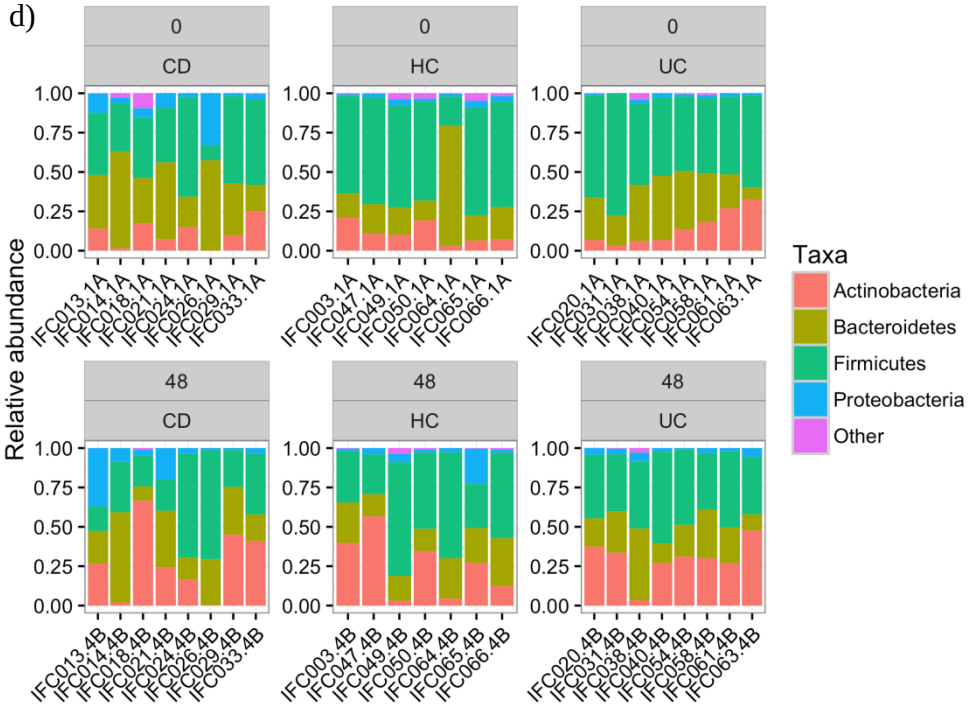
Figure 5.2 Taxa plots illustrating the relative abundance of bacterial phyla in individual CD patients, UC patients and healthy controls pre- and post *in vitro* 48hr fermentation with a) non-substrate conrol (blank), b) hi maize, c) apple pectin, d) raftilose, e) wheat bran, and f) mixed fibres



c)


0
CD
HC
UC
48
CD
HC
UC
Relative abundance
1.00
0.75
0.50
0.25
0.00
IFC013.1A
IFC014.1A
IFC018.1A
IFC021.1A
IFC024.1A
IFC026.1A
IFC029.1A
IFC033.1A
IFC003.1A
IFC047.1A
IFC049.1A
IFC050.1A
IFC064.1A
IFC065.1A
IFC066.1A
IFC020.1A
IFC031.1A
IFC038.1A
IFC040.1A
IFC054.1A
IFC058.1A
IFC061.1A
IFC063.1A
IFC013.3B
IFC014.3B
IFC018.3B
IFC021.3B
IFC024.3B
IFC026.3B
IFC029.3B
IFC033.3B
IFC003.3B
IFC047.3B
IFC049.3B
IFC050.3B
IFC064.3B
IFC065.3B
IFC066.3B
IFC020.3B
IFC031.3B
IFC038.3B
IFC040.3B
IFC054.3B
IFC058.3B
IFC061.3B
IFC063.3B
Taxa
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Other

d)


0
CD
HC
UC
48
CD
HC
UC
Relative abundance
1.00
0.75
0.50
0.25
0.00
IFC013.1A
IFC014.1A
IFC018.1A
IFC021.1A
IFC024.1A
IFC026.1A
IFC029.1A
IFC033.1A
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IFC020.1A
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IFC040.1A
IFC054.1A
IFC058.1A
IFC061.1A
IFC063.1A
IFC013.4B
IFC014.4B
IFC018.4B
IFC021.4B
IFC024.4B
IFC026.4B
IFC029.4B
IFC033.4B
IFC003.4B
IFC047.4B
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IFC064.4B
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IFC031.4B
IFC038.4B
IFC040.4B
IFC054.4B
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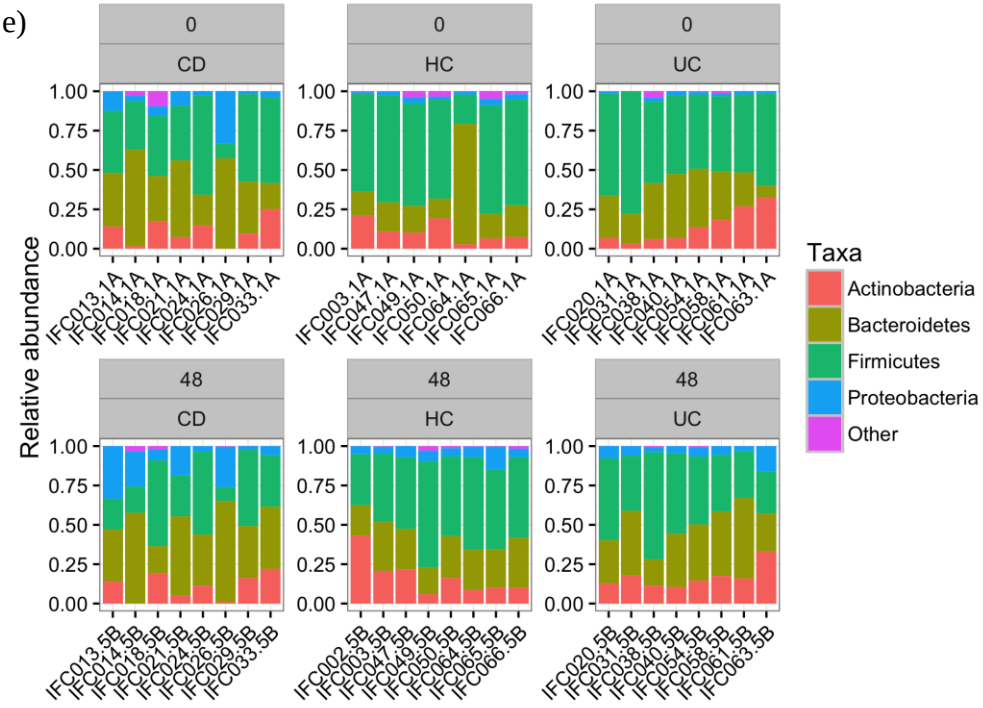
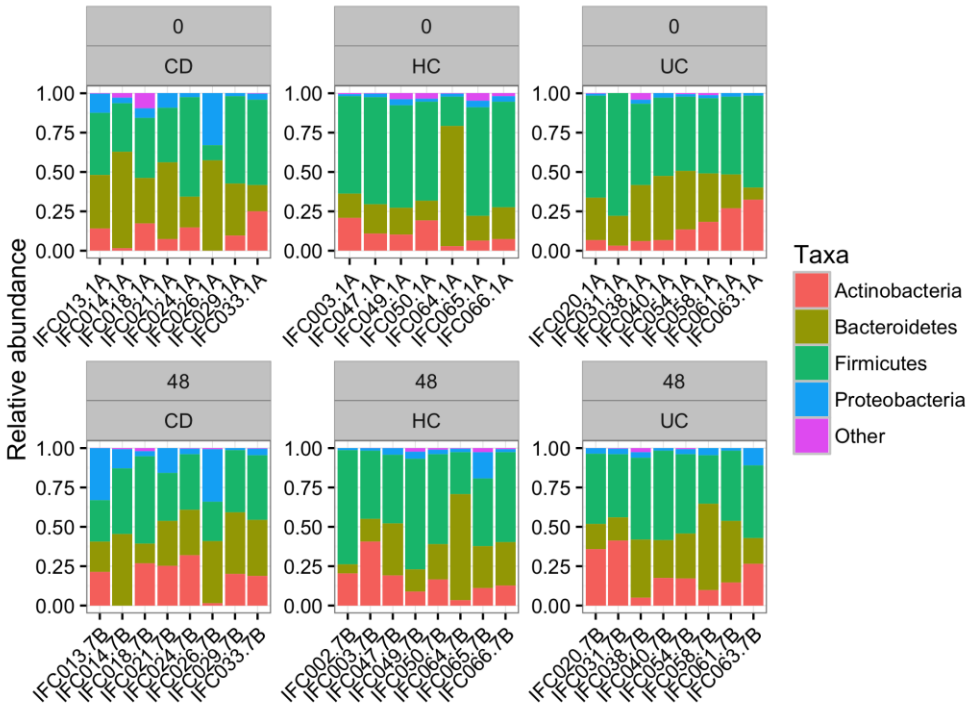
IFC061.4B
IFC063.4B
Taxa
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Other

e)



f)



0; 0hr, 48: 48hr, CD; Crohn's Disease, UC; Ulcerative Colitis, HC; healthy control
Each bar represents an individual participant. IFC labelling refers to individual participants

5.4.4 Changes in microbial community structure following 48 –hour fermentation

5.4.4.1 Response of the microbial community structure of all participants to 48 hours fermentation with various fibres

The PCA plots in Figure 5.3 illustrate the change in microbial community structure in participants following fermentation with the chosen fibrous substrates, considering all participants as one group (p-values shown). It can be deduced that fermentation with all fibres except pectin (p=0.101) and mixed fibres (p=0.379) significantly altered community structure (fibre-free control; p=0.01, hi maize; p = 0.036, raftilose; p=0.034, wheat bran; p= 0.001).

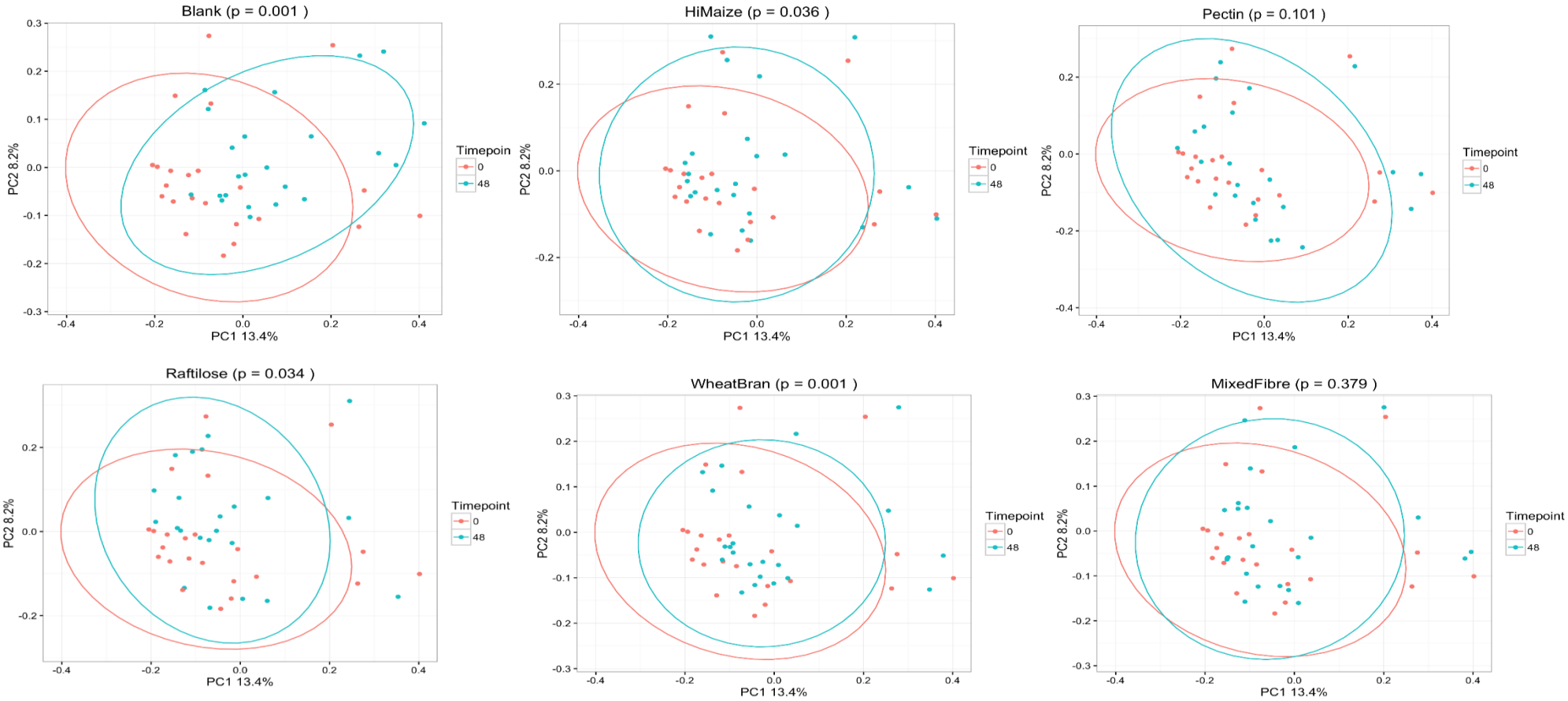
5.4.4.2 Response of the microbial community structure of CD patients, UC patients and healthy controls to 48 hours fermentation with various fibres

Changes in both baseline and post-fermentation community structure according to CD patients, UC patients and healthy controls is shown in Figure 5.4. This again highlights that there is significant changes in community structure after 48 hours of fermentation with hi maize (p=0.016), raftilose (p=0.021), wheat bran (p=0.002) and fibre-free control (p= 0.001) in each group; however when the groups are assessed separately, a significant difference is also detected after fermentation with pectin (p=0.046). The difference between community structure before and after fermentation with mixed fibre in any group remained insignificant (p=0.291).

For all fibres and time points, the difference between community structures of the three participant groups was significant (p=0.001) as tested by one-way ANOVA. However, on further analysis using t-test, this difference only remained significant between the healthy controls and UC patients A large inter-individual variation in community structure existed between the CD patients, particularly at baseline, which may explain the lack of significance in this group.

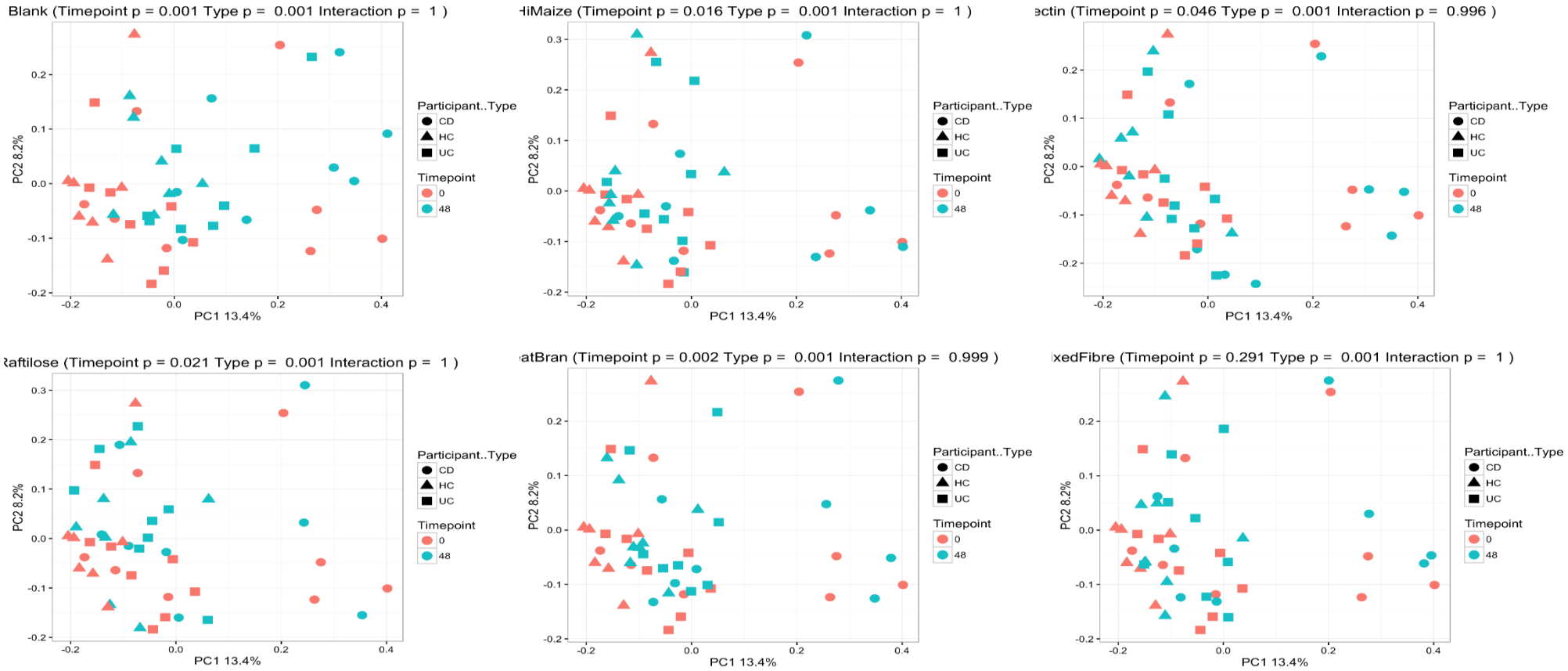
The interaction between participant groups, which indicates the difference in response to fermentation between groups, was close to 1.00 for all fibres, showing that the microbial structure of all groups responded to fibre in a similar fashion and that this change was not dependant on the groups.

Figure 5.3 Principal Component Analysis (PCA) plots displaying the community structure of all participants pre- and post-48 hours *in vitro* fermentation with various fibrous substrates (p-values from paired Wilcoxon test shown)



0; 0hr, 48: 48hr, CD; Crohn's Disease, UC; Ulcerative Colitis, HC; healthy control
Each dot represents an individual participant.

Figure 5.4 Principal Component Analysis (PCA) plots displaying the community structure of CD patients, UC patients and healthy controls before and after 48 hours in vitro fermentation with various fibrous substrates (p-values from paired Wilcoxon test shown)



0; 0hr, 48: 48hr, CD; Crohn's Disease, UC; Ulcerative Colitis, HC; healthy control
Each dot represents an individual participant.

5.5 Discussion

This study aimed to add to ongoing investigations regarding the role of the gut bacteria in IBD. Whilst characterising baseline bacteria is important, this study also investigated the subsequent changes in bacteria following fermentation with dietary fibre using an *in vitro* fermentation model to get an indication of the ability of the innate gut bacteria of IBD patient to utilise dietary fibre. This study provides further indication that the diversity and structure of the bacterial community of CD and UC patients is distinct from that of people with a healthy GIT and whilst it goes on to suggest that these differences can be somewhat overcome by the provision of dietary fibre, IBD patients still display a reduced ability to ferment these fibres *in vitro*.

With advancements in metagenomics technology and databases, research has been implemented aimed at characterising the microbial profile of IBD patients in the hope of shedding some light on the pathophysiology of these diseases. However, although there is an increasing number of studies focusing in this area, an IBD-specific microbial community is yet to be identified as there is much inter-study variation. This study found that, although there were no striking differences between the baseline community of healthy controls and UC patients, CD patients tended to display a higher relative abundance of both Proteobacteria and other species from unidentified phyla, although the power of this study was not high enough and number of participants in each group too low to conduct statistical analysis. Nonetheless, this is an interesting implication as other studies [68] have reported an increase in pathogenic strains from the Proteobacteria phylum in IBD, such as *E.Coli* and *Shigella flexneri*, both of which are within the gammaproteobacteria class [223]. Likewise, Lepage and colleagues [224] found that UC patients had increased species within the Proteobacteria phylum compared to their own healthy twin; however the current study did not any report differences in microbial composition between UC patients and healthy controls at phylum level. Other studies have reported marked differences in the relative abundance of both Firmicutes and Bacteriodes in both in UC and CD [86, 217, 224]. However this was only found to a small extent in the baseline bacterial profile of CD patients in this study, who appeared to have a reduced relative abundance of Firmicutes. Studies have found that UC and CD display distinct dysbiosis [225] [126], with one at least one report suggesting that CD patients display increased counts of unidentified bacteria, particularly within the Verrucomicrobia and Bacteroidetes phylum [126]. Similarly, patients with CD in the current study appeared to have increased unidentified species at baseline. The presence of unknown species is a

plausible avenue of research, as these may be key players in the onset of CD and therefore it is important that genome sequencing is continued. However the current study probably did not have sufficient power to decipher statistical differences in phylum level between groups. This may be due to the lack of power within this study. Indeed, there is also likely to be increased variation below phylum level [217] and certainly many studies investigating bacterial differences between IBD patients and healthy controls comment on species level. In particular, many studies report a reduction in counts of butyrate-producing bacteria in IBD patients, such as *Clostridrium leptum* [86, 128], *Clostridrium coccoides* [128], Bifidobacterium [122, 131] and *Faecalibacterium prausnitzii* [128, 129, 132]. A recent study by Chung et al reported that the *in vitro* fermentation of inulin and pectin had very little impact at phylum level, but very obvious changes were noted at species level [197]. Thus further insight into this area may disclose differences between IBD and healthy controls.

Identifying an IBD-specific bacterial profile is challenging partly due to large inter-individual variation, and evidently there are discrepancies in the literature regarding disease-associated strains. However, there is a general consensus that IBD patients suffer from a reduced microbial diversity; a finding that was evident in this study whereby IBD patients, particularly those with CD, displayed an obvious trend of reduced baseline microbial diversity compared to healthy controls. Diversity throughout the microbial community is an important aspect of gut health, providing the vast range of species necessary for gut immunity as well as the breakdown of various organic compounds which liberates essential nutrients from these otherwise indigestible foods. In particular, the possession of an array of beneficial bacteria helps to resist the overgrowth of pathogenic strains [43] such as *Adherent-invasive E. Coli* and *Enterobacteria*, both of which have been found in higher counts in CD patients [217]. This reduction in diversity may play an influential role in IBD onset, however it is not entirely clear whether it is a cause or an effect of the disease. As the current study was conducted in patients with quiescent disease, this suggests that CD patients are more likely to have an underlying reduction in diversity and therefore this is not necessarily preceded by inflammation.

It may be plausible to rectify reduced diversity and stimulate growth of health-promoting strains via ingestion of dietary fibre. The degradation of non-digestible carbohydrates by the human gut bacteria is extremely complex both in healthy and inflamed GITs, dependant on factors such as gut transit time, the pH of the colonic environment, the availability of bacterial enzymes, and, importantly, the specificity of the ingested carbohydrate [80]. Both *in vivo* and *in vitro* human studies have been used to

investigate the preferential growth of bacteria in response to dietary fibre, partly to explore the viability of using diet as a modulator of the microbial community. Such studies have linked the fermentation of specific fibres to the growth or inhibition of bacterial strains. It has been repeatedly shown that resistant starch promotes the growth of species in the *Bacteriodes* [97, 98] *Xylonibacteria* [98] and *Actinobacteria* phylum such as *Bifidobacterium Adolescentis, Parabacteriodes Distasonis* [97] and *Ruminoccus Bromii* [226] whilst suppressing the growth of Firmicutes. [98, 226] Although there was not sufficient power to conduct statistical analysis, the results of the current study are in agreement with this evidence, indicating that after 48-hour fermentation with raftilose, an important resistant starch and prebiotic, supported the growth of Actinobacteria at the expense of Firmicutes. The results of this study and others thus highlight that the enzymes employed by bacteria to breakdown fibre are polyspecific, which endorses the notion that diet could be used to manipulate microbial composition by promoting the growth of beneficial species. However, this study did not report any notable changes in microbial composition following fermentation of hi maize which is also a form of resistant starch. This may be reflective of a lack of power in this study, and it is likely that more insight would be gained via the recruitment of more participants. Likewise, the fermentation of all other fibres investigated in this study did not appear to have any drastic changes on microbial composition in neither healthy controls nor IBD patients. Previous studies have implied that the fermentation of apple pectin stimulates the growth of both *B. thetaiotaomicron* and *F. Prausnitzii [227]*, whilst wheat bran promotes *Lachnospiraceae,* a species of the Firmicutes phylum [79]. These species are apparently reduced in the diseased state and thus the intake of these fibres may be beneficial.

The provision of dietary fibre during the batch culture fermentations of this study tended to reduce microbial diversity in all groups; however this reduction rarely reached significance in CD patients and not at all in UC patients. Healthy controls, however, displayed significant reductions in diversity in response to all fibres except the fibre-free control and wheat-bran, which is poorly fermented. As these well-controlled *in vitro e*xperiments exposed the gut microbiota to each fibre independently, it is not surprising that diversity was reduced as only specific strains would benefit from each fibre. However, the stunted response in IBD patients highlights a potential inefficiency in the fermentation of such fibres, underlying the plausible abnormal composition and/or functionality of the bacteria of IBD patients. Similarly, James et al (2015) [202] reported that despite a reduced intake of fibre, UC patients in remission had increased faecal concentrations of starch and non-starch polysaccharides indicating that there is an imbalance between intake, utilisation

and subsequent output. A reduction in microbial diversity may influence this, as harbouring less bacteria with the specific CAZymes necessary for the degradation of dietary fibre could result in limited fermentation. Suboptimal utilisation of dietary fibre is likely to negatively impact colonic health, reducing the production of beneficial SCFA and therefore muting their effects on immunoregulation. The investigation reported in Chapter 3 of this thesis reported a tendency for reduced *in vitro* SCFA production in IBD, which may be related to the reduced microbial diversity reported in this Chapter.

The results of this study question the ability of dietary fibre to improve clinical symptoms of IBD. Whilst the direction of change in terms of IBD microbial diversity was similar to that of a healthy gut microbiome, the magnitude of change was not sufficient. There is discrepancies within the literature regarding the medicinal properties of dietary fibre in IBD, with some [154-156, 158] but not others [204] reporting improvements in disease activity. Relief from clinical symptoms would likely be mediated by the beneficial modulation of gut bacteria, enhances in SCFA production and the subsequent restoration of normal immune homeostasis. However if, as this study and others [202] suggest, the gut microbiota of IBD have an abnormally subdued response to fibre, the perceived benefits of supplementation may not necessarily translate to improvements in disease activity. Nevertheless, an important finding of this study was that, although IBD patients did not respond to fibre as efficiently as healthy controls, the community structure of both UC and CD patients responded to *in vitro* fermentation in the same way that healthy controls did despite initial differences between UC patients and healthy controls. This finding highlights that the consumption of dietary fibre is still invaluable in IBD as it promotes a swing towards a more stable, healthy microbial structure. Therefore, whilst it is unlikely that dietary fibre can fully treat disease, its consumption is still useful in the movement towards restoring gut homeostasis.

In summary, this study found that whilst the gut bacteria of IBD patients with quiescent disease are less efficient in the fermentation of various dietary fibres *in vitro*, anaerobic fermentation did induce a similar response in patients compared to controls in terms of community structure. This underlines the importance of maintaining dietary fibre intake in such patients. Although a fibre-free diet is successful in the treatment of active CD, it is likely that the intake of dietary fibre is important during remission in order to maintain a healthy microbial community, which may prevent future flare-ups. Further research should investigate the bacterial species involved in the fermentation of fibre in

IBD, as this may highlight discrepancies between patients and controls that were not elucidated in this study.

Chapter 6 : Discussion, general conclusions and proposed future research

The multifaceted aetiology of IBD and the impractical accessibility to the colon makes the study of these diseases in situ incredibly complicated; however with the ever increasing emergence of both UC and CD globally [13], it is imperative that research in this area is continued. Whilst genetics clearly plays a role in the onset of IBD, the pace at which the prevalence of these diseases is rising suggests that external factors must have a strong influence in their onset. Logically, it is likely that diet influences the condition of the GIT, and this is evident via the therapeutic effect of EEN in paediatric CD. Furthermore, there is mounting evidence to suggest that the dynamics of the microbial community is prominent in both the onset and treatment of IBD. The development of molecular techniques and expansive sequencing of bacterial genomes has greatly enhanced our understanding of the gut community and its impact on human health. As this eco-system is dependent on the host's dietary components for survival, the roles of the gut bacteria and diet in IBD are likely intertwined and therefore their relationship in the diseased state should be studied.

This thesis was aimed at further elucidating this association between dietary fibre and gut bacteria; to explore the composition and the efficacy of the innate gut bacteria of IBD patients in the hydrolysis of typical dietary polysaccharides and subsequent production of metabolites that have known benefits to both colonic and general health. Whilst research regarding SCFA production in IBD that has been of interest for decades, this study benefited from two benefits in its study design; the employment of an *in vitro* fermentation model, and subsequent modern sequencing techniques to identify the global microbes involved, an aspect which previous culture dependent techniques or taxon specific molecular microbiology techniques were unable to address. Utilising these study methods had allowed us to have a greater understanding of the colonic environment, in IBD. The aims of the studies within this thesis are as follows.

The main study within this thesis investigated the *in vitro* production of SCFA in adult UC and CD patients in remission in order to get an insight into the fibre fermentation capacity of colonic bacteria in the in the absence of inflammation. Studying this in quiescent disease allows us, as researchers, to get a clearer understanding of a cause-effect relationship in IBD, as it is hard to decipher whether a reduced production of these metabolites is a cause of disease, or rather a collateral impact of colonic inflammation. The baseline microbial composition of a subset of these adult patients was also investigated, as were the bacterial communities after 48 hours *in vitro* fibre fermentation. The selective stimulation of specific bacteria in response to the provision of single dietary fibres would

be expected to alter microbial structure, and it was of interest to assess if these changes were the same in IBD patients as in healthy controls.

A further project within this study aimed to investigate the functional capacity of the gut bacteria of treatment naïve, newly diagnosed paediatric CD patients and compare this to both that of paediatric CD patients on treatment and to matched healthy children. The investigation of SCFA production in active disease allows us to establish whether the activity of the gut bacteria of patients is different in newly presented untreated patients with CD, and if this differs to people who were treated.

6.1 *In vitro* production of SCFA in CD and UC patients, and it's implication in disease onset, treatment and maintenance of remission

The use of an *in vitro* fermentation model is instrumental in the assessment of the ability of colonic bacteria to perform anaerobic saccharolytic activity; the main outcome of interest in this study of which was the production of SCFA. SCFA, namely acetate, propionate and butyrate, provide a wealth of beneficial properties throughout the whole body. Due to their health-promoting characteristics, the role of SCFA production in IBD has been actively researched in many cross-sectional studies, with many reporting a reduction in both CD [122, 129, 131] and UC [128, 131, 132, 166, 201]. However most of these gauge SCFA production by concentrations within faecal samples. The majority of SCFA are either quickly absorbed by the lumen and transported to the systematic circulation or utilised as fuel by colonocytes and thus faecal concentrations do not necessarily represent production. Also as patients with IBD experience an increased number of diarrhoeal episodes, it is likely that reduced SCFA concentration is the result of a dilution effect rather that reflective of impaired SCFA. Therefore, it is of interest to assess the ability of the faecal bacteria of IBD patients to utilise dietary fibre in an *in vitro* system, and to compare this to the ability of bacteria obtained from a healthy GIT, fermented in the same controlled conditions.

The results of the adult cohort of this study indicated a tendency for reduced total and individual SCFA production in the disease state, even whilst in remission. Of prime interest is the reduced capacity of the gut bacteria of IBD patients to produce SCFA in response to the prebiotics, hi maize and raftilose. As foodstuffs that have been specifically designed to improve bacterial composition and thus inevitably enhance SCFA production, it is interesting that IBD patients cannot utilise prebiotics as efficiently as healthy controls. Nevertheless, the provision of dietary fibre *in vitro* did stimulate SCFA production and it is therefore plausible that patients require enhanced dietary fibre within their diet to optimise

SCFA production in order to overcome this limitation. A reduced capacity for the innate gut microbiota to utilise dietary fibre even in the absence of gut inflammation suggests that patients may not fully benefit from the protective, anti-inflammatory properties of SCFA. Optimising production via dietary supplementation may therefore promote colonic homeostasis, prolonging the remission period. Indeed there have been a few studies investigating the clinical efficacy of prebiotics for IBD patients in remission, most of which report improvements in inflammatory markers and functional symptoms [130, 154, 158]. Some IBD patients are known to avoid the intake of fibre due to potential unpleasant side effects such as abdominal distention from increased gas production. However this study did not report any differences in gas production between patients and healthy controls and would therefore suggest that the detrimental side effects of the fibres within this study would be negligible. Thus, this study endorses current guidelines that IBD patients in remission should aim to meet the recommended intakes of dietary fibre (30g/day), ensuring that a high proportion of this quota comes from fermentable fibres such as prebiotics.

The investigation of *in vitro* SCFA production in children in this study echoed previous studies which found an indirect relationship between disease activity and SCFA production [184] [131]. There was a trend of reduced SCFA in newly diagnosed, treatment-naive patients, which was increased in those who were on medication although not to the same level as healthy controls. Physiologically, it makes sense that the anti-inflammatory SCFA are reduced in the inflamed state; however it must be remembered that the faecal samples of patients with active disease are likely to be diarrhoea, which may confound results. The use of the *in vitro* systems helps to overcome this issue by not only assessing faecal concentrations, but fermenting in well-controlled conditions. However it would be important to assess bacterial load in baseline samples as this would be expected to affect SCFA production.

The results of this study may suggest that enhancing SCFA in active disease is important in the induction of remission; however it is known from previous studies that the successful treatment of paediatric CD with EEN coincides with a reduction in faecal butyrate concentrations [122], which may be due to the omission of dietary fibre. Therefore, although this study and others have found evidence of an impaired SCFA production in IBD, it is unlikely that this is instrumental to disease onset. Thus, the relationship between dietary fibre, SCFA production and IBD is evidently complicated; why is dietary fibre/SCFA apparently beneficial in remission, yet detrimental in active disease? The answer may lie in the extensive microbial community. There is evidence to

suggest that even commensal gut bacteria stimulate an unnecessary immune response in IBD patients, and it is possible that there is a breakdown in the tolerance towards these commensal bacteria that is enjoyed in a healthy GIT. Studies in the 1990s imply that when peripheral and lamina propria mononuclear cells from IBD patients are exposed to commensal bacteria cultivated from the same patient *in vitro*, cytokine production is induced [228]. This response was not evident in healthy controls, highlighting a disruption of immune homeostasis in IBD. Further studies found that both IgG [229] and T-lymphocyte [230] production is up-regulated in inflamed IBD intestinal tissue when stimulated by microbial antigens compared to both non-inflamed tissues and healthy controls. In human patients, a widely implemented and largely successful treatment is the administration of infliximab, a chimeric monoclonal antibody biologic which neutralises the pro-inflammatory cytokine TNF-α [29]. A further, more invasive strategy of alleviating the symptoms of IBD is the diversion of the intestinal flow from the inflamed area [231, 232]. It is possible that this allows the restoration of immune homeostasis in these areas due to the removal of cross-talk between the immune cells and microbial antigens. If the immune system of such patients is hyper-responsive, the arrival of further foreign antigens in the form of dietary fibre may exacerbate inflammation further. A study by Van Den Bogaerde et al [233] reported that peripheral blood lymphocytes of CD patients experienced more proliferation than healthy controls in response to food antigens that contained dietary fibre. These studies may partly explain why EEN is effective in the restoration of colonic homeostasis. The starvation of the gut microbiota likely ameliorates inflammatory response, improving both clinical, histological and functional symptoms.

Although successful, it is likely that the reduction in SCFA whilst on EEN is unsustainable. Therefore, optimising SCFA production once inflammation has been ameliorated is important. Gerasimidis and his team reported that following the return back to normal diet, faecal butyrate concentrations were returned to pre-EEN levels, whilst another study found that butyrate levels were significantly increased compared to baseline values after treatment on EEN [234]. Maintaining fermentable food within the diet may therefore help to promote and sustain production once remission has been induced.

In conclusion, the results of this study imply that SCFA production is restricted in IBD, both in active and quiescent disease. Although the production of these organic compounds is reportedly indirectly related to disease activity [128], enhancing production via the intake of dietary fibre is currently only advised in remission [146]. The implications of these studies and others are that, although SCFA production in compromised in active IBD, the priority in treating these diseases is to starve the gut bacteria, even though this

will inevitably reduce SCFA production further. Once the gut bacteria have been deprived and inflammation has been subdued, it is important to then restore normal gut homeostasis and again promote the production of SCFA. This is probably key in the maintenance of clinical remission. Nevertheless, it is possible that fibre may also inevitably induce relapse and it is not quite understood what perturbs this intricate balance. Further clinical research should focus on identifying factors influencing the change from remission to relapse, however this is formidable given the unpredictable nature of these diseases. Intervention studies should also be conducted investigating the impact of dietary fibre in quiescent disease, assessing its association with bacteria, metabolites and the maintenance of remission in clinical practise.

6.2 The microbial composition of CD and UC patients in remission and their response to *in vitro* stimulation with dietary fibre

There is mounting evidence implicating the involvement of the gut microbiota in IBD. As aforementioned, this may be due to a lack of immune tolerance towards either normal commensal bacteria or to the presence of pathogenic strains. It is important to identify key bacterial profiles involved in IBD in order for pathology to be further clarified, and therefore enhance the efficiency of treatment.

As reported in many other studies [86, 129, 131, 220],[125, 128, 131, 132, 220], the baseline microbial diversity of patients, particularly those with CD, was reduced. As patients of the current study were in remission, these results imply that compromised bacterial complexity is an underlying issue in IBD and not necessarily directly associated with inflammation. Diversity in the microbial community is essential in the protection against the overgrowth of pathogenic bacteria and the maintenance of gut homeostasis [43]. Therefore, despite being in clinical remission, patients may be vulnerable to detrimental perturbations in bacterial composition and/or imbalances in pro and anti-inflammatory cytokines which may cause further inflammation. In terms of maintaining remission, it would be advisable that such patients create a microbial environment that is rich in various different species and phyla, and the most practical and easy way of doing so is via diet. As we know, it is possible to manipulate bacterial profile via the introduction of fermentable foods which are specific to certain bacterial enzymes. This promotion of microbial diversity and a healthy, dynamic microbiome in quiescent IBD may highlight the mechanism by which the administration of prebiotics is known to lengthen remission period.

Microbial diversity is necessary in order to accommodate the hydrolysis of the large variety of dietary components which enter the colon. Whilst the profile of microbial composition may be important in disease pathophysiology, it is perhaps more interesting to assert the functional capacity of the innate species and their ability to respond to dietary stimuli. Although it is possible to alter bacterial profile through dietary changes, this study found that the baseline bacteria of IBD patients could not fully take advantage of the provision of dietary fibre *in vitro.* It was shown in the current study that the microbial diversity of healthy controls was significantly reduced following incubation with all fermentable fibres. This result is to be expected, as exposure to a single fibre would promote only the growth of specific bacteria. However, the microbial diversity of UC patients was not significantly altered following fermentation with any fibre, and only a few fibres induced a significant change in CD patients. It can be inferred that the gut bacteria present in the IBD faecal samples are therefore not apt in the selective fermentation of the fibres investigated, which may explain why another study [202] found an increased faecal concentration of non-starch polysaccharide in UC patients compared to healthy controls despite a reduced dietary intake. It is not entirely clear whether this reduced ability to hydrolyse fibre is due to a change in microbial composition or limitations in the functional capacity of the innate bacteria. Nevertheless, it is beneficial to ensure that the microbial community can efficiently utilise fibre in order to benefit from the therapeutic end-products of fermentation and this can again be achieved via exposure in diet.

Due to large inter-individual differences, science is yet to provide a clear IBD-specific bacterial profile, if in fact one does exist. This study only investigated differences in bacteria between patient groups and healthy controls at phylum level. Similar to other reports, it was found that CD patients tended to have increased representation of species within the Proteobacteria phylum [68] despite a reduced abundance of Firmicutes [86, 217, 224]. However, following 48-hour fermentation with various fibres, the differences in microbial composition between patients and controls were less distinct. Likewise, although the community structure of UC patients was significantly different to healthy controls, both communities responded to fibre fermentation in the same fashion. Therefore, although the microbiome of IBD patients is not as efficient as that of healthy controls in the fermentation of fibres *in vitro*, the pattern of change is similar and therefore the intake of dietary fibre may promote a microbial community similar to that of a healthy GIT. Whilst the baseline microbial composition of IBD patients and controls were similar at phylum level, there may be unknown discrepancies between groups at species level worth investigating.

As in Chapter 3, it is apparent that the gut microbiota has a reduced bacterial capacity than healthy controls. However, the provision of dietary fibre does induce changes in the bacterial profile that are similar to that of healthy bacteria. These results imply that the maintenance of dietary fibre within the diet of IBD remission is important in the promotion of a healthy microbiome, which will likely contribute gut homeostasis and prevent future inflammation. Thus, patients in remission should aim to incorporate the recommended dietary fibre into their diet.

6.3 The relevance of substrate availability during *in vitro* batch culture fermentations, and its potential implications in the current study

The batch culture fermentations utilised within this study have been well established and offer a practical, affordable and reliable representation into the ability of the gut microbiota. However, Chapter 3 of this thesis discusses the impact of increasing substrate availability during fermentations using stool samples from healthy participants. It was found in this study and in others [179, 180] that *in vitro* SCFA production becomes somewhat inhibited or saturated with increasing substrate availability. This relationship is not linear and it is not entirely clear at what point during fermentation this limitation becomes apparent. Certainly the production of SCFA acidifies the fermentation environment, and this may restrict optimal performance of the bacterial enzymes involved in the breakdown of substrates. Although a trend for reduced total SCFA production was seen in IBD patients, these differences were rarely found to be significant. This may be a true reflection of what occurs within the colon of patients, however it may also be due to the impact of increasing acidification within the *in vitro* fermentation vessels. Considering the results of the methodology chapter within this thesis, it should be considered that enzyme inhibition may have occurred during the i*n vitro* fermentations reported in Chapter 4 and 5 of this thesis. Thus, production of SCFA may have been hindered due to the reduction in pH caused by the ongoing fermentations. In this case, it is plausible that the bacteria of healthy controls hydrolysed the fibrous substrates at a quicker rate than patients, therefore becoming inhibited at an earlier stage. Thus, the bacteria of IBD may have displayed a reduced rate of production due to a lesser functional capacity, however given the circumstances were able to "catch-up" with the already inhibited enzymes of the healthy bacteria. It is therefore possible that a more significant difference in SCFA production may have been seen between IBD patients and healthy controls if there was intermittent removal of SCFA and other metabolites, as another study reported that inhibition was reduced when aliquots of faecal slurry were removed at specific intervals

[179]. The implications of these results would imply that establishing a rate of SCFA production by considering production at earlier time points may reveal important alterations between groups.

Despite the overall production of SCFA being restricted with substrate availability, butyrate production and its percentage contribution to total SCFA was increased with increasing fibre, both in this study and others [174]. It has been well documented within the literature that butyrogenic bacterial species such as Bifidobactera and Lactobacilli are able not only to tolerate an acidic extracellular environment [194], but to thrive in one [182, 212, 222-224]. Therefore, the reduction in pH in response the fibre fermentation reported following the in vitro experiments within this thesis may have accommodated the growth of butyrogenic bacteria, and therefore favoured the production of butyrate at the expense of acid-sensitive bacteria. However, the colonic environment has developed a physiological buffering response to acidification caused by SCFA production, with the majority of SCFA being absorbed by the lumen to be used locally or transported to the systematic circulation. Therefore, the production of butyrate recorded during the in vitro fermentations of this thesis may be a reflection of the culture environment rather than a true representation of microbial capacity in vivo. With anti-inflammatory properties and as the main fuel substrate of colonocytes, butyrate is essential in colonic health, yet has been speculated to be reduced in IBD [130, 131]. We therefore might have expected more differences in butyrate production in the disease state in Chapter 4 of this study. Whilst the findings may be a true representation of colonic activities, the lack of differences in butyrate production may be a reflection of the *in vitro* environment.

Nevertheless, the issues encountered in the batch culture fermentations would have been applicable to all participant groups and it is therefore still valid to make comparisons between patients and healthy controls. Whilst it is important to consider the implications of the *in vitro* environment, we also have to remember that the study of the gut microbiota is complicated, with many issues with both *in vivo* and *in vitro* study methodology. The batch culture experiments, not although without their drawbacks, were deemed the most appropriate for use in this thesis before we extend this work to human trials.

6.4 Study limitations

The major drawback of this study was the lack of power, both in the adult and paediatric cohorts. However there were large time constraints during the recruitment process, and the need to allocate time for laboratory analysis further restricted recruitment within the timeframe of a 12 month MSc by Research programme. It has been repeatedly discussed in

this thesis that the colonic bacteria can be manipulated via dietary means. Thus, it is a potential issue that this thesis did not consider the diet of patients in the days prior to the donation of stool samples, as it is likely that this influenced the baseline bacteria and their capacity to ferment fibre *in vitro*. Using a dietary assessment method, such as a food frequency questionnaire or weighed dietary records may have revealed important differences in the diet of patients and controls which could have impacted study results. Alternatively, participants could have been prescribed a standard diet for a few days prior to the donation of the stool in order to eliminate cofounding factors regarding baseline bacterial composition. However, both dietary assessment and prescribed diets are notoriously hard, suffering from low compliance and adherence. Furthermore, participants tend to change their habits and misreport during the recording period and their value is considered in large group assessments as opposed to per person or small group assessment [235]. This study did not incorporate the analysis of baseline bacterial load using qPCR and this is something to explore in future. Whilst Chapter 5 of this study investigated differences in microbial structure and diversity before and after fermentation, there was no consideration to the number of bacterial present in the initial samples. This may have impacted both SCFA production and bacterial response to fermentation.

As discussed in Section 6.3, it is advisable that future studies investigating this area should consider the rate of SCFA production in IBD rather than just quantify production following a certain period. This may reveal further discrepancies between the capabilities gut bacteria of IBD patients and that of heathy controls before fermentation is inhibited and production of metabolites reach a plateau.

Current guidelines advise that dietary fibre should be omitted in active IBD, yet encouraged in remission in order to promote microbial diversity, SCFA production and gut homeostasis [146]. However, there appears to be much confusion within patients regarding the optimal diet in IBD. Whilst recruiting adult patients with quiescent disease for this thesis, I observed that many were confused about what they should (and shouldn't) eat in order to preserve their remission status. Dietary fibre appeared to be a particular area of confusion and they were eager to hear more about the proposed study. Although this is just a personal observation from a small cohort of patients, I feel that this is an area to be formally investigated. To my knowledge, there is no current study investigating the knowledge and attitude of IBD patients in remission regarding dietary fibre; however understanding patient behaviour may help to identify areas in which to improve management of quiescent disease and prevent relapse. A well-structured, qualitative questionnaire could be administered to patients assessing their understanding and attitude

towards dietary fibre, and may have complemented the studies within this thesis well. Furthermore, a food frequency questionnaire may be used to indicate the habitual intake of dietary fibre in patients to gauge an estimation of fibre intake. If the data from such studies indicates IBD patients do not meet current guidelines, efforts should be made to promote good dietary practises in order to optimise remission the period. On the other hand, if it appears that patients tend to achieve guidelines, alternative methods may be necessary in order to further stimulate microbial symbiosis and SCFA production.

6.5 Conclusion

The implications derived from this thesis suggest that dietary fibre is advisable in quiescent IBD, as per current guidelines [146] . Patients in remission should attempt to overcome the innate reduction in microbial capacity to utilise dietary fibre and produce SCFA by ensuring they provide enough fermentable fibres to the gut microbiota. However, it is assumed that patients do not meet current guidelines and whilst this is a valid extrapolation from the general population, dietary practices in IBD patients in remission should be formally investigated. Furthermore, only a small number of studies have investigated the administration of dietary fibre during remission with varying success, thus more intervention studies are necessary in order to elucidate the effect of dietary fibre in quiescent IBD in clinical practice.

SCFA production in newly diagnosed paediatric patients with CD appears to be limited, yet somewhat restored with treatment. It is therefore evident that SCFA is indirectly associated with inflammation. The underlying reduction in SCFA production even whilst in remission suggests that this may be a cause of disease, however this contradictory to the clinical efficacy of fibre free EEN in paediatric CD. Therefore, whilst improving SCFA production may promote gut homeostasis and prolong the remission phase, this notion needs to be ascertained with clinical trials and it is unlikely that the reduction is a primary cause of disease. It is also plausible that changes in SCFA may simply reflect concomitant changes in other bacterial metabolites with pathogenic properties, and do not themselves cause inflammation. Although intervention studies are necessary to elucidate the relationship between dietary fibre, SCFA and disease onset/treatment *in vivo*, it is likely that the gut microbiota play a key role in the perpetuation of inflammation and thus ways in which microbial symbiosis can be established and maintained should be explored and promoted in IBD.

Appendices

Appendix 1; Patient Information Sheet

Participant Information Sheet

Glasgow gastroenterology additional research samples

You are due to undergo an endoscopic procedure (endoscopy or colonoscopy) within NHS Greater Glasgow and Clyde. In addition to providing clinical services, the Health Board and department are also committed to supporting medical research.

The purpose of this information sheet is to invite you to donate extra tissue samples at the time of your procedure for use in medical research.

However, before you decide whether or not to do this, we would like you to understand why the research is being done and what it would involve if you agreed. Please take time to carefully read the following information, which the gastroenterology team will also go through with you when you attend for your endoscopy. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information

What is the purpose of the study?

We want to better understand inflammation and inflammatory diseases.

The large intestine (or colon) can be affected by a number of common inflammatory disorders. The colon may also act a marker of inflammation in other parts of the body. As such, the colon offers a unique "window" on the workings of the immune system in these diseases and normal health. Studying immune cells and proteins in colonic biopsies, blood, stool, urine and saliva helps researchers better understand these processes and diseases. Understanding why and how people develop these common diseases allows researchers to develop better treatments and tests for people with these conditions.

The sample processing and storage, if applicable would be done by the Institute of Infection, Immunity, Inflammation Research Tissue Bank (I3IRTB), which is physically hosted by the Institute of Infection, Immunity and Inflammation at the Glasgow Biomedical Research Centre (GBRC), University of Glasgow and is overseen by the NHS Greater Glasgow and Clyde (NHSGGC) Biorepository. The samples will only ever be used in research applications which have been approved by a scientific review committee (more information below).

Why have I been asked to take part?

You will shortly be attending for an endoscopy and/or colonoscopy, during which small biopsy samples are often taken from the lining of the colon as part of your standard clinical care. We would like to collect additional biopsy samples during the procedure from patients with inflammatory disorders of the gut and from those who do not have such disorders in order to allow researchers to compare findings between these conditions.

Do I have to take part?

No, it is entirely up to you if you want to donate additional biopsies and/or blood, urine, stool or saliva samples. Whatever you decide, it will not affect the standard of care and treatment you will receive. You can also change your mind at any time, without giving a reason.

What will happen to me if I take part?

If you agree to take part, a small number of additional biopsy samples will be taken during your procedure. This takes only two to three minutes so does not significantly increase amount of time to do the procedure. You will not notice any other difference from your usual procedure.

We may also ask for a blood, urine, saliva or stool sample on the day of the test. If we only require one specific sample (eg urine) for a specific study, we would only ask you for this sample, but if we need to compare findings in different areas of the body, we may ask you to donate the other samples as well, in which case you will be able to choose if there are any of these you would prefer not to donate. We would anticipate that if you agree to take part, all required samples will be taken at the same time as your routine endoscopy.

Here is some information about each of these procedures:

1. *Blood test*. We may ask you to provide us with an extra blood sample, in addition to the sample required for your standard clinical care. Where possible, this blood sample will be taken as part of your routine intravenous cannulation for the endoscopic procedure. If you are not having a cannula or routine blood tests, then this could possibly mean an extra venepuncture (blood test).

2. We may also ask you to provide an extra *urine or saliva sample.*

3. On rare occasions for very specific studies, we may ask you for a *stool sample*. We can arrange to collect this from you on a separate day to the test if this is more convenient for you (eg when you return to clinic for the biopsy result).

4. We would also like to use some information from your *medical records* about your physical condition.

Your name, address and anything else that could identify you will be removed before allowing researchers to use any of your samples or data.

Occasionally we may need to understand how things change over time or with your current treatment, so we may ask if we can ask you for a repeat *blood or urine* sample. This would be no sooner than 4 weeks after your initial sample and will generally occur at your next clinic appointment. If you would prefer to only donate the initial sample but not the repeat sample, you can indicate this on the attached consent form.

If you take part, you will not be given any different medication, treatment or care from your standard NHS clinical care.

What are the possible benefits of taking part?

There will be no benefit for you personally, but the information from the studies carried out using these samples will help improve the treatment of people with similar and related conditions.

What are the possible disadvantages or risks of taking part?

The only possible disadvantages or risks relate to the biopsy and blood test.

You are having an endoscopic procedure already as part of your clinical care, so there will be no additional risk and taking the additional biopsy samples will not increase the procedure time significantly (2-3 minutes). Biopsies are often taken as part of routine clinical care and have a small risk of bleeding. Any risk from taking additional biopsies will be very small.

There is the possibility of some pain and bruising from the blood test. However we anticipate taking this as part of your routine intravenous cannulation for the endoscopic procedure and thus there would be no additional risk.

All biopsies and blood tests will be taken by people who are experienced and trained in these commonly performed procedures.

Where will researchers use my samples?

- The NHS
- Universities
- Research Institutions
- Commercial biotechnology or pharmaceutical companies

Samples may also be sent to these organisations abroad. You can be assured that before using your samples, all of the above must prove that they are following legal and ethical guidelines for their research. Any applications to use the samples will be reviewed by senior academic scientists to ensure the studies they are being used for are scientifically valid and justified. The tissue storage biobank has specific approved guidelines and monitoring processes in place for this. In addition, researchers working abroad will be required to sign a form agreeing to follow the same rules and regulations which apply in the UK.

How long will my samples be stored? What happens if I change my mind?

All samples you donate will either be used immediately or stored securely in the approved Institute of Infection, Immunity and Inflammation Research Tissue Bank (I3IRTB) until they are all used or you withdraw consent. If you change your mind in future and withdraw consent, you can tell a member of your healthcare team, or contact us on the telephone number or email address in the "Further Information" section. Some of your samples may already have been used for research, but we would dispose of any remaining samples that have not been used yet.

Will my medical notes be used?

Researchers need to know the medical history of the person who donated the tissue in order to understand the results. We would like your permission to take this information from your medical notes now, and possibly in the future as a follow up. A trained health professional or authorised member of the tissue bank will do this in confidence.

Will my taking part and my information be kept confidential?

Yes, all information that is collected will be kept strictly confidential in keeping with the Data Protection Act 1998. Your name and any other information from which you could be identified will be kept separately from your samples and medical data. Any information or samples given to researchers will therefore have had your name, address, and any other personal information removed so that you cannot be recognised. Only your healthcare team and individuals directly involved running in the tissue bank will have access to identifiable data which will be kept on secure computers and in locked filing cabinets.

Authorised responsible individuals from regulatory authorities, including NHS Greater Glasgow and Clyde R&D and the University of Glasgow may also need to access this data and your medical records as part of monitoring to make sure that the research is being carried out correctly. All will have a duty of confidentiality to you as a research participant and will never reveal your identity to anyone not directly involved with the study.

Can researchers find new information about my health?

The research on your samples will normally have nothing to do with your own care or treatment. If anything is found that you need to know, your doctor will be told.

Will researchers carry out genetic tests on my tissue?

It may be appropriate for genetic tests to be carried out. A lot of research today focuses on the study of genetic material from people with known diseases compared to healthy individuals. This comparison helps researchers understand how genetic differences affect our health. This in turn helps in the development of new drugs and treatments. The results of these tests will only ever be used for research and cannot be traced back to you.

Will I get any money for taking part?

No, you will not be paid for donating these samples. In the unlikely event that any sample cannot be taken during your clinic or hospital visit, you would be reimbursed for reasonable travel expenses incurred in attending for the additional visit.

Will anyone make money from my tissues?

It is illegal to sell tissue for profit. The NHS or Tissue Bank may charge researchers a fee for your samples, but this is to cover the costs of collection, storage or supplying the samples. The NHS uses a considerable amount of staff knowledge and skills ("know-how") to explain what is wrong with you. This "know-how" is valuable information for researchers. The NHS will use money it gets from researchers and your tissue to improve care to its patients.

If researchers develop a new drug, treatment or test, a pharmaceutical company or other researcher may then make a profit. It will not be possible for you to claim any money because you donated samples. However, any new drug, treatment, or test would potentially help all of us in the future

What if there is a problem?

If you have a concern about any aspect of this research, you should speak to the research team in the first instance. If you are still unhappy, you have the right to pursue a complaint through NHS Greater Glasgow and Clyde which is acting as research sponsor. Details about this are available from the research team.

In the event that something goes wrong and you are harmed during research and this is due to someone's negligence, you may have grounds for a legal action for compensation against NHS Greater Glasgow and Clyde, but you may have to pay your legal costs. The normal NHS complaints mechanisms will still be available.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the West of Scotland Research Ethics Committee 4.

Further information

If you have any further questions, please ask the person who is explaining this research to you. You may also wish to speak to your doctor or nurse, or can contact the research team on 0141-211 4290.

We will endeavour to supply this information in different languages and formats if requested.

Thank you for taking the time to read this Information Sheet and for considering taking part in this research study.

Appendix 2: Patient Consent Form

NHS
Greater Glasgow and Clyde

Affix patient ID Label

PARTICIPANT CONSENT FORM
Glasgow Gastroenterology research samples

Please initial all boxes

1. I confirm that I have read and understood the Participant Information Sheet dated **06/08/2013** (**version 1**) for the above study. I have had the opportunity to ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason, without my medical care or legal rights being affected. ☐

3. I declare that I have given my consent voluntarily to the donation and storage of the samples as indicated below. ☐

4. I agree that my donated sample(s) may be used by researchers, and may be used abroad for regulated medical research as described in the participant information sheet. ☐

5. I give my healthcare team and authorised biobank staff permission to use and givesome information (that cannot identify me) from my medical records to researchers. ☐

6. I understand that it may be appropriate for genetic tests to be carried out in order to determine whether genetic makeup has any connection with disease. ☐

7. I understand that you will not sell my tissue but costs will be recovered on a non-profit making basis. ☐

8. I understand that I will not benefit financially or be entitled to a share of any profit that might arise from the research ☐

9. I agree to provide additional colonoscopy biopsy samples.
Please circle YES or NO or NA (not applicable)

YES / NO / NA

10. I agree to provide an additionalblood sample. (Please circle) YES / NO / NA

11. I agree to provide an extra urine, stool or saliva samples. (Please circle)

YES / NO / NA

12. I agree to provide a repeat sample at my next visit. (Delete if not applicable)

YES / NO / NA

.....	.....	.....
Name of patient (please print)	***Signature***	***Date***

.....	.....	.....
Name of Person taking consent	***Signature***	***Date***

3 Copies: 1 to Participant, 1 to Biobank, 1 to Patient's records

Appendix 3: Participant Information Sheet

Participant Information Sheet

Title of study

Faecal samples for the study of *In vitro* metabolism 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 not clear 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 can 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, aged 18-60, non-smoker, in good general health, not taking any supplement or medication including antibiotics
ii) you do not suffer from any allergy or condition affecting bowel health

Do I have to take part?

It is up to you whether or not to take part. 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 a reason.

What will happen to me if I take part?

You will meet a member of the Research Team from the University of Glasgow who will explain the study at a convenient time. All transport costs as well as costs associated to the study will be reimbursed.

What do I have to do?

You will have the study explained by one of the research team. We will ask you for your age and gender and measure your height and weight. We will check your suitability for the study by asking some simple questions about your usual diet and any health problems and recent medications. Depending on the exact dietary ingredient we will study with your sample, you may be asked to eat a slightly restricted diet before providing us with a faecal sample. If required we will provide you with simple dietary advice to avoid either dietary fibre for two days, or avoid foods rich in plant polyphenolics. For some studies you may be able to stay on your normal diet. Each diet will last two days. Diet sheets will be

provided, and you will also be given a telephone number, to be able to call a member of the Research Team with any queries. You will be asked to tell us if you think you may have slipped up with any of the dietary advice.
At the end of the 2 day diet we will ask you to provide a faecal sample. You will be given full instructions on how to collect the sample and we will provided 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.

What are the possible disadvantages and risks of taking part?
There are no risks or disadvantages associated with this study other than time loss and

inconvenience of following dietary restrictions.

What are the possible benefits of taking part?
There are no direct benefits to the volunteers associated 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 which 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 Human Nutrition Group, at the University of Glasgow. The funding comes from a variety of sources including scholarships and some industrial sources. Details of each funding source involved in the study your samples are used for can be provided if you require.

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 Maizatul Omar at m.omar.1@research.gla.ac.uk.

Thank you for reading this information sheet.

Study Number:
Version no. 1 Date 29.11.11

Appendix 4: Healthy control consent form

(Form to be on headed paper)

Centre Number:

Study Number:

Subject Identification Number for this trial:

CONSENT FORM

Title of Project: Faecal samples for the study of *In vitro* metabolism by human faecal microbiota

Name of Researcher:

Please initial box

1. I confirm that I have read and understand the information sheet dated...28.11.11... (version...1) 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 subject Date Signature

_____ _____ _____

Name of Person taking consent Date Signature
(if different from researcher)

_____ _____ _____

Researcher Date Signature

1 for subject; 1 for researcher

Appendix 5 Adjusted p-values representing the change in OTUs before and after fermentation with diferent fibres for healthy controls, Crohn's Diseases patients (CD) and Ulcerative Colitis Patients (UC)

Partipant + fibre code	**Fibre**	**Participant Type**	P-value	log10FC	OTU	Adjusted p-value	Domain	Family	Genus
WheatBranHC.40	Wheat Bran	Healthy Control	0.02	1.04	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
WheatBranHC.68	Wheat Bran	Healthy Control	0.02	0.61	OTU_801	1	No blast hit	NA	NA
WheatBranHC.77	Wheat Bran	Healthy Control	0.02	0.52	OTU_741	1	Bacteria	Erysipelotrichaceae	Incertae Sedis
WheatBranHC.84	Wheat Bran	Healthy Control	0.03	-0.63	OTU_2	1	No blast hit	NA	NA
WheatBranHC.87	Wheat Bran	Healthy Control	0.04	-0.48	OTU_6	1	Bacteria	Lachnospiraceae	Dorea
WheatBranHC.149	Wheat Bran	Healthy Control	0.02	0.5	OTU_209	1	No blast hit	NA	NA
WheatBranHC.156	Wheat Bran	Healthy Control	0.04	-0.53	OTU_596	1	Bacteria	Coriobacteriaceae	uncultured
WheatBranHC.164	Wheat Bran	Healthy Control	0.04	-0.86	OTU_615	1	Bacteria	Eubacteriaceae	Anaerofustis
WheatBranHC.175	Wheat Bran	Healthy Control	0.03	0.43	OTU_116	1	Bacteria	Rikenellaceae	Rikenellaceae RC9 gut group

WheatBran HC.224	Wheat Bran	Healthy Control	0.04	0.74	OTU_684	1	Bacteria	Christensenellaceae	Christensenellaceae R-7 group
WheatBran HC.280	Wheat Bran	Healthy Control	0.04	0.57	OTU_129	1	No blast hit	NA	NA
WheatBran HC.289	Wheat Bran	Healthy Control	0.02	-0.44	OTU_652	1	Bacteria	Christensenellaceae	uncultured
WheatBran HC.329	Wheat Bran	Healthy Control	0.05	0.65	OTU_232	1	Bacteria	Christensenellaceae	Christensenellaceae R-7 group
WheatBran HC.351	Wheat Bran	Healthy Control	0.03	0.11	OTU_426	1	No blast hit	NA	NA
WheatBran HC.366	Wheat Bran	Healthy Control	0.02	-0.84	OTU_900	1	Bacteria	Ruminococcaceae	Ruminococcaceae UCG-013
WheatBran HC.371	Wheat Bran	Healthy Control	0.02	0.29	OTU_907	1	Bacteria	Aeromonadaceae	Aeromonas
WheatBran HC.384	Wheat Bran	Healthy Control	0.02	0.63	OTU_517	1	Bacteria	Lachnospiraceae	[Eubacterium] ventriosum group
WheatBran HC.420	Wheat Bran	Healthy Control	0.02	0.26	OTU_765	1	Bacteria	Clostridiales vadinBB60 group	uncultured bacterium

WheatBranHC.480	Wheat Bran	Healthy Control	0.02	0.79	OTU_301	1	Bacteria	Lachnospiraceae	Coprococcus 1
WheatBranHC.575	Wheat Bran	Healthy Control	0.02	-0.95	OTU_56	1	No blast hit	NA	NA
WheatBranHC.604	Wheat Bran	Healthy Control	0.02	-0.75	OTU_630	1	Bacteria	Bacillaceae	Bacillus
WheatBranHC.672	Wheat Bran	Healthy Control	0.03	0.42	OTU_913	1	Bacteria	Rikenellaceae	Alistipes
WheatBranHC.680	Wheat Bran	Healthy Control	0.02	-0.79	OTU_74	1	Bacteria	Ruminococcaceae	Oscillibacter
WheatBranHC.692	Wheat Bran	Healthy Control	0.04	-0.86	OTU_14	1	No blast hit	NA	NA
WheatBranHC.696	Wheat Bran	Healthy Control	0.03	-0.49	OTU_10	1	Bacteria	Erysipelotrichaceae	Holdemanella
WheatBranHC.707	Wheat Bran	Healthy Control	0.04	-0.72	OTU_779	1	Bacteria	Desulfovibrionaceae	Desulfovibrio
WheatBranHC.746	Wheat Bran	Healthy Control	0.04	-1.23	OTU_89	1	Bacteria	Ruminococcaceae	Ruminococcus 1
WheatBranHC.842	Wheat Bran	Healthy Control	0.03	-0.46	OTU_536	1	Bacteria	uncultured bacterium	NA
WheatBranHC.844	Wheat Bran	Healthy Control	0.04	-0.69	OTU_101	1	Bacteria	Lachnospiraceae	Lachnospiraceae UCG-005

WheatBranHC.867	Wheat Bran	Healthy Control	0.04	0.59	OTU_43	1	No blast hit	NA	NA
WheatBranHC.869	Wheat Bran	Healthy Control	0.04	-0.88	OTU_41	1	Bacteria	Ruminococcaceae	Oscillospira
WheatBranHC.887	Wheat Bran	Healthy Control	0.02	0.76	OTU_218	1	No blast hit	NA	NA
WheatBranHC.895	Wheat Bran	Healthy Control	0.05	-0.33	OTU_199	1	Bacteria	Ruminococcaceae	Ruminiclostridium 5
WheatBranHC.899	Wheat Bran	Healthy Control	0.02	-1.41	OTU_195	1	Bacteria	Halieaceae	OM60(NOR5) clade
WheatBranHC.941	Wheat Bran	Healthy Control	0.04	0.62	OTU_690	1	No blast hit	NA	NA
WheatBranHC.1014	Wheat Bran	Healthy Control	0.04	-0.88	OTU_545	1	Bacteria	Lachnospiraceae	Lachnospiraceae UCG-004
WheatBranHC.1015	Wheat Bran	Healthy Control	0.02	-0.8	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
WheatBranCD.84	Wheat Bran	CD	0.04	-0.39	OTU_2	1	No blast hit	NA	NA
WheatBranCD.149	Wheat Bran	CD	0.01	0.42	OTU_209	1	No blast hit	NA	NA
WheatBranCD.164	Wheat Bran	CD	0.04	-1.12	OTU_615	1	Bacteria	Eubacteriaceae	Anaerofustis

WheatBranCD.225	Wheat Bran	CD	0.04	-0.44	OTU_685	1	Bacteria	Ruminococcaceae	[Eubacterium] coprostanoligenes group
WheatBranCD.303	Wheat Bran	CD	0.02	-0.37	OTU_153	1	Bacteria	Coriobacteriaceae	uncultured
WheatBranCD.347	Wheat Bran	CD	0.02	0.09	OTU_343	1	Bacteria	Lachnospiraceae	[Eubacterium] hallii group
WheatBranCD.448	Wheat Bran	CD	0.04	-0.45	OTU_91	1	Bacteria	Desulfovibrionaceae	Bilophila
WheatBranCD.680	Wheat Bran	CD	0.04	-1.16	OTU_74	1	Bacteria	Ruminococcaceae	Oscillibacter
WheatBranCD.740	Wheat Bran	CD	0.02	-0.57	OTU_174	1	No blast hit	NA	NA
WheatBranCD.846	Wheat Bran	CD	0.02	0.23	OTU_103	1	Bacteria	Clostridiales vadinBB60 group	uncultured bacterium
WheatBranCD.887	Wheat Bran	CD	0.04	0.81	OTU_218	1	No blast hit	NA	NA
WheatBranCD.924	Wheat Bran	CD	0.04	1.15	OTU_361	1	Bacteria	Lachnospiraceae	uncultured
WheatBranCD.937	Wheat Bran	CD	0.04	-0.48	OTU_445	1	No blast hit	NA	NA

WheatBranCD.941	Wheat Bran	CD	0.02	0.2	OTU_690	1	No blast hit	NA	NA
WheatBranCD.965	Wheat Bran	CD	0.04	0.79	OTU_968	1	No blast hit	NA	NA
WheatBranCD.1015	Wheat Bran	CD	0.01	-0.93	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
WheatBranUC.40	Whear Bran	UC	0.02	1.19	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
WheatBranUC.68	Whear Bran	UC	0.02	0.74	OTU_801	1	No blast hit	NA	NA
WheatBranUC.81	Whear Bran	UC	0.04	0.34	OTU_9	1	Bacteria	Lachnospiraceae	Lachnoclostridium
WheatBranUC.149	Whear Bran	UC	0.04	0.64	OTU_209	1	No blast hit	NA	NA
WheatBranUC.308	Whear Bran	UC	0.02	-0.6	OTU_63	1	Bacteria	Ruminococcaceae	Ruminiclostridium 5
WheatBranUC.330	Whear Bran	UC	0.03	-1.47	OTU_231	1	Bacteria	Bifidobacteriaceae	Parascardovia
WheatBranUC.452	Whear Bran	UC	0.04	0.79	OTU_283	1	No blast hit	NA	NA
WheatBranUC.745	Whear Bran	UC	0.04	0.69	OTU_179	1	No blast hit	NA	NA
WheatBranUC.887	Whear Bran	UC	0.02	0.9	OTU_218	1	No blast hit	NA	NA
MixedFibreHC.64	Mixed Fibre	HC	0.04	-0.39	OTU_809	1	No blast hit	NA	NA

MixedFibre HC.86	Mixed Fibre	HC	0.03	-0.32	OTU_1	1	Bacteria	Ruminococcaceae	Ruminiclostridium 5
MixedFibre HC.140	Mixed Fibre	HC	0.04	-0.68	OTU_200	1	No blast hit	NA	NA
MixedFibre HC.156	Mixed Fibre	HC	0.04	-0.78	OTU_596	1	Bacteria	Coriobacteriaceae	uncultured
MixedFibre HC.164	Mixed Fibre	HC	0.04	-0.92	OTU_615	1	Bacteria	Eubacteriaceae	Anaerofustis
MixedFibre HC.187	Mixed Fibre	HC	0.04	-1.23	OTU_291	1	Bacteria	Lachnospiraceae	Blautia
MixedFibre HC.224	Mixed Fibre	HC	0.04	0.33	OTU_684	1	Bacteria	Christensenellaceae	Christensenellaceae R-7 group
MixedFibre HC.303	Mixed Fibre	HC	0.03	-0.32	OTU_153	1	Bacteria	Coriobacteriaceae	uncultured
MixedFibre HC.351	Mixed Fibre	HC	0.02	0.29	OTU_426	1	No blast hit	NA	NA
MixedFibre HC.366	Mixed Fibre	HC	0.02	-0.61	OTU_900	1	Bacteria	Ruminococcaceae	Ruminococcaceae UCG-013
MixedFibre HC.680	Mixed Fibre	HC	0.02	-0.59	OTU_74	1	Bacteria	Ruminococcaceae	Oscillibacter
MixedFibre HC.692	Mixed Fibre	HC	0.04	-1.39	OTU_14	1	No blast hit	NA	NA
MixedFibre HC.746	Mixed Fibre	HC	0.04	-0.58	OTU_89	1	Bacteria	Ruminococcaceae	Ruminococcus 1

MixedFibre HC.842	Mixed Fibre	HC	0.05	-0.41	OTU_536	1	Bacteria	uncultured bacterium	NA
MixedFibre HC.844	Mixed Fibre	HC	0.04	-0.04	OTU_101	1	Bacteria	Lachnospiraceae	Lachnospiraceae UCG-005
MixedFibre HC.869	Mixed Fibre	HC	0.04	-0.87	OTU_41	1	Bacteria	Ruminococcaceae	Oscillospira
MixedFibre HC.887	Mixed Fibre	HC	0.03	0.42	OTU_218	1	No blast hit	NA	NA
MixedFibre HC.899	Mixed Fibre	HC	0.03	-1.33	OTU_195	1	Bacteria	Halieaceae	OM60(NOR5) clade
MixedFibre HC.933	Mixed Fibre	HC	0.04	-0.43	OTU_441	1	Bacteria	Streptococcaceae	Lactococcus
MixedFibre HC.935	Mixed Fibre	HC	0.02	-0.19	OTU_443	1	Bacteria	Prevotellaceae	Prevotella 6
MixedFibre HC.937	Mixed Fibre	HC	0.02	-0.3	OTU_445	1	No blast hit	NA	NA
MixedFibre HC.1015	Mixed Fibre	HC	0.02	-1.01	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
MixedFibre CD.40	Mixed Fibre	CD	0.01	0.25	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
MixedFibre CD.164	Mixed Fibre	CD	0.04	-1.49	OTU_615	1	Bacteria	Eubacteriaceae	Anaerofustis
MixedFibre CD.196	Mixed Fibre	CD	0.04	-0.49	OTU_470	1	Bacteria	Pasteurellaceae	Haemophilus

MixedFibre CD.303	Mixed Fibre	CD	0.02	-0.45	OTU_153	1	Bacteria	Coriobacteriaceae	uncultured
MixedFibre CD.347	Mixed Fibre	CD	0.04	0.24	OTU_343	1	Bacteria	Lachnospiraceae	[Eubacterium] hallii group
MixedFibre CD.680	Mixed Fibre	CD	0.04	-0.82	OTU_74	1	Bacteria	Ruminococcaceae	Oscillibacter
MixedFibre CD.937	Mixed Fibre	CD	0.04	-0.61	OTU_445	1	No blast hit	NA	NA
MixedFibre CD.1015	Mixed Fibre	CD	0.01	-0.78	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
MixedFibre UC.40	Mixed Fibre	UC	0.04	0.99	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
MixedFibre UC.502	Mixed Fibre	UC	0.01	-0.49	OTU_410	1	Bacteria	Lachnospiraceae	Dorea
Blank HC.40	NSC	HC	0.03	1.43	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
Blank HC.64	NSC	HC	0.02	0.3	OTU_809	1	No blast hit	NA	NA
Blank HC.68	NSC	HC	0.05	-0.38	OTU_801	1	No blast hit	NA	NA
Blank HC.84	NSC	HC	0.03	-0.2	OTU_2	1	No blast hit	NA	NA
Blank HC.156	NSC	HC	0.04	-0.6	OTU_596	1	Bacteria	Coriobacteriaceae	uncultured
Blank HC.163	NSC	HC	0.03	0.36	OTU_599	1	No blast hit	NA	NA

Blank HC.289	NSC	HC	0.03	-0.58	OTU_652	1	Bacteria	Christensenellaceae	uncultured
Blank HC.306	NSC	HC	0.02	-0.69	OTU_156	1	Bacteria	NA	NA
Blank HC.308	NSC	HC	0.03	-0.37	OTU_63	1	Bacteria	Ruminococcaceae	Ruminiclostridium 5
Blank HC.329	NSC	HC	0.02	0.68	OTU_232	1	Bacteria	Christensenellaceae	Christensenellaceae R-7 group
Blank HC.339	NSC	HC	0.03	-0.32	OTU_360	1	No blast hit	NA	NA
Blank HC.384	NSC	HC	0.04	-0.6	OTU_517	1	Bacteria	Lachnospiraceae	[Eubacterium] ventriosum group
Blank HC.420	NSC	HC	0.02	0.64	OTU_765	1	Bacteria	Clostridiales vadinBB60 group	uncultured bacterium
Blank HC.445	NSC	HC	0.03	-0.43	OTU_288	1	Bacteria	Paenibacillaceae	Paenibacillus
Blank HC.448	NSC	HC	0.03	-0.38	OTU_91	1	Bacteria	Desulfovibrionaceae	Bilophila

Blank HC.480	NSC	HC	0.02	1.37	OTU_301	1	Bacteria	Lachnospiraceae	Coprococcus 1
Blank HC.573	NSC	HC	0.02	-0.76	OTU_54	1	Bacteria	Family XIII	uncultured
Blank HC.575	NSC	HC	0.02	-0.64	OTU_56	1	No blast hit	NA	NA
Blank HC.592	NSC	HC	0.05	0.48	OTU_182	1	Bacteria	Ruminococcaceae	Ruminococcaceae NK4A214 group
Blank HC.604	NSC	HC	0.05	-0.22	OTU_630	1	Bacteria	Bacillaceae	Bacillus
Blank HC.614	NSC	HC	0.05	-0.15	OTU_166	1	Bacteria	Coriobacteriaceae	Senegalimassilia
Blank HC.696	NSC	HC	0.03	-0.46	OTU_10	1	Bacteria	Erysipelotrichaceae	Holdemanella
Blank HC.740	NSC	HC	0.05	-0.19	OTU_174	1	No blast hit	NA	NA
Blank HC.746	NSC	HC	0.04	-0.34	OTU_89	1	Bacteria	Ruminococcaceae	Ruminococcus 1
Blank HC.794	NSC	HC	0.04	-0.34	OTU_402	1	Bacteria	Ruminococcaceae	Ruminococcaceae UCG-010
Blank HC.842	NSC	HC	0.02	-0.56	OTU_536	1	Bacteria	uncultured bacterium	NA

Blank HC.869	NSC	HC	0.04	-0.19	OTU_41	1	Bacteria	Ruminococcaceae	Oscillospira
Blank HC.887	NSC	HC	0.02	0.77	OTU_218	1	No blast hit	NA	NA
Blank HC.935	NSC	HC	0.02	-0.52	OTU_443	1	Bacteria	Prevotellaceae	Prevotella 6
Blank HC.937	NSC	HC	0.02	0.93	OTU_445	1	No blast hit	NA	NA
Blank HC.1015	NSC	HC	0.03	-0.54	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
Blank CD.40	NSC	CD	0.02	0.52	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
Blank CD.53	NSC	CD	0.02	0.45	OTU_430	1	No blast hit	NA	NA
Blank CD.81	NSC	CD	0.03	0.73	OTU_9	1	Bacteria	Lachnospiraceae	Lachnoclostridium
Blank CD.84	NSC	CD	0.01	-0.52	OTU_2	1	No blast hit	NA	NA
Blank CD.225	NSC	CD	0.04	-0.42	OTU_685	1	Bacteria	Ruminococcaceae	[Eubacterium] coprostanoligenes group
Blank CD.306	NSC	CD	0.02	-0.64	OTU_156	1	Bacteria	NA	NA
Blank CD.339	NSC	CD	0.04	-0.72	OTU_360	1	No blast hit	NA	NA

Blank CD.344	NSC	CD	0.04	0.39	OTU_346	1	Bacteria	Lachnospiraceae	Blautia
Blank CD.347	NSC	CD	0.01	-0.31	OTU_343	1	Bacteria	Lachnospiraceae	[Eubacterium] hallii group
Blank CD.394	NSC	CD	0.04	-0.75	OTU_258	1	No blast hit	NA	NA
Blank CD.448	NSC	CD	0.04	-0.57	OTU_91	1	Bacteria	Desulfovibrionaceae	Bilophila
Blank CD.696	NSC	CD	0.02	-0.36	OTU_10	1	Bacteria	Erysipelotrichaceae	Holdemanella
Blank CD.740	NSC	CD	0.01	-0.58	OTU_174	1	No blast hit	NA	NA
Blank CD.924	NSC	CD	0.02	2.04	OTU_361	1	Bacteria	Lachnospiraceae	uncultured
Blank CD.965	NSC	CD	0.04	1.41	OTU_968	1	No blast hit	NA	NA
Blank CD.1015	NSC	CD	0.01	-0.3	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
Blank UC.40	NSC	UC	0.02	1.71	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
Blank UC.81	NSC	UC	0.02	0.94	OTU_9	1	Bacteria	Lachnospiraceae	Lachnoclostridium
Blank UC.163	NSC	UC	0.04	0.25	OTU_599	1	No blast hit	NA	NA

Blank UC.196	NSC	UC	0.02	1.67	OTU_470	1	Bacteria	Pasteurellaceae	Haemophilus
Blank UC.306	NSC	UC	0.04	-0.31	OTU_156	1	Bacteria	NA	NA
Blank UC.308	NSC	UC	0.02	-0.5	OTU_63	1	Bacteria	Ruminococcaceae	Ruminiclostridium 5
Blank UC.329	NSC	UC	0.04	0.83	OTU_232	1	Bacteria	Christensenellaceae	Christensenellaceae R-7 group
Blank UC.339	NSC	UC	0.02	-0.48	OTU_360	1	No blast hit	NA	NA
Blank UC.408	NSC	UC	0.02	0.52	OTU_28	1	Bacteria	Family XIII	[Eubacterium] brachy group
Blank UC.420	NSC	UC	0.04	0.67	OTU_765	1	Bacteria	Clostridiales vadinBB60 group	uncultured bacterium
Blank UC.445	NSC	UC	0.04	-0.37	OTU_288	1	Bacteria	Paenibacillaceae	Paenibacillus
Blank UC.448	NSC	UC	0.03	-0.74	OTU_91	1	Bacteria	Desulfovibrionaceae	Bilophila
Blank UC.452	NSC	UC	0.02	1.17	OTU_283	1	No blast hit	NA	NA

Blank UC.480	NSC	UC	0.02	1.1	OTU_301	1	Bacteria	Lachnospiraceae	Coprococcus 1
Blank UC.502	NSC	UC	0.01	-0.62	OTU_410	1	Bacteria	Lachnospiraceae	Dorea
Blank UC.696	NSC	UC	0.04	-0.64	OTU_10	1	Bacteria	Erysipelotrichaceae	Holdemanella
Blank UC.740	NSC	UC	0.04	-0.28	OTU_174	1	No blast hit	NA	NA
Blank UC.745	NSC	UC	0.04	0.97	OTU_179	1	No blast hit	NA	NA
Blank UC.767	NSC	UC	0.01	0.49	OTU_311	1	Bacteria	Christensenellaceae	Christensenellaceae R-7 group
Blank UC.896	NSC	UC	0.01	1.46	OTU_196	1	Bacteria	Ruminococcaceae	Ruminococcaceae UCG-014
HiMaize HC.40	Hi Maize	Healthy Control	0.02	1.14	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
HiMaize HC.90	Hi Maize	Healthy Control	0.04	-1.47	OTU_5	1	Bacteria	Enterobacteriaceae	Enterobacter
HiMaize HC.101	Hi Maize	Healthy Control	0.04	-0.52	OTU_212	1	Bacteria	Lachnospiraceae	Dorea
HiMaize HC.156	Hi Maize	Healthy Control	0.04	-0.68	OTU_596	1	Bacteria	Coriobacteriaceae	uncultured

HiMaize HC.164	Hi Maize	Healthy Control	0.04	-0.46	OTU_615	1	Bacteria	Eubacteriaceae	Anaerofustis
HiMaize HC.187	Hi Maize	Healthy Control	0.04	-1.07	OTU_291	1	Bacteria	Lachnospiraceae	Blautia
HiMaize HC.196	Hi Maize	Healthy Control	0.05	-0.63	OTU_470	1	Bacteria	Pasteurellaceae	Haemophilus
HiMaize HC.248	Hi Maize	Healthy Control	0.04	-0.48	OTU_972	1	Bacteria	Christensenellaceae	Christensenellaceae R-7 group
HiMaize HC.339	Hi Maize	Healthy Control	0.03	-0.22	OTU_360	1	No blast hit	NA	NA
HiMaize HC.366	Hi Maize	Healthy Control	0.02	-0.56	OTU_900	1	Bacteria	Ruminococcaceae	Ruminococcaceae UCG-013
HiMaize HC.384	Hi Maize	Healthy Control	0.04	-0.78	OTU_517	1	Bacteria	Lachnospiraceae	[Eubacterium] ventriosum group
HiMaize HC.480	Hi Maize	Healthy Control	0.03	0.77	OTU_301	1	Bacteria	Lachnospiraceae	Coprococcus 1
HiMaize HC.573	Hi Maize	Healthy Control	0.02	-1.17	OTU_54	1	Bacteria	Family XIII	uncultured
HiMaize HC.604	Hi Maize	Healthy Control	0.02	-0.39	OTU_630	1	Bacteria	Bacillaceae	Bacillus
HiMaize HC.680	Hi Maize	Healthy Control	0.02	-0.74	OTU_74	1	Bacteria	Ruminococcaceae	Oscillibacter

HiMaize HC.692	Hi Maize	Healthy Control	0.04	-0.92	OTU_14	1	No blast hit	NA	NA
HiMaize HC.740	Hi Maize	Healthy Control	0.05	-0.6	OTU_174	1	No blast hit	NA	NA
HiMaize HC.746	Hi Maize	Healthy Control	0.04	-0.53	OTU_89	1	Bacteria	Ruminococcaceae	Ruminococcus 1
HiMaize HC.842	Hi Maize	Healthy Control	0.05	-0.47	OTU_536	1	Bacteria	uncultured bacterium	NA
HiMaize HC.867	Hi Maize	Healthy Control	0.04	-0.63	OTU_43	1	No blast hit	NA	NA
HiMaize HC.869	Hi Maize	Healthy Control	0.04	-0.87	OTU_41	1	Bacteria	Ruminococcaceae	Oscillospira
HiMaize HC.887	Hi Maize	Healthy Control	0.03	0.6	OTU_218	1	No blast hit	NA	NA
HiMaize HC.899	Hi Maize	Healthy Control	0.02	-1.42	OTU_195	1	Bacteria	Halieaceae	OM60(NOR5) clade
HiMaize HC.935	Hi Maize	Healthy Control	0.02	-0.7	OTU_443	1	Bacteria	Prevotellaceae	Prevotella 6
HiMaize HC.1015	Hi Maize	Healthy Control	0.02	-0.9	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
HiMaize CD.164	Hi Maize	CD	0.04	-0.91	OTU_615	1	Bacteria	Eubacteriaceae	Anaerofustis
HiMaize CD.303	Hi Maize	CD	0.02	-0.59	OTU_153	1	Bacteria	Coriobacteriaceae	uncultured

HiMaize CD.306	Hi Maize	CD	0.02	0.41	OTU_156	1	Bacteria	NA	NA
HiMaize CD.371	Hi Maize	CD	0.02	-0.15	OTU_907	1	Bacteria	Aeromonadaceae	Aeromonas
HiMaize CD.680	Hi Maize	CD	0.04	-0.64	OTU_74	1	Bacteria	Ruminococcaceae	Oscillibacter
HiMaize CD.740	Hi Maize	CD	0.01	-0.63	OTU_174	1	No blast hit	NA	NA
HiMaize CD.1015	Hi Maize	CD	0.01	-0.93	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
HiMaize UC.40	Hi Maize	UC	0.02	1.06	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
HiMaize UC.77	Hi Maize	UC	0.02	0.34	OTU_741	1	Bacteria	Erysipelotrichaceae	Incertae Sedis
HiMaize UC.330	Hi Maize	UC	0.02	-1.21	OTU_231	1	Bacteria	Bifidobacteriaceae	Parascardovia
HiMaize UC.394	Hi Maize	UC	0.02	-0.34	OTU_258	1	No blast hit	NA	NA
HiMaize UC.452	Hi Maize	UC	0.01	0.87	OTU_283	1	No blast hit	NA	NA
HiMaize UC.696	Hi Maize	UC	0.01	-0.6	OTU_10	1	Bacteria	Erysipelotrichaceae	Holdemanella
HiMaize UC.887	Hi Maize	UC	0.01	0.71	OTU_218	1	No blast hit	NA	NA
Pectin HC.40	Apple Pectin	Healthy Control	0.05	0.76	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella

Pectin HC.73	Apple Pectin	Healthy Control	0.04	-1.01	OTU_806	1	No blast hit	NA	NA
Pectin HC.90	Apple Pectin	Healthy Control	0.04	-1.23	OTU_5	1	Bacteria	Enterobacteriaceae	Enterobacter
Pectin HC.140	Apple Pectin	Healthy Control	0.04	-0.79	OTU_200	1	No blast hit	NA	NA
Pectin HC.156	Apple Pectin	Healthy Control	0.04	-0.67	OTU_596	1	Bacteria	Coriobacteriaceae	uncultured
Pectin HC.164	Apple Pectin	Healthy Control	0.04	-0.99	OTU_615	1	Bacteria	Eubacteriaceae	Anaerofustis
Pectin HC.187	Apple Pectin	Healthy Control	0.04	-1.24	OTU_291	1	Bacteria	Lachnospiraceae	Blautia
Pectin HC.196	Apple Pectin	Healthy Control	0.03	-0.75	OTU_470	1	Bacteria	Pasteurellaceae	Haemophilus
Pectin HC.248	Apple Pectin	Healthy Control	0.04	-1.12	OTU_972	1	Bacteria	Christensenellaceae	Christensenellaceae R-7 group
Pectin HC.280	Apple Pectin	Healthy Control	0.04	0.24	OTU_129	1	No blast hit	NA	NA
Pectin HC.303	Apple Pectin	Healthy Control	0.02	-0.67	OTU_153	1	Bacteria	Coriobacteriaceae	uncultured
Pectin HC.309	Apple Pectin	Healthy Control	0.04	-0.47	OTU_155	1	No blast hit	NA	NA
Pectin HC.335	Apple Pectin	Healthy Control	0.04	-0.39	OTU_726	1	Bacteria	Ruminococcaceae	Ruminococcaceae UCG-011

Pectin HC.366	Apple Pectin	Healthy Control	0.02	-0.77	OTU_900	1	Bacteria	Ruminococcaceae	Ruminococcaceae UCG-013
Pectin HC.461	Apple Pectin	Healthy Control	0.04	-0.7	OTU_460	1	No blast hit	NA	NA
Pectin HC.470	Apple Pectin	Healthy Control	0.05	-0.36	OTU_264	1	Bacteria	Alcaligenaceae	Sutterella
Pectin HC.573	Apple Pectin	Healthy Control	0.02	-1.26	OTU_54	1	Bacteria	Family XIII	uncultured
Pectin HC.604	Apple Pectin	Healthy Control	0.03	-0.57	OTU_630	1	Bacteria	Bacillaceae	Bacillus
Pectin HC.639	Apple Pectin	Healthy Control	0.03	-0.55	OTU_355	1	Bacteria	Ruminococcaceae	Ruminiclostridium 5
Pectin HC.680	Apple Pectin	Healthy Control	0.02	-1.14	OTU_74	1	Bacteria	Ruminococcaceae	Oscillibacter
Pectin HC.707	Apple Pectin	Healthy Control	0.04	-0.4	OTU_779	1	Bacteria	Desulfovibrionaceae	Desulfovibrio
Pectin HC.739	Apple Pectin	Healthy Control	0.03	-0.16	OTU_173	1	Bacteria	Bacteroidales S24-7 group	uncultured bacterium
Pectin HC.746	Apple Pectin	Healthy Control	0.04	-1.09	OTU_89	1	Bacteria	Ruminococcaceae	Ruminococcus 1
Pectin HC.844	Apple Pectin	Healthy Control	0.04	-0.12	OTU_101	1	Bacteria	Lachnospiraceae	Lachnospiraceae UCG-005

Pectin HC.869	Apple Pectin	Healthy Control	0.04	-0.81	OTU_41	1	Bacteria	Ruminococcaceae	Oscillospira
Pectin HC.899	Apple Pectin	Healthy Control	0.02	-1.32	OTU_195	1	Bacteria	Halieaceae	OM60(NOR5) clade
Pectin HC.933	Apple Pectin	Healthy Control	0.04	-0.6	OTU_441	1	Bacteria	Streptococcaceae	Lactococcus
Pectin HC.935	Apple Pectin	Healthy Control	0.02	-1.01	OTU_443	1	Bacteria	Prevotellaceae	Prevotella 6
Pectin HC.1015	Apple Pectin	Healthy Control	0.05	-0.61	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
Pectin CD.164	Apple Pectin	CD	0.04	-1.29	OTU_615	1	Bacteria	Eubacteriaceae	Anaerofustis
Pectin CD.196	Apple Pectin	CD	0.03	-0.78	OTU_470	1	Bacteria	Pasteurellaceae	Haemophilus
Pectin CD.284	Apple Pectin	CD	0.03	-0.84	OTU_125	1	Bacteria	Ruminococcaceae	Anaerotruncus
Pectin CD.303	Apple Pectin	CD	0.02	-0.73	OTU_153	1	Bacteria	Coriobacteriaceae	uncultured
Pectin CD.306	Apple Pectin	CD	0.02	-0.49	OTU_156	1	Bacteria	NA	NA
Pectin CD.339	Apple Pectin	CD	0.03	-0.68	OTU_360	1	No blast hit	NA	NA
Pectin CD.371	Apple Pectin	CD	0.04	-1.02	OTU_907	1	Bacteria	Aeromonadaceae	Aeromonas

Pectin CD.611	Apple Pectin	CD	0.04	-0.33	OTU_90	1	Bacteria	Lachnospiraceae	Lachnospiraceae NK4A136 group
Pectin CD.680	Apple Pectin	CD	0.04	-0.89	OTU_74	1	Bacteria	Ruminococcaceae	Oscillibacter
Pectin CD.740	Apple Pectin	CD	0.01	-0.53	OTU_174	1	No blast hit	NA	NA
Pectin CD.937	Apple Pectin	CD	0.04	-0.79	OTU_445	1	No blast hit	NA	NA
Pectin CD.1015	Apple Pectin	CD	0.01	-0.74	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
Pectin UC.77	Apple Pectin	UC	0.04	0.32	OTU_741	1	Bacteria	Erysipelotrichaceae	Incertae Sedis
Pectin UC.303	Apple Pectin	UC	0.04	-0.59	OTU_153	1	Bacteria	Coriobacteriaceae	uncultured
Pectin UC.306	Apple Pectin	UC	0.04	-0.51	OTU_156	1	Bacteria	NA	NA
Pectin UC.339	Apple Pectin	UC	0.02	-0.58	OTU_360	1	No blast hit	NA	NA
Pectin UC.445	Apple Pectin	UC	0.02	-0.56	OTU_288	1	Bacteria	Paenibacillaceae	Paenibacillus
Pectin UC.502	Apple Pectin	UC	0.02	-0.52	OTU_410	1	Bacteria	Lachnospiraceae	Dorea

Pectin UC.842	Apple Pectin	UC	0.03	0.53	OTU_536	1	Bacteria	uncultured bacterium	NA
Pectin UC.887	Apple Pectin	UC	0.04	0.6	OTU_218	1	No blast hit	NA	NA
Pectin UC.1014	Apple Pectin	UC	0.04	1.06	OTU_545	1	Bacteria	Lachnospiraceae	Lachnospiraceae UCG-004
Raftilose HC.40	Raftilose	Healthy Control	0.02	1.2	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
Raftilose HC.64	Raftilose	Healthy Control	0.04	-0.29	OTU_809	1	No blast hit	NA	NA
Raftilose HC.156	Raftilose	Healthy Control	0.04	-0.71	OTU_596	1	Bacteria	Coriobacteriaceae	uncultured
Raftilose HC.164	Raftilose	Healthy Control	0.04	-0.72	OTU_615	1	Bacteria	Eubacteriaceae	Anaerofustis
Raftilose HC.187	Raftilose	Healthy Control	0.04	-0.88	OTU_291	1	Bacteria	Lachnospiraceae	Blautia
Raftilose HC.196	Raftilose	Healthy Control	0.03	-0.45	OTU_470	1	Bacteria	Pasteurellaceae	Haemophilus
Raftilose HC.248	Raftilose	Healthy Control	0.04	-0.97	OTU_972	1	Bacteria	Christensenellaceae	Christensenellaceae R-7 group
Raftilose HC.303	Raftilose	Healthy Control	0.02	-0.51	OTU_153	1	Bacteria	Coriobacteriaceae	uncultured

Raftilose HC.306	Raftilose	Healthy Control	0.05	-0.35	OTU_156	1	Bacteria	NA	NA
Raftilose HC.335	Raftilose	Healthy Control	0.04	-0.5	OTU_726	1	Bacteria	Ruminococcaceae	Ruminococcaceae UCG-011
Raftilose HC.351	Raftilose	Healthy Control	0.02	0.3	OTU_426	1	No blast hit	NA	NA
Raftilose HC.366	Raftilose	Healthy Control	0.02	-0.44	OTU_900	1	Bacteria	Ruminococcaceae	Ruminococcaceae UCG-013
Raftilose HC.384	Raftilose	Healthy Control	0.04	-0.74	OTU_517	1	Bacteria	Lachnospiraceae	[Eubacterium] ventriosum group
Raftilose HC.573	Raftilose	Healthy Control	0.02	-1.32	OTU_54	1	Bacteria	Family XIII	uncultured
Raftilose HC.575	Raftilose	Healthy Control	0.02	-0.57	OTU_56	1	No blast hit	NA	NA
Raftilose HC.589	Raftilose	Healthy Control	0.04	-0.4	OTU_181	1	Bacteria	Ruminococcaceae	Ruminococcaceae UCG-014
Raftilose HC.604	Raftilose	Healthy Control	0.02	-0.56	OTU_630	1	Bacteria	Bacillaceae	Bacillus
Raftilose HC.639	Raftilose	Healthy Control	0.04	-0.51	OTU_355	1	Bacteria	Ruminococcaceae	Ruminiclostridium 5
Raftilose HC.680	Raftilose	Healthy Control	0.02	-0.46	OTU_74	1	Bacteria	Ruminococcaceae	Oscillibacter

Raftilose HC.692	Raftilose	Healthy Control	0.04	-0.84	OTU_14	1	No blast hit	NA	NA
Raftilose HC.867	Raftilose	Healthy Control	0.04	-0.48	OTU_43	1	No blast hit	NA	NA
Raftilose HC.869	Raftilose	Healthy Control	0.04	-0.66	OTU_41	1	Bacteria	Ruminococcaceae	Oscillospira
Raftilose HC.899	Raftilose	Healthy Control	0.02	-1.12	OTU_195	1	Bacteria	Halieaceae	OM60(NOR5) clade
Raftilose HC.937	Raftilose	Healthy Control	0.03	-0.38	OTU_445	1	No blast hit	NA	NA
Raftilose HC.999	Raftilose	Healthy Control	0.05	0.4	OTU_663	1	Bacteria	Porphyromonadaceae	Parabacteroides
Raftilose HC.1015	Raftilose	Healthy Control	0.03	-0.77	OTU_544	1	Bacteria	Ruminococcaceae	Ruminococcus 1
Raftilose CD.225	Raftilose	CD	0.04	-0.34	OTU_685	1	Bacteria	Ruminococcaceae	[Eubacterium] coprostanoligenes group
Raftilose CD.284	Raftilose	CD	0.02	-0.69	OTU_125	1	Bacteria	Ruminococcaceae	Anaerotruncus
Raftilose CD.371	Raftilose	CD	0.02	-0.99	OTU_907	1	Bacteria	Aeromonadaceae	Aeromonas
Raftilose CD.680	Raftilose	CD	0.04	-0.64	OTU_74	1	Bacteria	Ruminococcaceae	Oscillibacter

Raftilose CD.846	Raftilose	CD	0.04	-0.21	OTU_103	1	Bacteria	Clostridiales vadinBB60 group	uncultured bacterium
Raftilose CD.937	Raftilose	CD	0.04	-0.62	OTU_445	1	No blast hit	NA	NA
Raftilose UC.40	Raftilose	UC	0.02	0.87	OTU_395	1	Bacteria	Lachnospiraceae	Tyzzerella
Raftilose UC.303	Raftilose	UC	0.04	-0.47	OTU_153	1	Bacteria	Coriobacteriaceae	uncultured
Raftilose UC.347	Raftilose	UC	0.02	0.3	OTU_343	1	Bacteria	Lachnospiraceae	[Eubacterium] hallii group
Raftilose UC.394	Raftilose	UC	0.02	-0.85	OTU_258	1	No blast hit	NA	NA
Raftilose UC.445	Raftilose	UC	0.04	-0.47	OTU_288	1	Bacteria	Paenibacillaceae	Paenibacillus
Raftilose UC.480	Raftilose	UC	0.02	0.29	OTU_301	1	Bacteria	Lachnospiraceae	Coprococcus 1

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