Investigating the influence of dietary fibre on intestinal health

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"Dis-moi ce que tu manges, je te dirai ce que tu es."

– Anthelme Brillat-Savarin, 1826

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Declaration

I hereby certify that the work presented in this thesis titled “Investigating the influence of dietary fibre on intestinal health” is an original piece of research and is the result of my own work except where appropriately acknowledged.

This work has not been submitted as part of the requirements for a degree to any other university or institution other than Macquarie University. I consent to a copy of this thesis being available in the Macquarie University library.

Raymond Wei Wern Chong

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Statement of Manuscript Authorship

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R. W. W. Chong was responsible for the conception and design of the experiments, collection of data, and preparation of the manuscript. M. Ball, C. McRae and N.H. Packer contributed to the interpretation of the data and edited the final manuscript.

Chapter 3

Gamage, H.K., Tetu, S.G., **Chong, R.W.**, Bucio-Noble, D., Rosewarne, C.P., Kautto, L., Ball, M.S., Molloy, M., Packer, N.H. and Paulsen, I.T., 2018. **Fibre Supplements Derived from Sugarcane Stem, Wheat Dextrin And Psyllium Husk Have Different In Vitro Effects On The Human Gut Microbiota.** *Frontiers in Microbiology*, *9*, p.1618.

R. W. W. Chong contributed the quantitative determination of short chain fatty acids in supernatants obtained following the *in vitro* digestion of dietary fibres and incubation with human faecal samples. I also contributed the chemical composition data of NutriKane, psyllium husk, and Benefibre as determined by chemical analysis.

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R. W. W. Chong contributed the quantitative determination of short chain fatty acids in supernatants obtained following the *in vitro* digestion of cereal products and incubation with human faecal samples.

Chapter 4

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Abstract

Increasing the consumption of dietary fibre has long been implicated in the maintenance of intestinal health and the reduced risk of non-communicable diseases including type 2 diabetes, cardiovascular disease and colorectal cancer. As a category, dietary fibre encompasses a variety of molecules that comprise a diverse range of physical properties and physiological activities. To examine the effect of chemical composition on physiological activity, three dietary fibres were characterised and compared. NutriKane™, a fibre derived from sugarcane was an insoluble fibre and contained chromium and manganese, which are rarely found in foods, and phenolic compounds with antioxidant activity. In comparison, Benefiber™ and psyllium husk, while both soluble fibres exhibited drastically different physical properties due to their characteristic carbohydrate content. To further investigate the effect of dietary fibre on intestinal health, we compared the effects of these fibres on the gut microbiota and mucus layer using *in vitro* and *in vivo* systems respectively. In contrast to Benefiber™ and psyllium husk, which were readily fermented *in vitro*, NutriKane™ was less effective in stimulating short chain fatty acid production emphasizing significant differences in the metabolic activity of soluble and insoluble dietary fibres. We next characterised the glycosylation of MUC2, the major component of the mucus layer, from C57BL/6 mice given 60% fat diets modified with dietary fibre. We found that dietary fibre modification, but not high fat content is able to alter MUC2 glycosylation highlighting a novel interaction between diet and the host mucus layer. Our findings demonstrate that not all dietary fibres are equal and variations in carbohydrate content contribute to the unique physical properties and metabolic activity of fibres. Overall our findings highlight the complexity of interactions between diet, the gut microbiota, and the host mucus layer and support the development of intuitive dietary interventions for the improvement of intestinal health.

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Abbreviations

AES	Atomic emission spectroscopy
ANOVA	Analysis of variance
AX	Arabinoxylan
BF	Benefibre
BGL	Blood glucose level
BMI	Body mass index
C1GalT1	Core 1 β1-3-galactosyltransferase
C2GnTs	Core 2 β1-6-*N*-acetylglucosaminyltransferases
C3GnT	Core 3 β1-3- *N*-acetylglucosaminyltransferase
CAZymes	Carbohydrate active enzymes
COX 2	Prostaglandin-endoperoxide synthase 2
CRP	C-reactive protein
CVD	Cardiovascular diseases
DCM	Dichloromethane
DF	Dietary fibre
DNA	Deoxyribonucleic acid
EI	Electron ionisation
ESI	Electrospray ionisation
FBGL	Fasting blood glucose level
FID	Flame ionisation detection
GC	Gas chromatography
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
GLUT4	Glucose transporter type 4
HCl	Hydrochloric acid
HDL-c	High density lipoprotein cholesterol
HF	High fat diet
HF-NK	High fat diet modified with Nutrikane (sugarcane fibre)
HF-BF	High fat diet modified with Benefibre (wheat dextrin)
HPLC	High performance liquid chromatography
IL	Interleukin
iNOS	Nitric oxide synthase
IDF	Insoluble dietary fibre
LC	Liquid chromatography
LDL-c	Low density lipoprotein cholesterol
NC	Normal chow

NCDs	Noncommunicable diseases
NF-κB	Nuclear Factor kappa-light-chain-enhancer
NK	NutriKane
MS	Mass spectrometry
MP-AES	Microwave plasma atomic mission spectroscopy
mRNA	Messenger ribonucleic acid
MUC	Mucin
MUC2	Mucin 2
Myd88	Myeloid differentiation factor 88
OTU	Operational taxonomic unit
PGC	Porous graphitised carbon
PH	Psyllium husk
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RS	Resistant starch
SC	Sugarcane fibre
SCFA	Short chain fatty acid
SD	Standard deviation
SDF	Soluble dietary fibre
SEM	Standard error of the mean
T1DM	Type 1 Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
VNTR	Variable number of tandem repeats
TFA	Trifluoroacetic acid
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
WHC	Water holding capacity

Glycan monosaccharide abbreviations

Fuc	Fucose
Gal	Galactose
GalNac	*N*-acetylgalactosamine
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
Hex	Hexose
NeuAc	Sialic acid
Sulf	Sulfate

Chapter 1: Introduction

1.1 The influence of diet on human health and disease

Despite rapid technological progress and improvements to living standards and modern medicine, development towards modern societies has caused significant shifts in diet and lifestyle conditions (Cordain et al., 2005). These shifts have coincided with a dramatic increase in the occurrence of chronic non-communicable diseases (NCDs) including type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVDs), respiratory disease, hypertension, stroke, and some specific cancers (Amine et al., 2002; Beaglehole et al., 2011). In 2015, dietary risks such as high consumption of sodium, sugar, trans and polyunsaturated fats, and low consumption of fruit, vegetables, whole grains, nuts and seeds, became the leading risk factors contributing to disease burden and premature death across the globe (Forouzanfar et al., 2016).

In comparison with rural African societies, where NCDs such as CVDs and colon cancer are virtually non-existent, African Americans experienced similar rates of NCDs to Caucasian Americans, ruling out most genetic effects (Burkitt, 1969). In addition, epidemiologists have long observed that NCD rates among migrants from low-risk countries tend to increase upon migration to high-risk countries (Hastings et al., 2016; Kato et al., 1973; Marmot et al., 1975). This trend strongly suggests that environmental factors, such as diet and lifestyle patterns, instead of genetic factors, are the primary determinants of NCDs (Willett et al., 2006). These findings are significant because they demonstrate that NCDs are not the inevitable consequences of modern society but rather the result of accumulated dietary and lifestyle risk factors that are identifiable and can be modified. Despite the decline of infectious disease related death, the total annual number of deaths resulting from NCDs is projected to increase from 28 million in 2012 to 52 million by 2030 (Mendis, 2014), thus the development of effective dietary and lifestyle interventions is imperative to relieving global disease burden.

1.1.1 An overview of non-communicable diseases affected by dietary intake

As their name suggests, non-communicable diseases (NCDs) are a category of diseases that are not capable of being passed from one person to another, either by viral, fungal or bacterial direct transmission or via a non-human vector, such as mosquitos. Instead, NCDs are often acquired through the accumulation of environmental factors, genetic predisposition, gender, age, and can even be influenced by socioeconomic status and geography (Beaglehole et al., 2011; Miranda et al., 2008). The NCD category encompasses a range of disease groups including CVDs, cancers, diabetes, chronic respiratory disease, asthma, chronic kidney disease, arthritis, and even mental illnesses (Beaglehole et al., 2011). Using disability-adjusted life years (DALYs), a statistical metric developed to quantify the number of healthy years of life lost, global disease burden due to chronic disease can be measured and compared to define associated risk factors (Murray, 1994). Just four NCDs (CVDs, cancers, respiratory diseases, and diabetes) account for over 80% of global disease burden (Forouzanfar et al., 2016)(**Figure 1-1**). Three of these (CVDs, diabetes, and cancers) are strongly associated with dietary risks and were the largest contributors to disease burden in 2015, highlighting the severe impact of poor diet on human health and disease.

1.1.1.1 Cardiovascular diseases

Cardiovascular diseases (CVDs) are a group of diseases concerned with complications of the heart and blood vessels. The total number of CVD related deaths is projected to increase from 17.5 million in 2012, to 22.2 million in 2030 (Mendis, 2014). According to the WHO, CVDs are the leading cause of death globally with an estimated 17.9 million people died from CVDs in 2016, of which 85% were due to heart attack and stroke (World Health Organization, 2017). The most common CVDs include hypertension, atherosclerosis, stroke, heart attack, congestive heart failure, and coronary heart disease. The leading cause of CVD related death, coronary heart disease, is characterised by narrowed arteries and reduced blood flow to the heart due to the build-up of fatty plaque material (atherosclerosis), ultimately resulting in heart attack.

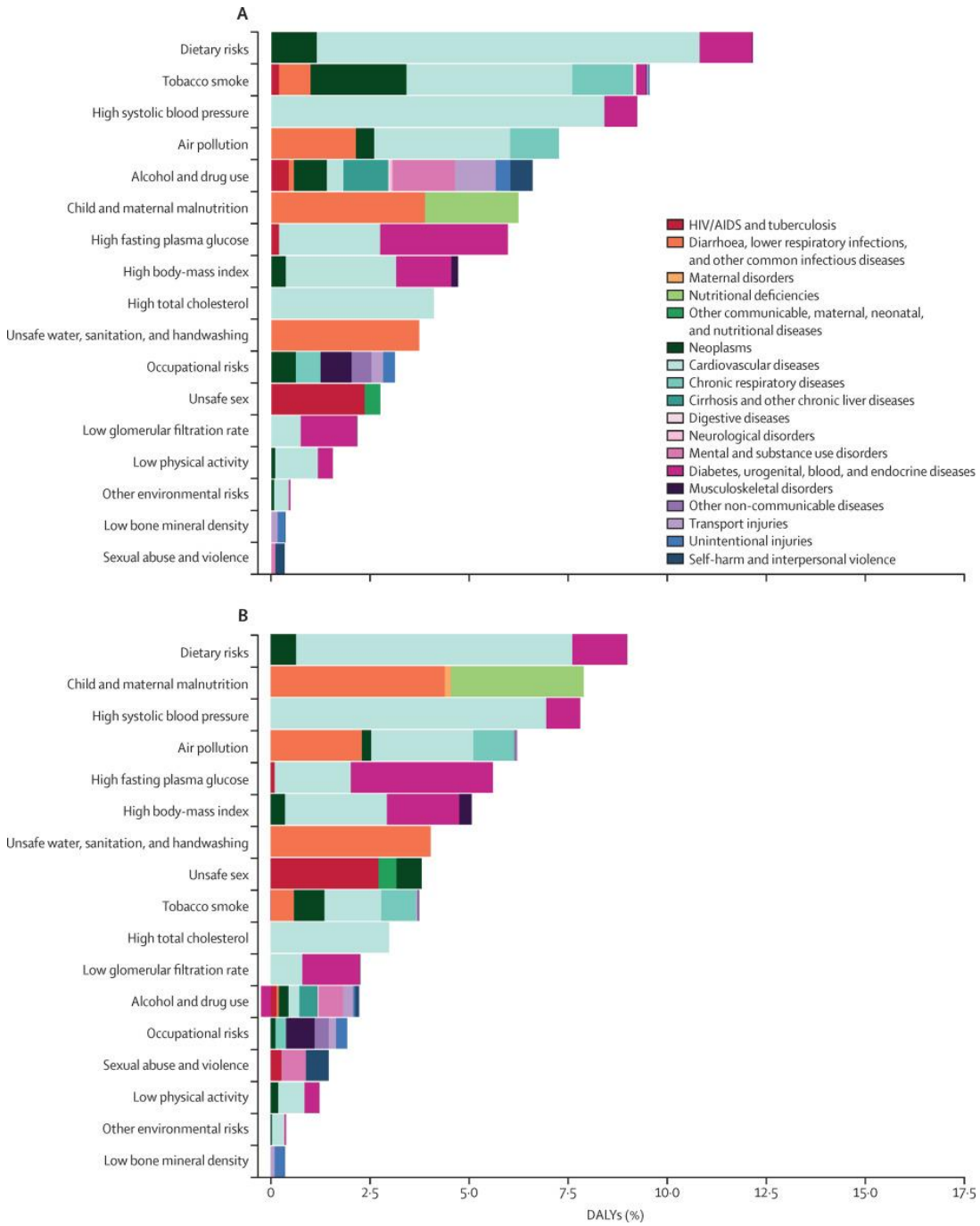

Figure 1-1. Global disability-adjusted life-years (DALYs), a measure of overall disease burden attributable to major risk factors for (A) men and (B) women in 2015. Dietary risks are the leading cause of overall disease burden attributed to CVDs, diabetes and cancer (labelled as neoplasms in legend)[1].

[1]Reproduced from Forouzanfar et al. 2016. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet*. 388:1659-172 . This article is available under the terms of the Creative Commons Attribution License Attribution 4.0 International (CC BY 4.0).

Blood pressure itself is determined by just two variables: peripheral vascular resistance and cardiac output. The precise genetic and physiological mechanism resulting in hypertension involves an extensive list of genes and pathways (Padmanabhan et al., 2015). Atherosclerosis is most commonly associated with elevated levels of low-density lipoprotein cholesterol (LDL-c) >3.36 mmol/L (130 mg/dL) and reduced levels of high-density lipoprotein cholesterol (HDL-c) <1.03 mmol/L (40 mg/dL) (Martin et al., 2014). The presence of LDL-c followed by an oxidation event to oxLDL-c is thought to induce the expression of proinflammatory genes and cell adhesion molecules resulting in the accumulation of circulating LDL and macrophages to form a fatty plaque (Scott, 2004). Risk factors for CVDs include genetic predisposition, dietary risks, physiological factors like excess body weight, and lifestyle patterns including lack of physical activity, smoking, and excessive alcohol intake (Lackland and Weber). Specific dietary risks such as high sodium and cholesterol intake have been directly linked to an increased risk of developing cardiovascular disease and reducing sodium and cholesterol intake is associated with a decreased risk (Anderson et al., 1987; Cook et al., 2007; Hastings et al., 2016).

1.1.1.2 Diabetes mellitus

Diabetes mellitus occurs when insulin, the hormone that regulates the concentration of glucose in the blood, is either inadequately produced and secreted by the pancreas or cannot effectively be used by the body due to resistance to endogenous insulin (American Diabetes Association, 2014). Global prevalence of diabetes was estimated to increase from 285 million in 2010 to 439 million in 2030 with considerable variation depending on the socioeconomic status of individual countries (Shaw et al., 2010). According to the WHO, an estimated 1.6 million deaths were directly caused by diabetes in 2016 (World Health Organization, 2018b).

There are two types of diabetes. Type 1 diabetes mellitus (T1DM) accounts for 5 – 10% of diabetes cases and is characterised by little to no insulin secretion resulting from autoimmune destruction of the β-cells of the pancreas (American Diabetes Association, 2014). This causes periods of acute

hyperglycaemia (increases in blood sugar level) leading to ketoacidosis, which results in dangerous levels of ketones in the blood that are potentially fatal without immediate treatment by insulin supplementation. There are many genetic predispositions to developing T1DM while environmental risk factors are still poorly defined (American Diabetes Association, 2014). Furthermore, obesity is rarely associated with T1DM suggesting low levels of dietary risk. Type 2 diabetes mellitus (T2DM) accounts for 90 – 95% of diabetes cases and is characterised by initial insulin resistance followed by relative insulin deficiency as the disease progresses (American Diabetes Association, 2014). This form of diabetes often goes undiagnosed for many years and does not require insulin supplementation in the early stages.

The primary cellular mechanism for insulin resistance is thought to involve defective activation of insulin receptor substrate-1 (IRS-1), the initial member of the insulin action cascade, by decreased tyrosine and increased serine phosphorylation, which attenuates the insulin signal (Leahy, 2005). As the condition progresses, β-cell function deteriorates due to a combination of glucose toxicity, whereby elevated levels of glucose alter β-cell metabolic pathways, enzymes, and genes; and β-cell exhaustion, when β-cell function becomes impaired by unsustainable periods of compensatory insulin secretion (Leahy, 2005). While genetic predisposition is believed to influence the acquisition and progression of T2DM, specifics of the genetics are still unclear. Though risk generally increases with age, T2DM is most commonly associated with the obese phenotype indicating a strong influence of physical inactivity, and dietary risks such as low fruit, vegetable, wholegrain, nut and seed intake, and high sodium, red meat, and processed meat intake (American Diabetes Association, 2014). A large body of evidence in animal models strongly suggests an association between high dietary fat and insulin impairment (Winzell and Ahrén, 2004). Prospective cohort studies in human males suggest that the Western dietary pattern characterised by high consumption of red meat, processed meat, refined grains, high-fat dairy products, high-sugar desserts and drinks is associated with substantial risk for T2DM (van Dam et al., 2002; Williams et al., 2000).

1.1.1.3 Cancer

Cancer encompasses the large group of potentially lethal diseases characterised by the presence of neoplasms, defined as abnormal growths of tissue and classified according to the area of origin and by the extent they have spread to other systems of the body. In 2012, there were an estimated 14 million new cases of cancer and 8.2 million deaths (Ferlay et al., 2015) and these numbers are expected to increase to 26 million new cases and 17 million deaths by 2030 (Are et al., 2013). According to the WHO, cancer is the second leading cause of death globally with an estimated 9.6 million deaths in 2018 spread across lung (2.09 million), breast (2.09 million), colorectal (1.80 million), prostate (1.28 million), skin cancer (non-melanoma) (1.04 million), and stomach cancers (1.03 million) (World Health Organization, 2018a).

The pathogenesis of cancer is a complicated process and is dependent on a vast number of genetic and environmental factors. The formation of the initial abnormal growth (neoplasm or tumour), typically develops through the acquisition of genetic mutations from exposure to mutagens such as tobacco smoke and ultraviolet light, or random events during cellular replication. These initial mutations are called “driver” genes that grant a selective growth advantage allowing the neoplasm to outgrow surrounding epithelial cells, becoming a small adenoma (Vogelstein et al., 2013). Adenomas are considered benign since they lack the ability to invade surrounding tissues. Further accumulation of genetic mutations results in the progression of an adenoma to an adenocarcinoma (carcinoma), which are now capable of penetrating the basement membrane and are considered malignant (Vogelstein et al., 2013). Malignant carcinomas that have migrated away from their site of origin have “metastasized” and are the main cause of cancer related death. Metastasis to vital organs or surgically inaccessible sites, such as the liver, brain, lung, or bone, present a particularly poor prognosis and survival rate since uncontrolled tumour growth causes blockages and can compromise the function of these organs.

Despite the valid assumption that there exists a set of genetic alterations responsible for metastasis, intensive investigation has yet to identify a common and distinguishing set of metastatic genes. Strong correlations between cancer incidence and human development have been identified with lung, breast, colorectal cancer and prostate cancer, which are commonly considered “Western” diseases, more prevalent in developed countries suggesting a contribution of lifestyle factors to cancer incidence (Jemal et al., 2010). While exposure to mutagens is a major risk factor for cancer, dietary risks are estimated to account for 20 – 30% of all cancers (Key et al., 2002). Following exposure to tobacco smoke, dietary pattern and alcohol intake were the second and third leading cause of global DALYs resulting from neoplasms in men in 2015 (Forouzanfar et al., 2016)(**Figure 1-1**). A landmark intervention study by O'Keefe et al. (2015) found that exchanging the high-fibre, low-fat diet of rural South Africans with a low-fibre, high-fat diet typical of African Americans resulted in a significant increase in biomarkers of colorectal cancer risk, while reciprocal changes to a high-fibre, low-fat diet in African Americans reversed these changes. Systematic review of prospective studies has shown an inverse association between high intake of total dietary fibre, cereal fibre, and whole grains, with a 10% reduction in risk of colorectal cancer for each 10 g intake of fibre daily (Aune et al., 2011). High consumption of red meat and processed meat has also been associated with an increase in colorectal cancer (Chan et al., 2011; Larsson and Wolk, 2006).

1.1.1.4 Intestinal health

Despite their widespread use in the scientific literature, the terms ‘gut health’ and ‘intestinal health’ are relatively undefined and lack clear means for objective assessment (Bischoff, 2011). The World Health Organisation (WHO) definition of 'health' from 1948 proposes a positive definition instead of 'the absence of diseases'. As such, intestinal health can be understood as a state of physical and mental well-being in the absence of intestinal complaints that require the consultation of a doctor. The main difficulty in defining gut health lies in the long list of potential complaints that are subjective in diagnosis and can include flatulence, bloating, regurgitation, heartburn,

nausea, vomiting, constipation, diarrhoea, food intolerance, incontinence, abdominal pain and cramps, loss of appetite, weight loss and blood in stool, and many of these symptoms can be indicative of both relatively harmless and life-threatening disease (Cummings et al., 2004). While the details of intestinal health are complex, there are several underlying mechanisms by which poor intestinal health can influence systemic health. These include impaired digestion and nutrition, inflammation, dysbiosis of the gut microbiota, and compromise of the intestinal barrier (Bischoff, 2011).

As the primary site of digestion, the intestine is responsible for the absorption of food, water, and minerals from the diet. Abnormal nutritional status includes the presence of food allergies (Chafen et al., 2010) or conditions such as coeliac disease (Tack et al., 2010) that prevent the consumption of specific types of foods. Impaired digestion or abnormal nutrition also contributes to irregular bowel movements such constipation and diarrhoea, and feelings of discomfort including flatulence, bloating, heartburn, and nausea.

The inflammatory response combats infection and tissue injury through the recruitment of innate immune cells such as macrophages, fibroblasts, mast cells, and dendritic cells, leukocytes, monocytes and neutrophils (Newton and Dixit, 2012). The presence of inflammation in the gut has several causes, mechanisms, intensities, and outcomes (Wirtz et al., 2017). These can include infection, environmental and dietary exposure, which result in binding of receptors such as Toll-like receptors (TLRs), tumour necrosis factor (TNF) and interleukins (IL), G-protein coupled receptors (GPCRs), integrins, selectins, and others to their ligands triggering the formation of multi-subunit signalling complexes. Inflammatory bowel diseases such as Crohn's disease (CD) and ulcerative colitis (UC) are characterized by chronic diarrhea and abdominal pain. Systemic low grade inflammation has also been associated with diseases such as obesity, diabetes, and cancer (Coussens and Werb, 2002; Mantovani et al., 2008).

The gastrointestinal tract is home to trillions of microorganisms that contribute to resistance to pathogenic infection, breakdown of non-digestible carbohydrates, and production of metabolites absorbed by the host (Macfarlane and Macfarlane, 1997). Dysbiosis describes a state of microbial imbalance that has been associated with many intestinal disorders including inflammatory bowel disease and has been implicated in extra-intestinal disorders include allergy, asthma, metabolic syndrome, cardiovascular disease, and obesity (Carding et al., 2015).

Rather than a static mechanical barrier, the intestinal barrier is in fact a functional entity comprising epithelial defence, a mucosal immune system, metabolic function and the site of communication between host and the luminal contents (Fasano and Shea-Donohue, 2005; Groschwitz and Hogan, 2009). Diet is a risk factor that drives symptoms of these disorders.

1.1.1.5 Obesity

The WHO classifies overweight and obese individuals according to body mass index (BMI) as shown in **Table 1-1**. In 2005, an estimated 937 million adults were considered overweight and 396 million were considered obese and this number is projected to increase to 1.35 billion overweight and 573 million obese individuals by 2030 (Kelly et al., 2008). According to the WHO, worldwide obesity has tripled since 1975 with an estimated 1.9 billion adults, 18 years and older, were overweight in 2016, of which 650 million were obese (World Health Organization, 2018c).

Before the year 2000, epidemiological studies had found that obesity was prevalent in developed countries and confined to individuals of higher socioeconomic status; however, by 2003 this was no longer the case as rates of obesity in lower to middle income economies and demographics began to rise (Monteiro et al., 2004). While the pathogenesis of obesity is a complex interaction between genetics, metabolism, and environmental factors, diet and physical activity level have a strong influence on the development of obesity.

The consumption of energy dense hypercaloric meals results in the storage of excess energy in the form of white adipose tissue as either subcutaneous fat around the abdomen, or visceral fat surrounding the organs (Berry et al., 2013). Additionally, low levels of physical activity decrease energy expenditure, disrupting the energy balance equation and resulting in overall weight gain.

Table 1-1. Classification of overweight and obese adults according to body mass index (BMI, height/weight^2).Berry et al. (2013)

Classification	BMI
Underweight	<18.5
Normal range	18.5 – 24.9
Overweight	>25.0
Pre-obese	25.0 – 29.9
Obese class I	30.0 – 34.9
Obese class II	35.0 – 39.9
Obese class III	>40.0

At the biochemical level, obesity is associated with increased expression of inflammatory markers including C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), leptin, and adiponectin (Das, 2001). Previous studies have also implicated the involvement of microbial communities in the gut as regulators of obesity (Ley et al., 2006b; Schwiertz et al., 2010; Turnbaugh et al., 2008). Studies in mice found that conventionally raised mice that harbored a normal microbiota contained significantly more total body fat than germ free mice devoid of any microorganisms. Furthermore, when transplanted with a normal microbiota, germ free mice experienced a dramatic increase in their total fat content, a result that was not limited to gender, age, or even strain (Bäckhed et al., 2004). While not considered a discrete NCD, obesity is a major risk factor for the main NCDs, particularly T2DM and cardiovascular disease, and also greatly increases the risk of developing metabolic syndrome (Furukawa et al., 2017).

1.1.1.6 Metabolic syndrome

Metabolic syndrome is used to describe the coexistence of several risk factors for CVD and T2DM including hypertension, low fasting HDL-c, hyperglycemia, high blood triglycerides, glucose intolerance and an overweight to obese BMI classification (Kaur, 2014). In 2009, a joint statement from International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity set out to establish a set of criteria for the diagnosis of metabolic syndrome (**Table 1-2**). The presence of any 3 out of 5 risk factors constitutes a diagnosis of metabolic syndrome.

Table 1-2. Criteria for clinical diagnosis of the metabolic syndrome. Taken from O'Neill and O'Driscoll (2015)

Measure	**Categorical Cut Points**
Elevated waist circumference	Population- and country-specific definitions
Elevated triglycerides	≥150 mg/dL (1.7 mmol/L)
Reduced HDL-c	<40 mg/dL (1.0 mmol/L) in males
	<50 mg/dL (1.3 mmol/L) in females
Elevated blood pressure	Systolic ≥130 and/or diastolic ≥85 mm Hg
Elevated fasting glucose	≥100 mg/dL

Worldwide prevalence of the metabolic syndrome is estimated to be anywhere between 10 and 84% of the total population depending on ethnicity, age, gender, and region (Kaur, 2014). High BMI and obesity are the most prevalent risk factors for metabolic syndrome indicating the powerful influence of diet and lifestyle. Since the pathogenesis of metabolic syndrome is a combination of the risk factors described for CVDS, T2DM, and obesity, several dietary risks are involved; these include low fruit, vegetable, wholegrain, nut and seed intake, high sodium and cholesterol intake, and consumption of energy dense meals.

1.1.2 Dietary intervention for the treatment and prevention of non-communicable diseases

It has become increasingly clear that diet exerts considerable influence on the quality of health and incidence of NCDs as described above. Dietary supplementation is an effective strategy that is convenient to adopt with relatively little change to diet or lifestyle. The influence of diet also presents an ideal target for intervention and preventative treatment. A WHO initiated review of dietary and lifestyle intervention programs found that the most effective strategies involved early intervention in schools with educational programs encouraging the consumption of fruits and vegetables (World Health Organization, 2009).

1.2 Food as medicine

The concept of food as medicine has been explored since antiquity with Hippocrates, the father of modern medicine, stating “Let food be thy medicine and medicine be thy food” almost 2500 years ago (Smith, 2004). Some of the earliest diseases to be treated with this philosophy were rickets, pellagra, scurvy, beriberi, and other diseases of deficiency. Treatment was relatively simple once the deficient substance was identified and in 1912, Casimir Funk described how these diseases could not only be completely cured but also prevented by supplementation of the diet with these deficient substances, which he called ‘vitamines’ and would later become known as vitamins (Funk, 1912). The consequence of these discoveries was the near complete eradication of deficiency diseases and a focus on improving nutritional guidelines for populations. Since then, dietary supplementation and food fortification has become an effective form of preventative medicine. The most prominent example can be seen in the case of folate or folic acid (vitamin B9) deficiency. In the late 1980s, a series of studies implicated pre-conception folate consumption with the prevention of neural tube defects in new-born infants. These findings were eventually confirmed in 1991 by the Medical Research Council Vitamin Study, which found that consumption of folic acid had a 72% protective effect when compared against a mixture of other vitamins

(1991). In response, widespread fortification of grain products was adopted in the United States, making folic acid one of the most successful dietary interventions to date (Crider et al., 2011).

1.2.1 Functional foods and nutraceuticals

With an ageing health-conscious population and increasing interest in the health benefits of unexplored foods, the demand for health-promoting food products has created an entire industry of food based preventative medicine. The terms ‘functional foods’ and ‘nutraceuticals’ have been created to describe these products. Functional foods are foods that resemble traditional foods but possess demonstrated physiological benefits beyond their nutritional properties (Gul et al., 2016). The European Food Information council states that functional foods must contain biologically active components that have the potential to optimize physical and mental well-being and which may also reduce the risk of disease. Functional foods can be novel preparations of traditional food, fortified foods, dietary supplements, or traditional foods with high quantities of biological active compounds. For example, grain products fortified with folic acid as mentioned above can be considered functional foods. On the other hand, the term nutraceutical was coined in 1989 by Stephen DeFelice as a portmanteau of the words, nutrition and pharmaceutical (Kalra, 2003). A nutraceutical was defined as “a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease”. Nutraceuticals are distinguished from functional foods in that they need not appear as traditional foods and can be delivered as extracts or concentrates in a non-food matrix and are usually marketed in a similar fashion to medications.

With the rise of NCDs like CVD and T2DM that are strongly associated with dietary risk, it is somewhat paradoxical that in the wake of an epidemiological era marred by diseases of undernutrition, we are now facing the looming threat of diseases of overnutrition that is fast approaching pandemic proportions. As such, the field of functional foods and nutraceuticals is attracting significant attention and experiencing a period of accelerated growth in response to the demands of an ageing population that is becoming increasingly aware of the dangerous and

potentially lethal consequences of poor diet and lifestyle factors (Daliri and Lee, 2015; Espín et al., 2007; Gul et al., 2016). In 2013, the net worth of the entire functional food and nutraceutical market was estimated at $175 billion (US dollars) and has been projected to increase to $250 billion in 2018, and $278 billion by 2021 (Daliri and Lee, 2015). The appeal of functional foods and nutraceuticals is twofold: naturally derived products are perhaps mistakenly believed to be more beneficial to health in the eyes of the consumer, and less stringent regulatory constraints regarding food products allow these products to reach market considerably faster and cheaper than pharmaceutical drugs (Siró et al., 2008).

1.2.2 Dietary fibre as a promoter of health

Many epidemiological studies have shown that increased consumption of fruits and vegetables is inversely associated with mortality and incidence of chronic diseases, especially those implicated with high dietary risk (**Table 1-3**). This association is frequently attributed to the presence of significant quantities of dietary fibre, plant based secondary metabolites, and the low energy-density of fruits and vegetables. The inverse relationship between dietary fibre consumption and risk factors for NCD incidence, particularly CVD and T2DM is well established (Brownlee et al., 2017; Kendall et al., 2010). An increase in dietary fibre intake of 7 grams per day has been shown to decrease the risk of CVD by at least 9% (Threapleton et al., 2013). A strong inverse association has been identified between dietary fibre intake and incidence of diabetes (Meyer et al., 2000). A similar inverse association between intake of dietary fibre, cereal fibre, and whole grains, and risk of colorectal cancer has also been identified (Aune et al., 2011). Interest in the health benefits of dietary fibre is steadily increasing as it represents an abundantly available, chemically diverse yet easily obtainable fraction of plants that is relatively straightforward to employ in the context of food as preventative medicine.

1.2.3 Defining dietary fibre

The earliest definition of dietary fibre (DF) was proposed by Hugh Trowell as "the skeletal remains of plant cells that are resistant to digestion by enzymes of man" (Trowell, 1972). This definition distinguished DF from crude fibre, which was the portion of plant carbohydrate that resisted extraction by acid and subsequent base (Van Soest, 1963). The need to distinguish dietary from crude fibre was due to the hypothesis of Trowell in that DF but not crude fibre was associated with protection from diseases such as diverticular disease, appendicitis, and cancer of colon. Shortly after, in response to reports that guar gum, a polysaccharide extracted from guar beans, possessed cholesterol and blood sugar level reducing activity, a redefinition was proposed to include all cell wall associated polysaccharides and not just structural polysaccharides (Trowell et al., 1976).

Table 1-3. Summary of epidemiological studies investigating the effect of fruit and vegetable consumption on mortality and incidence of disease

Study intake parameters	Study outcome	Ref
More than:		
27 servings per month	20% reduced stroke mortality	(Hjartåker et al., 2015)
	8–10 % reduced all-cause mortality	
3 servings per day	27% reduced stroke incidence	(Bazzano et al., 2002)
	15% reduced all-cause mortality	
Increase intake by:		
150g per day	6–28% reduction in cancer incidence	(van't Veer et al., 2007)
	6–22% reduction in CVD related death	
1.15 servings per day	14% reduced in T2DM incidence	(Carter et al., 2010)
1.86 servings per day	25% reduced risk of obesity	(He et al., 2004)
1 serving per day	6% reduced risk of ischemic stroke	(Joshipura et al., 1999)

Since then, there have been many revisions to expand the definition of dietary fibre, which often coincided with the development of new measurement techniques (McCleary, 2007). The definition

of dietary fibre was discussed for almost two decades by the CODEX Alimentarius Commission, which was founded by the United Nations' Food and Agriculture Organisation (FAO) for the purpose of global standardization and harmonization. A consensus was eventually reached on the current and most widely accepted definition of dietary fibre, quoted below (Jones, 2014):

Dietary fibre means carbohydrate polymers[1] with 10 or more monomeric units[2], which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories:

1. *Edible carbohydrate polymers naturally occurring in the food as consumed.*
2. *Carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities*
3. *Synthetic carbohydrate polymers, which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities*

Footnote 1 states, "when derived from a plant origin, dietary fibre may include fractions of lignin and/or other compounds associated with polysaccharides in the plant cell walls. These compounds also may be measured by certain analytical method(s) for dietary fibre.

Footnote 2 states that, "Decision on whether to include carbohydrates of 3 to 9 monomeric units should be left up to national authorities."

The CODEX definition has been successful in highlighting the key feature of dietary fibre as resistance to enzymatic digestion, while remaining broad enough to encompass any biologically relevant compounds that may be identified in the future. Footnote 1 is significant in that it allows the inclusion of non-carbohydrate compounds under an otherwise carbohydrate centric definition. However, challenges remain in complete harmonization; the inclusion of footnote 2 itself is

disruptive towards a universally accepted definition of dietary fibre because national authorities can decide whether to include carbohydrate of 3 to 9 monomeric units.

1.2.4 The chemical complexity of dietary fibre

As suggested by the CODEX definition, DF most frequently refers to carbohydrate polymers (polysaccharides) that are structurally complex due to the nature of their composition and synthesis. Polysaccharides are comprised of monosaccharides, which contain between 3 and 9 carbons with a single carbonyl functional group, and one hydroxyl group on each of the remaining carbons. Monosaccharides are grouped together by the number of carbons they contain; a monosaccharide with six carbons is a hexose and a monosaccharide with five is a pentose. Since monosaccharides with the same number of carbons are essentially stereoisomers that have the same chemical formula but different structural configuration, different monosaccharides are distinguished by the stereochemical orientation of their hydroxyl groups (**Figure 1-2**). Further variation is generated when monosaccharides are combined as two monosaccharides forming a disaccharide, chains of three to ten forming oligosaccharides, and chains larger than 10 being polysaccharides. Linkage of monosaccharides occurs covalently by the formation of an *O*-glycosidic bond between the anomeric carbon (named position 1) of one monosaccharide to the hydroxyl group of another monosaccharide or compound. Linkage position is defined by the carbon of the involved hydroxyl group and is numbered according to the distance from the anomeric carbon. Furthermore, since each carbon on a monosaccharide is a chiral centre, asymmetry around the anomeric carbon results in two possible configurations called the α- and β-anomers that determine linkage type.

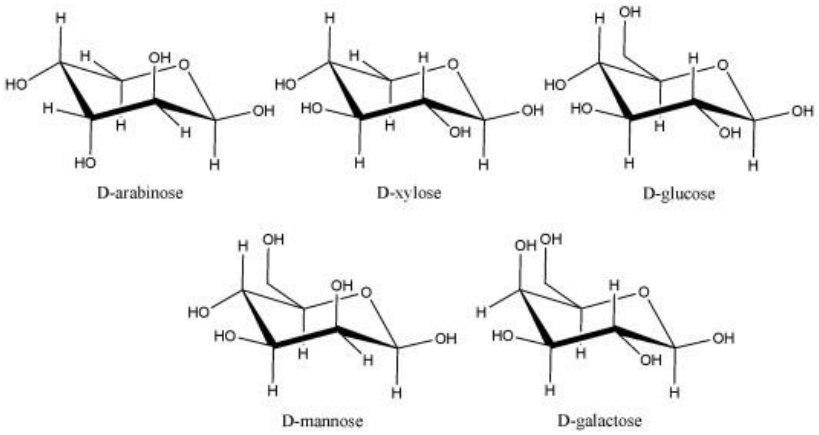

Figure 1-2. Chair conformation of some common monosaccharides. The pentoses arabinose and xylose are stereoisomers containing five carbons while the hexoses glucose, mannose and galactose are stereoisomers containing six[2].

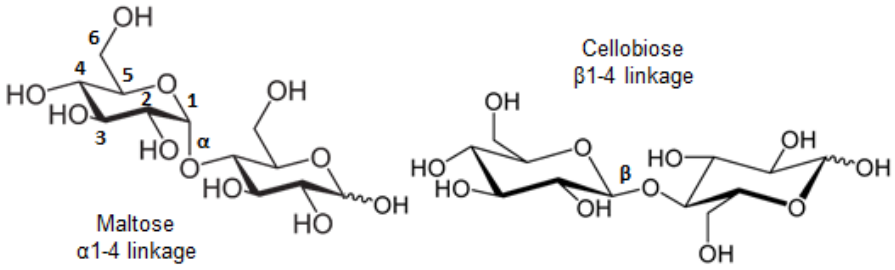

Figure 1-3. An example of α- and β-type linkage orientation. Maltose and cellobiose are both disaccharides comprised of two glucose (Glc) residues connected at the same position that are differentiated by linkage type. Carbon position numbering begins at 1 at the location of the glycosidic bond. The position of each carbon is shown numerically on maltose.

For example, though maltose and cellobiose share the same 1-4 linkage position, α- and β-linkage results in two different disaccharides being formed (**Figure 1-3**). The structural complexity of carbohydrates contributes to the diversity in physical properties and physiological activity of different polysaccharides. Solubility is determined by the degree of order across adjacent polysaccharide molecules (Guillon and Champ, 2000). Linear molecules such as cellulose are insoluble in water since they form highly ordered sheets of tightly packed ribbons favouring hydrogen bonding between hydroxyl groups and creating a rigid and highly stable structure (Cui, 2005). Conversely, branched molecules such as amylopectin are highly soluble in water since the

[2] Reproduced with permission from Elsevier, Domingues, D.S., E.D. Pauli, J.E.M. de Abreu, F.W. Massura, V. Cristiano, M.J. Santos, and S.L. Nixdorf. 2014. Detection of roasted and ground coffee adulteration by HPLC by amperometric and by post-column derivatization UV–Vis detection. *Food Chemistry*. 146:353-362. under licence number 4287480886775, 14-02-2018.

available space between adjacent molecules allows water molecules to interact with the hydroxyl groups (Cui, 2005). Linkage type and position are also important in determining the structural orientation of the polysaccharide chain and defining the extent of polysaccharide digestion due to the linkage and position specificity of Carbohydrate-Active enzymes (CAZymes); for example, 4-β-D-glucan 4-glucanohydrolase (CAZy entry EC 3.2.1.4) will only cleave before a glucose with β-linkage in the 1-4 position (Kaoutari et al., 2013).

1.2.4.1 Starch and non-starch polysaccharides

Starch is the most abundant storage polysaccharide in plants and is comprised of two glucose polysaccharides: amylose, a completely linear chain of α-(1-4)-linked glucose units, and amylopectin, a linear chain of α-(1-4)-linked glucose interspersed with glucose chains linked at the 6-position that generates a highly branched configuration (**Figure 1-4**) (Sajilata et al., 2006).

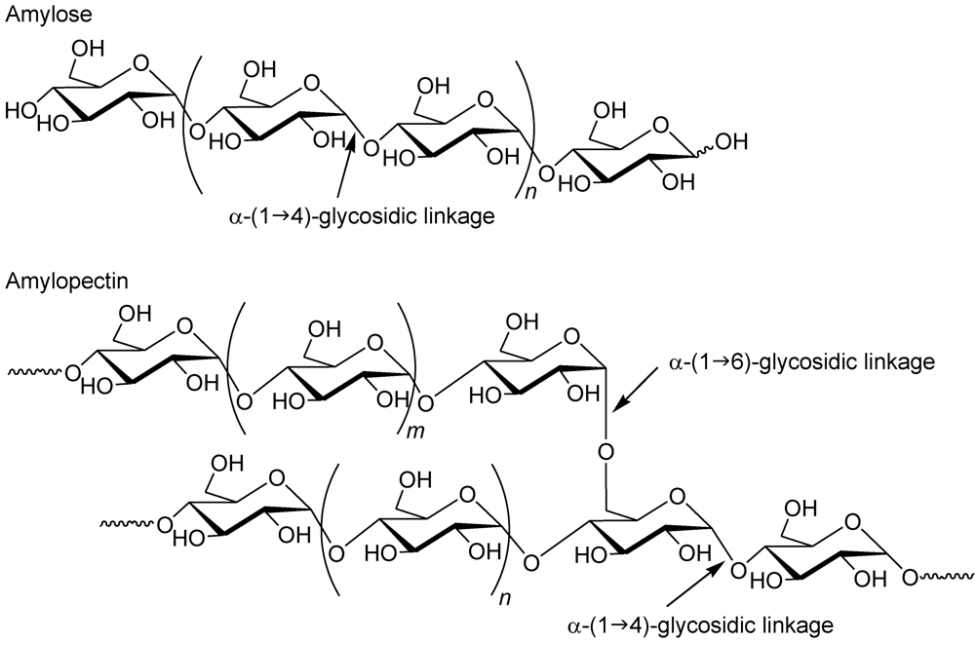

Figure 1-4. An example of the branching effect resulting from α-(1-6)-glycosidic linkage. The linear chains of amylose and amylopectin are comprised of α-(1-4)-linkage of glucose monomers. The branched structure of amylopectin is the result of α-(1-6)-glycosidic linkage[3].

[3] Reproduced with permission from Kadokawa, J.-i. 2012. Preparation and Applications of Amylose Supramolecules by Means of Phosphorylase-Catalyzed Enzymatic Polymerization. *Polymers*. 4:116.. This article is available under the terms of the Creative Commons Attribution License Attribution 3.0 Unported (CC BY 3.0)

Due to the secretion of α-amylase, which cleaves α-1-4 linked glycosidic bonds, starch is generally not considered a dietary fibre. With the presence of α-amylase in the human pancreas, starch rarely escapes the small intestine (Topping et al., 2008), however, a metabolically significant fraction known as resistant starch has been found in the large intestine of healthy individuals (Sajilata et al., 2006). Resistant starch (RS) is subdivided into four different forms based on the mechanism of resistance (**Table 1-4**). RS_1 is starch that is physically inaccessible as partially milled grains or seeds that prevent access of α-amylase to its substrate. RS_2 are the granular starches that are tightly packed and relatively dehydrated, commonly found in raw unprocessed foods that require cooking to expand and hydrate. RS_3 is starch that has been retrograded through heating and cooling allowing the re-association of starch chains that are completely resistant to digestion. Finally, RS_4 are the chemically modified starches. Due to their resistance to digestion, RSes can be major contributors to dietary fibre depending on the types of food being consumed and the means of preparation.

Table 1-4. Classification of resistant starches (RS).

Type of RS	Mechanism of resistance	Examples of occurrence
RS_1	Physically inaccessible	Partly milled grains and seeds
RS_2	Resistant granules	Raw potato, green banana high amylose starches
RS_3	Retrograded	Cooked and cooled starchy foods
RS_4	Chemically modified	Etherised or esterified processed starches

With the exception of resistant starches, dietary fibres generally fall under the category of non-starch polysaccharides (NSP) (Topping and Clifton, 2001) that encompasse all other polysaccharides that do not contain the α-1-4 glycosidic bonds found in starch. Due to the structural complexity of these compounds, NSPs are difficult to classify but can be generally be separated into one of three categories: cellulose, non-cellulosic polymers, and pectic polysaccharides (Choct, 1997).

1.2.4.1 Cellulose, hemicellulose, and pectic polysaccharides

Cellulose is a plant cell wall polysaccharide and is the most abundant organic compound in nature estimated to account for just over 50% of all carbon found in plants (Choct, 1997). Cellulose is a large polysaccharide comprised of between 7000 – 10000 β-(1-4)-glucose units that form tightly packed bundles that are insoluble in water, alkali, and weak acid (Bauer and Ibáñez, 2014). As the major plant cell wall associated polysaccharide, cellulose is incredibly resistant to enzymatic digestion, thus the main physiological activity of cellulose is thought to be mechanical in nature by increasing bulk faecal size and stimulating the gastrointestinal tract by friction; however, the identification of families of bacterial enzymes capable of breaking down cellulose in the human gut suggests this type of digestion may not be explicitly restricted to ruminants (Cantarel et al., 2012). Non-cellulosic polymers include the hemicelluloses, a diverse group of polysaccharides that are predominantly comprised of xylans, as well as β-glucans, mannans, glucomannans, galactomannans, and galactoglucomannans (Elleuch et al., 2011)(**Table 1-5**). Compared to cellulose, hemicellulose molecules tend to be smaller (500 – 3000 units) and far more heterogeneous and amorphous in structure by possessing a more diverse monosaccharide composition (Gibson, 2012).

Non-cellulosic polymers possess a diverse range of functional and physiological properties determined by monosaccharide and linkage composition. Pectic polysaccharides (pectins), are another diverse group of polysaccharides primarily composed of α-(1-4)-galacturonic acid polymers substituted with galactose and rhamnose residues. Pectins also undergo varying degrees of methyl esterification of free carboxyl groups, which alters the charge state of the molecule and has been shown to influence the structural integrity of plant tissues (Thakur et al., 1997).Within plants, pectins interact with cellulose and hemicellulose as part of a three-dimensional structure that is fundamental to cell wall integrity but more importantly, pectins are known for their ability to form gels in the presence of Ca^{2+}, sugar and acid, which is an important characteristic in the food industry (Thakur et al., 1997)

Table 1-5. Summary of NSPs and constitutive polysaccharide monomers and their plant origin. Adapted from Sinha et al. (2011) and Elleuch et al. (2011)

Plant source	NSP class	Main chain	Branch unit(s)	Ref.
All plants	Cellulose	β-(1-4)-glucose		(Elleuch et al., 2011)
Oats, Barley	β-glucan	β-(1-4)-glucose β-(1-3)-glucose		(Sinha et al., 2011)
Woody plants	Xylan	β-(1-4)-xylose		(Elleuch et al., 2011)
Psyllium Husk	Arabinoxylan	β-(1-4)-xylose	Xylose Ara-α-(1-3)-Xyl-β-(1-3)-Ara	(Fischer et al., 2004)
Ivory nut	Mannan	β-(1-4)-mannose		(Chanzy et al., 1979)
Guar bean Locust bean	Galactomannan	β-(1-4)-mannose	α-(1-6)-galactose	(Bourbon et al., 2010)
Sugar beet	Glucomannan	β-(1-4)-mannose	α-(1-3)-glucose	(Nishinari et al., 1992)
Most plants	Inulin	β-(2-1)-fructosyl-fructose		(Marchessault et al., 1980)

1.2.4.2 Lignin

Lignin is a non-carbohydrate plant cell wall polymer and is the second most abundant naturally occurring compound after cellulose, comprising approximately 25 – 30% of the dry weight of plants (Christopher et al., 2014). Lignin is crucial for the structural integrity of the plant cell wall and provides stiffness and strength to the cell wall by surrounding and crosslinking with the cellulose-hemicellulose matrix (Boerjan et al., 2003). Lignin biosynthesis involves the polymerization of the hydroxy cinnamyl alcohol monomers: sinapyl (S), coniferyl (G), and *p*-coumaryl (H) alcohols (**Figure 1-5**). The ratios of each alcohol are known to vary depending on the plant, cell type, and developmental factors. The polymerization process occurs via a series of enzyme catalyzed radical coupling reactions that create a complex lattice of phenylpropanoid units that is highly resistant to chemical degradation. Biochemical degradation of lignin is limited to a select number of bacterial and fungal families that possess the necessary peroxidase/and or laccase enzymes that are able to act on lignin polymers (de Gonzalo et al., 2016). High amounts of dietary

lignin have been associated with impaired digestibility of animal feeds (Knudsen, 1997; Schedle et al., 2008).

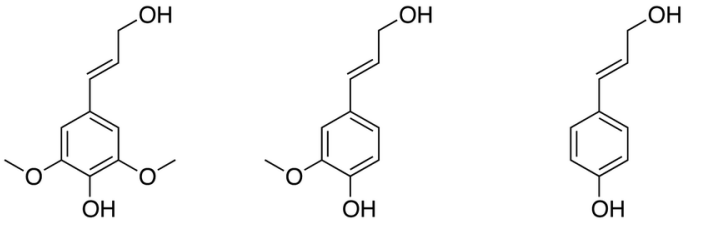

Figure 1-5. Monomers of lignin. Sinapyl (S), coniferyl (G), and *p*-coumaryl (H) undergo extensive crosslinking to form chemically stable networks due to the aromatic nature of the benzene ring[4].

1.2.4.3 Soluble and insoluble dietary fibre

Dietary fibres are subdivided into soluble and insoluble fibres based primarily on water solubility and effect on viscosity. Soluble fibres are generally soluble in water and are associated with increases in viscosity, such that fibres that form a mucilage or gel in the presence of water also fall under this category. In contrast, insoluble fibres are completely insoluble in water, tend to be comprised of structural polysaccharides and are typically low in density (Elleuch et al., 2011). The chemical differences between soluble and insoluble fibres have also been associated with contrasting physiological activities.

Soluble fibres, such as β-glucans, are generally well fermented by the resident gut bacteria and increase the production of metabolites that are absorbed and utilised by the host (Slavin et al., 2009). Gel forming soluble fibres, such as arabinoxylan from psyllium husk, possess high water holding capacity and swell in size contributing to feelings of satiety while also slowing transit time of the stool (McRorie, 2015). Soluble fibre is most commonly known to assist in the control of blood cholesterol levels, a property that has been shown to be diminished at lower particle sizes

[4] Reproduced with permission from Strassberger, Z., S. Tanase, and G. Rothenberg. 2014. The pros and cons of lignin valorisation in an integrated biorefinery. *RSC Advances*. 4:25310-25318.. This article is available under the terms of the Creative Commons Attribution License Attribution 3.0 Unported (CC BY 3.0)

and consequently lower viscosities, indicating a direct link between high viscosity and cholesterol lowering activity (Wolever et al., 2010).

In contrast, insoluble fibres are less readily fermented due to reduced accessible surface area and hydrogen bonding networks that hold carbohydrate chains together posing a significant challenge for microbial enzymes to degrade (Cockburn and Koropatkin, 2016)(Holscher, 2017). Insoluble fibres are thought to provide more mechanical effects by providing "roughage", which irritates the lining of the gastrointestinal tract, stimulating the secretion of mucus and inducing a laxative effect by faecal bulking, and are largely found relatively intact in faeces (Brownlee, 2011). Insoluble fibres have been associated with reductions in blood glucose and insulin levels by means of "meal dilution" whereby insoluble fibres increase faecal bulk while contributing zero calories to the diet (Macagnan et al., 2016), and by the inhibition of pancreatic enzymes *in vitro* thus decreasing the digestion and absorption of nutrients (Dunaif and Schneeman, 1981). Furthermore, insoluble fibres have also been proposed to act as vehicles to transport nutritionally important compounds such as essential minerals and polysaccharide associated polyphenols with antioxidant activity to the gut microbiota of the colon (Macagnan et al., 2016).

1.3 The gut microbiota in health and disease

The human body is a bioreactor housing trillions of microorganisms, collectively known as the microbiota or microbiome, which is unique to each individual (Grice and Segre, 2012; The Human Microbiome Project, 2012). Often used interchangeably, the human "microbiota" is defined as the microbial taxa associated with humans while the human "microbiome" is the catalogue of these microbes and their genes (Ursell et al., 2012). Recent advances have associated the genetic coding potential of the microbiota, which is estimated to exceed that of the host by 500-fold, with an important role in complementing the human genome and exerting influence on host metabolism, immunity, and health (Louis et al., 2014; Singh et al., 2017).

The influence of the gut microbiota on human metabolism and health occurs via numerous mechanisms with perhaps the most pertinent being the production of metabolites that are beneficial and often essential for the host (Nicholson et al., 2012). An early observation estimated that the absorption of microbial metabolites contributed up to 10% of the daily caloric intake of the human diet (McNeil, 1984). The gut microbiome integrates many important pathways, including those related to enterohepatic circulation of bile, cholesterol and phospholipids, and modulates host immunity, glucose, lipid, and energy metabolism, and choline availability (Kovatcheva-Datchary and Arora, 2013; Singh et al., 2017). Depletion of choline has since been associated with the progression of non-alcoholic fatty liver disease (Corbin and Zeisel, 2012; Dumas et al., 2006), while more comprehensive metabolomic analysis identified significantly elevated concentrations of glycerol, bile salts, and certain amino acids in the stools of patients with colorectal cancer compared to healthy adults (Weir et al., 2013). Furthermore, studies in mice have identified a relationship between the gut microbiota and an increased capacity for energy harvest and obesity (Turnbaugh et al., 2008; Turnbaugh et al., 2006). Since the microbiota inhabits a living host, selective pressures exist to promote the fitness of the host at the risk of decreasing the number of potential microbial habitats or even complete loss of habitat. By this mechanism of natural selection, the microbiota is encouraged to promote cooperation between microbes and host in a system of mutualism that outcompetes opportunistic pathogens, thus decreasing host susceptibility to infection (Bäckhed et al., 2005).

While the precise mechanisms are still under debate, a link is being established between the diet, gut microbiota composition, and progression of diseases such as colorectal cancer (Louis et al., 2014; O'Keefe et al., 2015), inflammatory bowel disease (Ding et al., 2010), obesity (Ley et al., 2006a), and T2DM (Brunkwall and Orho-Melander, 2017; Tolhurst et al., 2012). Considering the associations between changes in gut microbiota and various disease states, it is still unclear whether certain gut microbiomes are either linked to these diseases or are the result of these diseases.

1.3.1 The composition of the human gut microbiota

The gastrointestinal tract is densely populated by a complex community of bacteria, viruses, fungi, protozoa, and archaea, which together is termed the microbiota (The Human Microbiome Project, 2012). The composition of the gut microbiota exhibits significant variation across the different regions of the gastrointestinal tract due to chemical gradients, region specific mucosa, and immune responses (Donaldson et al., 2015; Flint et al., 2007) (**Figure 1-6**). For example, oxygen gradients limit the growth of aerotolerant microbes, facultative and obligate anaerobes to specific regions of the gastrointestinal tract (Albenberg et al., 2014). The secretion of anti-microbials such as bile salts and peptides also drive the selection of bile salt resistant microbes, such as *Lactobacillus* and *Bifidobacterium*, to primarily inhabit the small intestine (Ruiz et al., 2013).

Culture-based studies suggest that most healthy adults share a core microbiota comprised of the same gut bacterial species (Lozupone et al., 2012). The human gut microbiota is dominated by five bacterial phyla: a large proportion of the gut bacteria are members of the *Firmicutes* and *Bacteroidetes*, with the remaining represented by members of *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (Eckburg et al., 2005). The *Firmicutes* are a phylum of mostly Gram-positive bacteria that includes the genera *Bacillus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus* with the majority of species falling under the family *Clostridium* (Duncan et al., 2007).

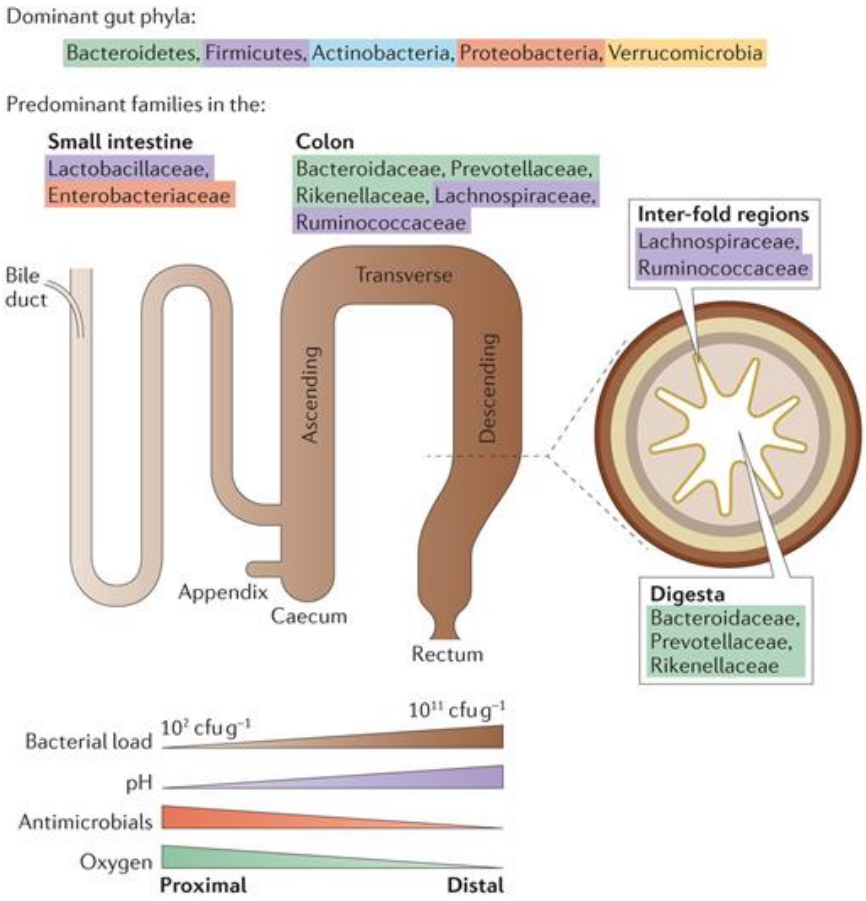

Figure 1-6. Simplified representation of the microbial variation along the gastrointestinal tract. Bacterial load increases from the small to large intestine with the greatest density found in the colon. Localisation of different bacterial families reflects physiological gradients of factors such as pH, antimicrobials, and oxygen, which creating ecological niches. While most gut bacteria reside in lumen and associate with the digesta, some bacteria adhere and interact closely with the mucosal layer[5].

This phylum accounts for the vast majority colonic or faecal bacteria with great variation between studies determined by sampling and storage methods, DNA/RNA extraction, sequencing techniques, and individual variation. Bacteria in the family *Clostridium* play a major role in the fermentation of carbohydrates in the gut and contribute to the production of short chain fatty acids that contribute to dietary energy intake and are intimately involved in the maintenance of overall gut function (Lopetuso et al., 2013). Emerging evidence also suggests that the *Firmicutes* include some highly specialised degraders of insoluble plant cell wall polysaccharides (Flint et al., 2014).

[5] Reproduced and altered with permission from Springer Nature, Donaldson, G.P., S.M. Lee, and S.K. Mazmanian. 2015. Gut biogeography of the bacterial microbiota. *Nature Reviews Microbiology*. 14:20. under licence number 4265200640716, 10-01-2018

The *Bacteroidetes* are a phylum of Gram-negative bacteria that includes the genera *Bacteroides*, *Prevotella*, *Parabacteroides*, *Porphyromonas* and *Alistipes*, accounting for roughly a quarter of colonic or faecal bacteria (Duncan et al., 2007). Detailed investigations on human colonic *Bacteroides* isolates show that these organisms possess the capability to utilise a very wide range of substrates of both host and dietary origin allowing them to fulfil both generalist and specialist roles within the gastrointestinal tract (Johnson et al., 2017). Compared to bacteria of other phyla, *Bacteroidetes* possess a proportionally higher number of CAZymes capable of digesting both complex plant polysaccharides and host derived glycans (Kaoutari et al., 2013; Thomas et al., 2011).

The gut microbiota is a dynamic population, particularly during the early years of human development. As the initial point of inoculation, birth mode via either vaginal delivery or caesarean section has a profound impact on the microbiota resulting in significantly different compositions (Dominguez-Bello et al., 2010). Within the first 6 months of life, infants rapidly acquire bacteria such as *Bifidobacteria* and *Bacteroides* that allow utilisation of lactate found in breast milk or milk formula (Koenig et al., 2011). By the end of the first 12 months, bacterial composition begins to resemble an adult-like microbiota, marked by colonisation of *Firmicutes* and *Bacteroidetes*, is completely settled by 2.5 years of age and remains relatively stable until old age (Koenig et al., 2011). However, environmental factors such as diet, antibiotic use, and pathogenic infection are known to influence gut microbial composition, potentially causing dysbiosis and disruption of the balanced gut microbiota (Nicholson et al., 2012). Several human studies have demonstrated that a high ratio of *Firmicutes* to *Bacteroidetes* in the gut microbiota, with *Firmicutes* significantly increased in obese people compared to lean people that consequently decrease with weight loss (Barlow et al., 2015; Koliada et al., 2017; Ley et al., 2006b). However, this matter is highly contested since some studies have produced conflicting results and failed to find significant differences in the *Firmicutes* to *Bacteroidetes* ratio between lean and obese humans at both baseline level and after the weight loss (Duncan et al., 2008; Million et al., 2013; Turnbaugh et al.,

2009a). These differences are most likely due to different environmental influences, including diet, physical activity, as well as socio-economic impacts (Dugas et al., 2016).

1.3.2 The influence of diet on microbiota composition

Despite the relative stability of the gut microbiota, human studies have shown that changes in diet can induce significant changes in the composition of the gut microbiota that occur rapidly and reproducibly (David et al., 2013; Desai et al., 2016; Turnbaugh et al., 2009b). These diet driven changes occur in response to two major mechanisms: a) the capacity for microbial species to utilize dietary and host derived susbtrates, and b) the tolerance of microbial species to environmental factors in the gut such as pH, salt, and micronutrient concentrations (Flint et al., 2014).

In a human study comparing vegetarian and omnivorous diets, consumption of either a plant-based diet or an animal-based diet induced significant changes in microbiota composition within 24 hours (David et al., 2013). The animal-based diet induced a notable reduction in carbohydrate fermenters while amino acid fermenters were enriched indicating that changes in microbial composition occur in response to the availability of nutrients (David et al., 2013; Turnbaugh et al., 2009b). Unsurprisingly, the consumption of complex plant polysaccharides and dietary fibre has been shown to increase the abundance of microbes possessing carbohydrate active enzymes (CAZymes) such as those within the phyla Bacteroidetes and Firmicutes (Cantarel et al., 2012; Flint et al., 2012; Kaoutari et al., 2013). In study involving a gnotobiotic mouse model, the replacement of dietary fibre with dietary fat induced significant changes to the gut microbiota characterised by an increase in bacterial species capable of degrading host derived glycans (Desai et al., 2016). These species showed greater capacity for switching between dietary energy sources and clearly demonstrate the influence of substrate utlisisation on gut microbiota composition.

Emerging research suggests that the microbial response to dietary interventions is highly variable among individuals with some taxa either responsive or resilient to dietary change (McOrist et al., 2011; Salonen et al., 2014). Interindividual variability has been attributed to baseline gut

microbiota composition and habitual dietary intake (Healey et al., 2017). For examples, studies have shown that individuals with lower baseline Bifidobacteria concentrations experience a more pronounced increase in Bifidobacteria after an inulin intervention (De Preter et al., 2008; Tuohy et al., 2001). Variability in the types and amounts of available fermentable substrates has a major impact on gut microbiota composition and functional capacity. Individuals with low dietary fibre intake risk losing bacterial taxa capable of utilising fermentable substrates due to fibre deficiency (Wu et al., 2011). Conversely, individuals with higher dietary fiber intakes may foster bacterial taxa better equipped to utilise fermentable subtrates, leading to a greater microbial response (Desai et al., 2016). These factors make it difficult to predict an individual response or benefit to a given dietary intervention

1.3.3 Short chain fatty acids as the major metabolites of the gut microbiota

The gut microbiota interacts with the host primarily through the production of microbial metabolites that are absorbed and utilised by the host or assist in modulating the luminal environment. While the microbiota is capable of producing a diverse repertoire of metabolites, the short chain fatty acids (SCFAs) are the most abundant (Koh et al., 2016; Rooks and Garrett, 2016). Short chain fatty acids are a group of carboxylic acids with an aliphatic chain consisting of between 1 and 6 carbons, that may be linear or branched (Cummings, 1981; Miller and Wolin, 1996).

As a prerequisite step to fermentation, complex plant polysaccharides must first be hydrolysed into simple monosaccharides (hexoses or pentoses) by the concerted saccharolytic action of CAZyme-containing microbes (Kaoutari et al., 2013). Once in monosaccharide form, fermentation follows various metabolic pathways to give the final SCFA products, predominantly as acetate, propionate, and butyrate (**Figure 1-7**). In general, the catabolism of these monosaccharides follows the Embden–Meyerhof–Parnas (EMP pathway) of glycolysis for hexoses or the pentose-phosphate pathway for pentoses, which convert monosaccharides to phosphoenolpyruvate (PEP) (Miller and Wolin, 1996; Peretó, 2011). Acetate, the most abundant SCFA, is either formed from Acetyl-

coenzyme A (Acetyl-CoA) or carbon dioxide (CO_2) via the Wood-Ljungdahl pathway (Miller and Wolin, 1996; Ragsdale and Pierce, 2008). Propionate can be formed by three different pathways: the acrylate, succinate, and propanediol pathways (**Figure 1-7**). The acrylate and succinate pathways produce propionate from hexoses and pentoses via lactate and succinate intermediates respectively while the propanediol pathway converts deoxy-sugars such as fucose and rhamnose to propionate. The formation of butyrate begins with the condensation of two molecules of Acetyl-CoA followed by a reduction step in which butyryl-CoA is reduced to butyrate.

The production of SCFAs directly reduce the pH of the luminal space, which in turn influences the composition of the gut microbiota by affecting the growth of pH sensitive bacteria (Ríos-Covián et al., 2016). SCFAs are well absorbed by the host in exchange for bicarbonate creating an equilibrium that further controls the luminal pH along the digestive tract (den Besten et al., 2013b). As such, the decrease in pH from ileum to cecum is a result of higher SCFA concentrations while the gradual increase in pH from the proximal to distal colon is due to declining SCFA concentration following absorption (Cummings et al., 1987). While most SCFAs are transported to the liver and other organs to be utilised as substrates or signal molecules in energy and lipid metabolism, butyrate is preferentially utilised by the colonocytes as a primary energy source (den Besten et al., 2013b; Yin et al., 2001). In humans, SCFAs contribute approximately 10% of the daily caloric requirements (Bergman, 1990). The involvement of SCFAs in lipid, glucose, and cholesterol metabolism has garnered significant attention regarding the potential role of SCFA in the control of obesity, T2DM, and the metabolic syndrome (Schwiertz et al., 2010; Tolhurst et al., 2012). While the mechanism is still being elucidated, the protective effect of SCFAs against diabetes is thought to involve the regulation of host metabolism by the stimulating the secretion of glucagon-like peptide 1 and 2 (GLP-1 and GLP-2), which modulate gut barrier function to reduce uptake of inflammatory compounds and enhance secretion of insulin (Tolhurst et al., 2012).

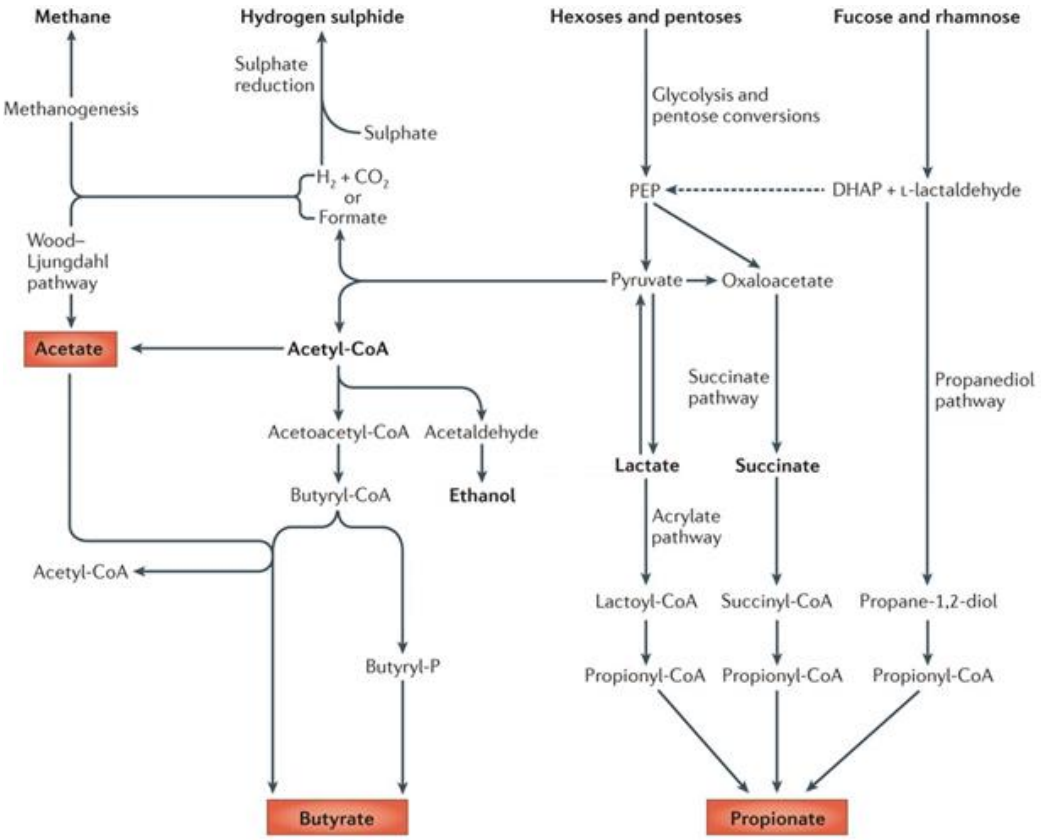

Figure 1-7. The metabolic pathways involved in the production of the short chain fatty acids. The fermentative production of acetate and butyrate involves Acetyl-CoA while propionate is produced via lactate, succinate, or propanediol through multiple pathways[6].

SCFAs are recognised by the G protein-coupled receptors (GPCRs) GPR41, GPR43 and GPR109A, which facilitate microbiota induced regulation of host metabolism, inflammatory pathways, and innate immunity (Le Poul et al., 2003; Tremaroli and Bäckhed, 2012). SCFA–GPR43 signalling is one of the molecular pathways whereby commensal bacteria regulate immune and inflammatory responses (Maslowski et al., 2009). GPR43, which is activated by SCFAs with decreasing affinity by acetate>propionate>butyrate, is the sole functional receptor for SCFAs on neutrophils indicating an active role of SCFAs in the innate immune system and inflammatory pathways (Le Poul et al., 2003). SCFAs have been demonstrated to regulate the production of leptin, a hormone predominantly produced by adipose cells that is crucial in regulating energy homeostasis by inhibiting feelings of hunger (Gabriel, 2018). Treatment of adipose tissue with

[6] Adapted with permission from Springer Nature, Louis, P., G.L. Hold, and H.J. Flint. 2014. The gut microbiota, bacterial metabolites and colorectal cancer. Ibid. 12:661. under licence number 4275810342256, 25-01-2018

propionate significantly reduced obesity associated inflammation by downregulating the production of tumour necrosis factor (TNF)-α and Chemokine (C-C motif) ligand 5 CCL5 by macrophages, and by altering metabolism by increasing the expression of lipoprotein lipase and Glucose transporter type 4 (GLUT4), which are associated with lipogenesis and glucose uptake, respectively (Al-Lahham et al., 2012).

SCFAs have been shown to influence gene expression in the Nuclear Factor kappa-light-chain-enhancer (NF-κB) pathway resulting in downregulation of the proinflammatory mediators such as nitric oxide synthase (iNOS), prostaglandin-endoperoxide synthase 2 (COX2), TNF-α, interleukin (IL) 1β, IL-6 IL-12 (Chang et al., 2014; Maslowski et al., 2009). Butyrate is also negatively associated with tumour formation and has been shown to promote cellular differentiation in immunity regulating T cells (Furusawa et al., 2013) and induce apoptosis in tumour cells through the inhibition of histone deacetylases (Hague et al., 1996; McBain et al., 1997). Consequently, butyrate has received significant attention as a target for the prevention of colorectal cancer (Aune et al., 2011; Louis et al., 2014; Scheppach et al., 1995).

1.4 The gastrointestinal mucosa and intestinal health

The gastrointestinal mucosa is the interface between the contents of the gastrointestinal tract (lumen), and the tissue of the gastrointestinal tract. The gastrointestinal mucosa is a complex structure comprised of a variety of epithelial cells in the gut lining that is coated by a protective layer of mucus secreted by specialized goblet cells (Donaldson et al., 2015). The mucus layer has been implicated in the maintenance of luminal pH, preventing digestive enzymes from accessing the epithelial cells, regulating small molecule transport, and providing a physical barrier between the gut microbiota and the host (Allen and Snary, 1972; Johansson et al., 2013). The thickness of this mucus layer has been shown to vary significantly between different regions of the gastrointestinal tract relative to the density of the gut microbiota and the colon exhibits both the highest density of microbes and the thickest mucus layer (Atuma et al., 2001).

The mucus layer of the colon is unique in being comprised of two distinct layers: one densely packed and firmly attached to the epithelia and the other more loosely attached (Brownlee et al., 2003; Johansson et al., 2008). While the inner layer is devoid of microbes (Johansson et al., 2008), the outer mucus layer is populated by numerous commensal bacteria; however, defining the composition of this layer is difficult due to regional differences along the gastrointestinal tract, interspecies differences in comparing rats, mice, and humans, and the phylogenetic spread of bacteria across many different families (Tailford et al., 2015). While the primary role of the mucus layer is to provide chemical and physical protection for underlying epithelial cells from luminal factors, there is increasing evidence to suggest that the mucosal layer interacts with both diet and the microbiota and plays a more dynamic role in intestinal health and homeostasis (Desai et al., 2016; Montagne et al., 2003).

1.4.1 The mucus layer and MUC2

The mucus layer is predominantly comprised of mucins, a class of high molecular weight glycoproteins typically in the range of 1 – 20 megadaltons. At least 20 different MUC encoding genes have been identified in humans, which are differentially expressed across different regions of the body as membrane bound or secreted entities (Brockhausen and Stanley, 2017; Ma et al., 2017). MUC2 is the major secreted mucin of the small intestine and colon while MUC5AC and MUC6 are expressed in the stomach (Tailford et al., 2015; Tytgat et al., 1994). Mucin proteins are typically differentiated by the variable number of tandem repeat regions (VNTRs) in the protein core. These VNTR regions are rich in serine, threonine and proline and are heavily *O*-glycosylated through the sequential enzymatic attachment of single monosaccharides to the OH- group of serine and threonine residues typically beginning with attachment of *N*-acetylgalactosamine (*O*-GalNAc). Further elongation of the GalNAc produces *O*-glycan chains consisting of anywhere between 5 to 15 monosaccharides in length. Mucins are so heavily glycosylated that carbohydrates can comprise between 50 to 80% by weight (Allen et al., 1998).

The expression of terminal residues, such as fucose or sialic acid, generates significant glycan diversity that influences the innate immunity of the gastrointestinal tract (Magalhães et al., 2016; Struwe et al., 2015; Xu et al., 2004). While these glycans act primarily as binding sites for commensal and pathogenic microbes they are also degraded by specialist microbes under certain nutritional conditions such as periods of low consumption of plant-based carbohydrates (Desai et al., 2016). Another important feature of mucins is their ability to polymerise to form large multimers due to cysteine rich domains at the amino and carboxy terminals that allow disulfide bond formation (Allen et al., 1998). The extensive *O*-glycosylation of MUC2 imparts high water-binding capacity and the polymeric potential imparts gel forming properties due to the CysD domains that engage in non-covalent dimeric interactions allowing adjacent molecules to from stratified sheets and giving mucus the majority of its physical properties (**Figure 1-8**) (Allen et al., 1998; Lang et al., 2007).

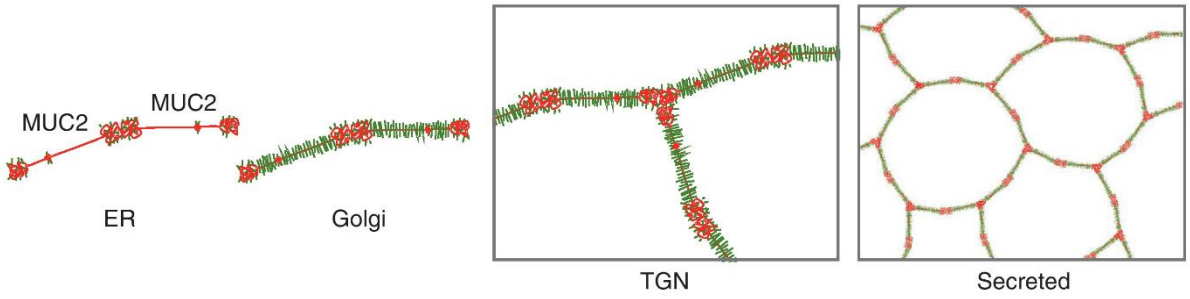

Figure 1-8. Assembly and secretion of MUC2. MUC2 dimers linked by CysD domains (protein = red) are first assembled in the endoplasmic reticulum (ER) then glcosylated (glycans = green) in the Golgi apparatus. In the trans Golgi network (TGN), MUC2 is further assembled into branched trimers that coalesce to form the secreted MUC2 polymer[7].

1.4.2 The influence of diet and the SCFAs on the mucus layer

Diet and microbiota composition have been shown to directly influence the secretion, structure and composition of gastrointestinal mucus (Sharma et al., 1995). Increased secretion is commonly attributed to the loss of mucus by "sloughing" from the abrasive passage of dietary fibre complemented by an increase in mucin secreting goblet cells (Enss et al., 1994). The consumption

[7] Adapted with permission from Elsevier, Hansson, G.C. 2012. Role of mucus layers in gut infection and inflammation. *Current Opinion in Microbiology*. 15:57-62., under licence number 4279040286798, 30-01-2018

of incremental doses of dietary fibre from wheat bran, psyllium husk, and citrus, was shown to produce linear dose-dependent increases in mucus secretion, while no effect was observed with guar gum or soy fibre (Brownlee et al., 2003; Montagne et al., 2003). Dietary fibre composition appears have a profound influence on mucus secretion; rats that were fed diets rich in only pectin or cellulose actually resulted in a decrease in mucus thickness suggesting that mixtures of plant polysaccharides are more effective than purified diets (Brownlee et al., 2003).

The influence of SCFAs on mucus secretion has also been explored. Administration of acetate (100 mM) and butyrate (5 mM) has been shown to induce secretion of mucus in an isolated rat colon (Barcelo et al., 2000). Using LS174T, a cell line derived from human mucin secreting goblet cells, propionate and butyrate (1 mM) were able to induce an increase in MUC2 mRNA levels *in vitro* (Burger-van Paassen et al., 2009). At higher concentrations of propionate (5 – 15 mM) MUC2 mRNA levels remained high while increased concentrations of butyrate (5–15 mM) caused a return to baseline MUC2 mRNA levels (Burger-van Paassen et al., 2009).

1.4.3 The role of MUC2 in intestinal health and disease

MUC2 has been established to be a vitally essential component of the gastrointestinal tract. As the first line of defence against invading enteric pathogens, the integrity of the outer mucosal layer is a critical factor in host innate immunity. Initial observations in patients with inflammatory bowel disease found significant amounts of bacteria in contact with the colonic epithelial cell wall compared to practically none in healthy controls indicating that invasion of the mucus layer is the first step to the progression of bacterial induced inflammation (An et al., 2007; Bergstrom et al., 2010; Schultsz et al., 1999; Van der Sluis et al., 2006).

Deletion of the MUC2 encoding gene in mice has revealed much regarding the role of MUC2 in innate immunity. MUC2 deficient mice suffered from growth retardation, rectal bleeding, chronic diarrhea and severe colitis attributed to direct contact between the gut bacteria and epithelial cells of the gastrointestinal tract (Johansson et al., 2008; Van der Sluis et al., 2006). The absence of

MUC2 also induced increased cell proliferation, decreased apoptosis, and increased migration of intestinal epithelial cells causing intestinal tumour formation and progression to carcinoma (Velcich et al., 2002). Furthermore, MUC2 deficient mice infected with *Citrobacter rodentium*, a mouse specific pathogen analogous to human *E. coli*, were highly susceptible to infection and developed lethal colitis (Bergstrom et al., 2010). These studies were crucial in confirming the hypothesis that a resilient and stable mucosal barrier plays a significant role in intestinal health (Rhodes, 1996).

The mucosal layer is known to be populated by bacteria with mucin-degrading activity that, while not inherently pathogenic, have been hypothesised to at least influence the integrity of the mucus layer (Martens et al., 2008; Tailford et al., 2015). Intriguingly, studies on *Bacteroides thetaiotaomicron*, a prominent human mutualist (Bäckhed et al., 2005; Xu et al., 2003), has implied a link between dietary composition, the gut microbiota, and the mucosal layer. As opposed to mucin-degrading specialists such as *Akkermansia muciniphila* (Derrien et al., 2004), genomic analysis of *B. thetaiotaomicron*, has revealed a significant adaptive advantage due to the functional diversity of CAZymes in the genome and the ability to switch between dietary and host mucin *O*-glycans based on nutrient availability (Martens et al., 2009). While the presence of mucin-degrading bacteria would appear to be counterproductive to host innate immunity, it is important to note that the mucus layer is continuously replaced through inducible goblet cell secretion, host glycan diversity is dynamic and responsive to highly regulated glycosyltransferase expression, and mucin-associated microbes appear to be highly adapted to this niche environment. These factors create a system that applies selective pressure to support a mutualistic gut microbiota that prioritises the maintenance of a stable "healthy" mucus layer.

More recent work has proposed a mechanism by which dietary composition can influence the stability of the mucus layer by interaction with gut microbiota (Desai et al., 2016). Using a gnotobiotic mouse model inoculated with a synthetic humanized microbiota, fibre deprivation was

shown to trigger the upregulation of mucin-degrading enzymes resulting in significant degradation of the colonic mucosal layer (Desai et al., 2016). When challenged with *C. rodentium* infection, fibre deprived mice were more susceptible to infection than germ free mice fed the same diet thus highlighting the significance of the gut microbiota in degrading the mucus layer and regulating pathogen susceptibility. While this study succeeds at implicating dietary composition with gut microbiota metabolism, mucus barrier stability, and infection susceptibility, there remains a lack of information regarding the functional role of host mucin glycosylation.

1.5 Thesis aims

Increasing the consumption of dietary fibre has been implicated in reducing the risk of non-communicable diseases including cardiovascular disease, type 2 diabetes, metabolic syndrome and colorectal cancer. While dietary fibre has a direct effect on the gastrointestinal tract, little is known about how different types of dietary fibre influence the delicate balance between the metabolism of the gut microbiota and the host colonic mucus layer to contribute to intestinal health. The overarching aim of this project was thus to investigate the effect of different dietary fibres on intestinal health through its influence on the gut microbiota and the colonic mucus layer using a combination of *in vitro* and *in vivo* model systems.

Specific Aims

1) Characterise the chemical composition of a dietary fibre derived from sugarcane in comparison with commercially available dietary fibres

2) Determine the effect of different dietary fibres on the human gut microbiota metabolism by measuring the production of short chain fatty acids using an *in vitro* gut mimicking system

3) Investigate the effect of dietary fibres on the host intestinal mucus layer by characterising changes *in vivo* on the glycosylation of MUC2, the major component of intestinal mucus, as induced by different diets and bacterial infection.

Chapter 2: Exploring sugarcane as a source of dietary fibre

2.1 Introduction

The plant kingdom has provided researchers with a relatively untapped resource in the pursuit of novel functional foods and nutraceuticals. As an economically significant crop, sugarcane (*Saccharum* sp.) represents an ideal candidate for further exploration since it is grown globally on an industrial scale (Food and Agriculture Organization of the United Nations) to produce sucrose, is readily available, and is positioned to benefit from research that adds value complementary to sucrose production. The historical use of sugarcane in the traditional medicine of many ancient cultures (Akber et al., 2011; Karthikeyan and Samipillai, 2010) also suggests the presence of biologically active compounds and this has been supported by the extraction and identification of polyphenolic compounds in sugarcane that possess antioxidant, antimicrobial, and anti-tumour bioactivity (Heleno et al., 2015). Within the last decade, sugarcane has been the subject of intense investigation as a suitable feedstock for bioethanol production due to the presence of significant amounts of lignocellulosic biomass in sugarcane stalk (de Souza et al., 2013; Dias et al., 2009; Masarin et al., 2011; Sindhu et al., 2016). These studies have focused on the characterisation and development of pre-treatment processes to aid in the release of structural polysaccharides from bagasse, the residual plant material waste from sucrose production, to generate suitable substrates for fermentation reactions (de Souza et al., 2013).

Despite abundant evidence indicating significant quantities of complex plant polysaccharides and bioactive compounds, which are valuable to the food industry, research into sugarcane outside the scope of sucrose or bioethanol production is scarce. The development of new dietary fibre products has been gaining interest as the food industry adapts to the demands of consumers who desire safe, palatable, and convenient products with proven health benefits to combat the rise of major NCDs such as CVD, obesity, T2DM, and colorectal cancer (Elleuch et al., 2011). The chemical and nutritional composition of food product is essential knowledge in the determination of functional

and physiological properties, assessment of quality and nutrient contribution, and for assigning appropriate applications. Benefiber and psyllium husk were included in the characterisation of dietary fibres to compare NutriKane, a dietary fibre derived from while sugarcane stalk, with commercially available dietary fibres. Benefiber is a wheat dextrin that is marketed as a water-soluble dietary fibre that relieves constipation and promote gut health. Psyllium husk is a dietary fibre derived from the seed husk of the *Plantago ovata* plant that is an effective laxative that swells in the presence of water and increases the bulk of stools making them softer and easier to pass. Both Benefibre and psyllium husk are soluble dietary fibres that have received widespread adoption in the field of health food products.

2.2 Chemical characterisation and comparison of dietary fibres (Paper 1)

Raymond W. W. Chong, Malcolm Ball, Christopher McRae, Nicolle H. Packer**.** *Comparing the chemical composition of dietary fibres prepared from sugarcane, psyllium husk and wheat dextrin.* Manuscript submitted to Food Chemistry.

The following manuscript presents the chemical characterisation of NutriKane, a dietary fibre derived from sugarcane stalk, in comparison with Benefiber and psyllium husk, which commercially available dietary fibres. Benefiber is a modified wheat dextrin soluble fibre. Psyllium husk is soluble gel forming fibre with a high-water holding capacity. The characterisation of five different preparations of sugarcane derived fibre was also included to determine the impact of strain, harvest period, and processing conditions on the chemical composition of the final product, which are important considerations in development and production processes within the food industry. All data collection, analysis and interpretation were performed by R. W. W. Chong. The manuscript was prepared by R. W. W. Chong and was edited by M. Ball, C. McRae and N. H. Packer. This manuscript has been submitted for publication to the Journal of Food Chemistry and is currently under review.

Comparing the chemical composition of dietary fibres prepared from sugarcane, psyllium husk and wheat dextrin

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Abstract

A dietary fibre prepared from sugarcane stalk was compared with psyllium husk and wheat dextrin. In contrast to the other dietary fibres, sugarcane fibre (SC) was found to be contain significant amounts of insoluble dietary fibre (73 – 86%), lignin (18.66 – 20.23%), and rare minerals such as chromium (0.67 – 2.54 mg/100 g) and manganese (1.07 – 2.34 mg/100g). Analysis of the ethanol extract also detected compounds with antioxidant activity. Characterisation of five sugarcane fibres prepared from selected strains, harvest periods, and processing conditions showed these factors influenced the final composition. Furthermore, using an *in vitro* digestion mimicking system we found that potassium, magnesium, chromium, and zinc in SCs was bio accessible. Meanwhile, sodium was shown to bind to the sugarcane fibre potentially indicating bile salt binding activity. The results of this study support the use of sugarcane as a source of dietary fibre functional foods.

Key words: Chemical composition**;** Dietary fibre; Sugarcane; Psyllium husk; Wheat dextrin,

Highlights

- The chemical compositions of sugarcane fibre, psyllium husk and wheat dextrin were determined
- Sugarcane fibre composition was influenced by strains, harvest period, and processing
- Sugarcane fibre contained more insoluble fibre and chromium than psyllium husk and wheat dextrin
- Sugarcane fibre exhibited antioxidant and salt binding activity

1. Introduction

The necessity for adequate dietary fibre intake is one of the most well studied aspects of a direct link between nutrition and human health (Kendall et al., 2010). Increased consumption of dietary

fibre has been associated with improvements in diet-associated diseases such as obesity, type 2 diabetes mellitus, cardiovascular disease, inflammatory bowel diseases and colorectal cancer (Dahl and Stewart, 2015). As such, researchers have begun to explore the value of agricultural byproducts such as maize straw (Lv et al., 2017) and citrus peels (Wang et al., 2015) in the preparation of novel dietary fibres.

Sugarcane (*Saccharum officinarum* L.) is one of the most economically important agricultural crops that is grown primarily as a source of sucrose. The crushed stalk, commonly known as bagasse, is high in lignocellulosic content and has received considerable attention as potential feedstock for the biofuel industry (Sindhu et al., 2016). Since lignocellulosic content is comprised of undigestible lignin, cellulose and hemicellulose, it represents a relatively underutilised source of dietary fibre. Sugarcane and its derivative products have also been investigated as a source of polyphenolic compounds that may be beneficial to human health (Duarte-Almeida et al., 2011; Feng et al., 2014; Zheng et al., 2017).

According to the CODEX Alimentarius, dietary fibre is broadly defined as the carbohydrate polymers, which are primarily the non-starch polysaccharides like cellulose, hemicelluloses, and pectins, as well as plant cell wall associated compounds that are not hydrolysed by the endogenous enzymes in the human digestive tract (Jones, 2014). Based on physicochemical properties, such as water solubility and gel-forming capacity, dietary fibre is subdivided into soluble and insoluble fibre, which are often associated with different physiological activities once consumed. For example, soluble fibres are generally well fermented in the gut and increase the viscosity of the luminal contents producing a laxative effect, while insoluble fibres have been shown to reduce digestibility and may adsorb potential carcinogens (Ferguson et al., 1995).

While the preparation of dietary fibre from sugarcane bagasse has been reported (Miao et al., 2016; Sangnark and Noomhorm, 2003), the application of fresh sugarcane stalk has yet to be explored. As an industrial waste material, bagasse is often contaminated by agricultural waste and requires

chemical treatment prior to human consumption. These treatments have been shown to alter the chemical properties of these products (Sangnark and Noomhorm, 2004). As an alternative to sugarcane bagasse, we investigated a fibre product prepared directly from whole sugarcane (SC) within 15 hours of harvest. The method of preparation was designed to remove sucrose using mechanical maceration and grinding followed by water steeping. This method aimed to prepare a product high in dietary fibre while preserving beneficial compounds, such as antioxidant polyphenolics, that would otherwise be destroyed through chemical treatment.

The aims of this study were to: (a) determine the chemical composition of five sugarcane fibre products selected from different strains, harvested at different stages of plant life cycle and different production procedures, to investigate the influence of genetics, plant life cycle and processing conditions on the chemical properties of the fibre product; (b) compare the chemical composition and physical properties of sugarcane fibre with psyllium husk (PH) and wheat dextrin (WD).

2. Material and Methods

2.1 Chemicals

Unless stated otherwise, all chemicals and reagents were purchased from Sigma Aldrich, Sydney, Australia. Sugarcane fibre samples were provided by Gratuk Technologies Pty. Ltd., Sydney, Australia. Sample labels and growing conditions are provided in Table 1. Wheat dextrin (Benefibre™ brand) and Psyllium husk (Macro brand) were purchased from a local supermarket. All solvents were of analytical grade or higher. Ultrapure water was obtained from a MilliQ system (Millipore, Sydney, Australia).

2.2 Preparation of sugarcane fibre products

Samples were prepared using a proprietary process. Briefly, sugarcane stalks were harvested green, washed in cold water then shredded and mechanically macerated to a pulp. The pulp was steeped in cold water then dried using a proprietary high-temperature short residence time drier.

The pulp was further mechanically ground to approximately 50 µm particles and air classified (size separated by a column of rising air), with larger particles returned for further grinding.

Five sugarcane fibre products (SCs) were selected based on availability. These products were prepared from different strains, harvest periods, and processing conditions to determine the effect of each condition on the chemical composition of the final fibre product (Table 1) Strains Q208^{A}, Q252^{A}, Q240^{A} were among the most recently released sugarcane varieties within the Herbert/Burdekin region in Queensland, Australia.

Table 1. Samples IDs and descriptions of the different strains, harvest period, and processing conditions used to prepare samples included in this study

Sample ID	Strain	Harvest period	Processing conditions
SC-NK (NutriKane)	Q208^{A}	Storage phase	Ripping saws
SC-8B3	Q208^{A}	Storage phase	Shearing forces
SC-954	Q252^{A}	Storage phase	Shearing forces
SC-3Q1	Q240^{A}	Storage phase	Shearing forces
SC-0T7	Q240^{A}	Growth phase	Shearing forces

2.3 Compositional analysis

Moisture and ash content of SC, WD, and PH samples were assessed simultaneously by thermogravimetric analysis (TGA) using a Thermogravimetric Analyzer 2050 (TA Instruments) in triplicate. Moisture was calculated as the weight lost while the temperature was held at 110 °C under a nitrogen atmosphere for 10 mins or until no further mass was lost. Ash content was calculated as the residue remaining after the temperature was raised to 800 °C in an oxygen atmosphere for 10 mins or until no further mass was lost. Total nitrogen content was determined by the Pregl-Dumas method using a Series II CHNS/O Analyzer 2400 (Perkin Elmer) in duplicate. Protein content was calculated by multiplying Nitrogen content by a factor of 6.25 (Fujihara et al., 2008). Total fat content was measured in duplicate according to AOAC 945.16 method by Soxhlet

extraction with petroleum ether for 16 hours. Water holding capacity was determined in triplicate according to Sangnark and Noomhorn (Sangnark and Noomhorm, 2003). Briefly, a dried sample (3 grams) was mixed with an excess of distilled water and allowed to hydrate for 2 hours. Excess water was drained by gravity filtration then the residual pellet was separated into three 1-gram portions (wet weight) for subsequent drying to constant weight in a 110 °C oven (dry weight). Water holding capacity was defined as the weight lost as follows:

$$Water\ holding\ capacity = \frac{wet\ weight - dry\ weight}{dry\ weight}$$

2.4 Total, insoluble, and soluble dietary fibre analysis

Total dietary fibre (TDF) was determined enzymatically according to AOAC 985.29 method. Dried samples of SC, WD, and PH underwent sequential enzymatic digestion by heat stable α-amylase (95–100 °C, pH 6.0, 15 min), Subtilisin A (60 °C, pH 7.5, 30 min), and amyloglucosidase (60 °C, pH 4.5, 30 min) to remove starch and protein. To determine TDF content, the enzyme digests were precipitated with four volumes of 95% (v/v) ethanol, filtered then washed with 78% (v/v) ethanol, acetone, then dried in a 110 °C oven. Insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) were determined according to AOAC 991.43. Dried samples underwent the same sequential enzymatic digestion as for TDF. For IDF, the enzyme digestate was filtered, and the residue was washed with warm water, dried in a 110 °C oven, and weighed. For SDF, the combined filtrates and washes were precipitated with 78% and 95% alcohol, filtered, dried in a 110 °C oven, and weighed. All measurements for TDF, SDF, and IDF residues were measured in duplicate and corrected for blank, ash and protein.

2.5 Determination of acid insoluble lignin

Acid insoluble lignin was measured by acid hydrolysis according to Willför et al. (2009) in triplicate. Approximately 1 gram of SC, WD, and PH samples were digested with 72% (v/v) sulfuric acid at room temperature for 1 hour, then diluted with water to 4% (v/v) sulfuric acid and autoclaved to a maximum temperature of 121°C for 30 minutes. Lignin hydrolysates were filtered

through glass Gooch crucibles packed with acid washed Celite™, washed with cold water, then dried and weighed.

2.6 Monosaccharide analysis by gas chromatography/flame ionisation detection (GC-FID)

Approximately 1 gram of SC, WD, and PH samples were hydrolysed according to the protocol for determination of acid insoluble lignin. The supernatant (1 mL) was neutralised with a saturated solution of barium hydroxide ($Ba(OH)_2$), desalted over Dowex 50W X8 hydrogen form cation exchange resin, then dried under nitrogen. Monosaccharides were reduced with 1 M sodium borohydride ($NaBH_4$) in 2 M ammonium hydroxide (NH_4OH) at room temperature for 2.5 hours. The reaction was quenched with glacial acetic acid and dried under nitrogen stream. To remove excess borate samples were repeatedly dissolved in methanol (250 µL) and dried under nitrogen. Acetylation was achieved by the addition of anhydrous acetic anhydride (250 µL) and incubation at 100° C for 2.5 hours. The reaction was quenched with ultrapure water (2 mL) and acetylated monosaccharides were extracted with dichloromethane (DCM). The organic DCM phase was collected and washed twice with equal parts of ultrapure water then dried by passing through a hand packed column of dry $MgSO_4$.

Acetylated samples were analysed in triplicate using a Shimadzu 17A gas chromatograph (GC) with flame ionisation detection (FID). One µL of DCM phase was injected by autosampler in split mode (split ratio 10:1) and separated on a 5% diphenyl dimethyl polysiloxane (BP5, 40 m × 0.25 mm × 0.25 25 µm film thickness, SGE) using a hydrogen carrier gas with a linear velocity of 50.0 cm/s. The temperature program was 80 °C for 2 mins, then ramped at 20 °C/min to 140°C, 4 °C/min to 250 °C and 40 °C/min to 300 °C, holding at 300 °C for 5 mins. The injector and detector temperatures were 280 and 300 °C respectively. Quantitation was performed by acetylation of a mixture of monosaccharide standards of known concentration with 2-deoxy-D-glucose as an internal standard, which was added to all samples at 100 ppm concentration, to allow calculation of response factors.

2.7 Scanning electron microscopy

Approximately 5 mg of SC, WD, and PH were mounted onto aluminium stubs using double sided carbon tape. Samples were then sputter-coated with gold in an EMITECH K550 to 20 nm thickness and viewed in a JEOL JSM-6480 LA scanning electron microscope. Images were captured with assistance from the Macquarie University Micropscopy Unit.

2.8 Mineral analysis

Determination of minerals was performed by microwave plasma atomic emission spectroscopy following acid digestion of SC, WD, and PH samples (MP-AES, 4200 MP-AES, Agilent Technologies, Santa Clara). Approximately 1 g of each sample was initially digested with 15 mL of concentrated nitric acid (70% v/v) at room temperature for 16 hours, then boiled for 1 hour. To complete digestion, perchloric acid (2mL, 70% v/v) was added and the volume reduced to 5 mL by boiling. Digested samples were diluted with 5% (v/v) nitric acid and filtered (0.45 µm Millipore membrane) before analysis. A periodic table mix of elements was used to generate external standard curves of Ca (393.366 nm), Cr (425.433 nm), Cu (324.754 nm), Fe (259.940 nm), K (766.491 nm), Mg (285.213 nm), Mn (403.076 nm), Na (588.995 nm), and Zn (213.857 nm) in the concentration range of 0.1 – 5 mg/L. Samples were analysed in technical triplicates.

2.9 Bioaccessibility of minerals by simulated *in vitro* digestion

Bioaccessibility of the minerals determined by comparing two extraction methods. Approximately 1 gram of SC, WD, and PH was extracted with water at 37 °C to remove water soluble minerals. A separate sample was digested *in vitro* with gastrointestinal fluids and enzymes to simulate the human digestive system with reference to the procedure of Minekus et al. (2014).

Approximately 1 gram of SC, WD, and PH was weighed separately and dispersed into 20 mL of ultrapure water and the pH adjusted to 3.0 with 6 M HCl. Considering the powdered nature of the samples the salivary phase was omitted and digestion was initiated in the gastric phase by the addition of 100 µL porcine pepsin solution (1 g in 10 mL water, final concentration 2000

Units/mL). Samples were incubated for 2 hours at 37 °C with shaking (150 rpm orbital). For the intestinal phase, the pH was raised to 7.0 with 1 M NaOH then bovine bile extract (2.5 g in 10 mL, final concentration 2.5% w/v), trypsin (final concentration 100 Units/mL), chymotrypsin (25 Units/mL), pancreatic amylase (final concentration 75 Units/mL), porcine lipase (final concentration 2000 Units/mL), and porcine colipase (final concentration 4000 Units/mL) were added. Samples were incubated for 2 hours at 37 °C with shaking (150 rpm orbital). Digestion was stopped by placing samples on ice and adjusting the pH to 7.2 with 1 M sodium bicarbonate ($NaHCO_3$). Samples were centrifuged at 8000 rcf for 10 minutes at 4 °C, then the supernatants removed. The sample residues were washed three times by adding 20 mL aliquots of ultrapure water, centrifuging at 8000 rcf for 10 minutes at 4 °C and removing the supernatants.

Once extracted with water or digested *in vitro*, fibre samples were washed with water, mineralised with acid and the mineral content of the residual fibres determined by MP-AES. The loss of minerals following *in vitro* digestion was considered indicative of bioaccessibility. SC-8B3 was used as a representative sample due to its high mineral content among the SC samples. Considering its water solubility WD was excluded.

2.10 Extraction and fractionation of antioxidant compounds

Extraction of dried samples of SC, WD, and PH was performed by ultrasound assisted extraction. Approximately 1 g of each sample was extracted with 52% (v/v) ethanol at a 14 mL/g solvent to sample ratio, at 60 °C for 1 hour in an ultrasonic water bath (40 kHz) (Feng et al., 2014). The extraction was performed three times with fresh samples. Ethanolic extracts were filtered through 0.45 µm membranes (Millipore) and freeze dried. Dried extracts were fractionated by solvent partitioning. Approximately 100 mg of each extract was re-suspended in 10 mL ultrapure water then partitioned sequentially with three volumes of petroleum ether, ethyl acetate, and 1-butanol. Partitions were combined then dried under nitrogen to yield the petroleum ether (PE), ethyl acetate (EA), 1-butanol (BU), and aqueous (AQ) fractions.

2.11 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant activity of ethanol extracts and liquid partitioned fractions was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as described by Musa et al. (2013). Samples were analysed in triplicate.

2.12 Ferric reducing antioxidant power assay (FRAP)

The ferric reducing antioxidant power (FRAP) assay was used to estimate antioxidant activity by the reduction of an Fe^{3+} complex to Fe^{2+}. The FRAP assay was performed in a 96 well plate format according to Bolanos de la Torre et al. (2015). Samples were analysed in triplicate.

2.13 Total phenolic content (TPC)

Total phenolic content was determined using the Folin-Ciocalteu assay adapted for smaller volumes in a 1 mL cuvette (Singleton et al., 1999). Dried ethanolic extracts were dissolved in ethanol at a concentration of 10mg/mL for colourimetric analysis. In a 1 mL cuvette, 100 µL of diluted sample was mixed with 600 µL ultrapure water and 100 µL Folin-Ciocalteu's phenol reagent. The mixture was incubated at room temperature for 5 mins then 200 µL of 20% (w/v) Na_2CO_3 was added. The mixture was incubated at room temperature in the dark for 20 mins. The absorbance at 735 nm was measured using a FLUOstar Galaxy Multifunctional Microplate Reader (BMG technologies) and compared against a standard curve of gallic acid between the concentrations of 50 and 1000 µM. Samples were analysed in triplicate.

2.14 Ultra-high-pressure liquid chromatography/high resolution mass spectrometry (UPLC-HRMS)

For identification of antioxidant compounds, the ethyl acetate fraction was dissolved in 1 mL of methanol, filtered through a 0.45 µm membrane then analysed by high resolution mass spectrometry using the QExactive Plus mass spectrometer (ThermoFisher, Waltham, MA, USA) coupled to a Thermo UltiMate 3000 HPLC system (ThermoFisher, Waltham, MA, USA). The analytes were resolved using a Waters Acquity BEH C18 column (2.1 × 100 mm, 1.7 µm) at a flow rate of 400 µL/min. Solvent A was 0.1% formic acid, Solvent B was methanol and the

gradient conditions were: 10% to 80% B, 24 min.; hold at 80% B, 0.5 min.; 80% to 10% B, 1.5 min.; and re-equilibration to 10% B for 4 min. The QExactive Plus mass spectrometer was operated using electrospray ionization in negative ion mode with a capillary voltage of -3.75 kV and capillary temperature of 320 °C. Nitrogen gas was used as the collision gas. The instrument was calibrated by external calibration using standards supplied by the manufacturer. Samples were analyzed using full scan mode from m/z 100–1000 at a resolution of 35 000 FWHM. Compound identification was achieved by comparing retention time and spectral matching against a set of external calibration standards.

2.15 Statistical Analysis

All experiment analyses were performed in technical triplicates. Data was analysed using GraphPad Prism 7.03. Ordinary one-way analysis of variance (ANOVA) and post-hoc testing with Tukey's test was performed to determine statistically significant differences between samples with 95% ($P < 0.05$) confidence limits.

3. Results and Discussion

3.1 Effect of strain, harvest period, and processing conditions on sugarcane fibres

3.1.1 Proximate composition

Protein content, which ranged between 0.54 – 1.38 g/100g, was highest in SC-3Q1 and lowest in SC-NK (**Table 2**). Protein content of samples prepared from strain $Q240^{A}$ (SC-3Q1 and SC0T7) was significantly higher than samples prepared from strains $Q252^{A}$ (SC-954, p = <0.0001 and 0.0012) and $Q208^{A}$ (SC-NK, p = <0.0001 and <0.0001, and SC-8B3, p = 0.0004 and 0.0077). These results suggest that strain is a main contributor to protein content. There was no difference in moisture, protein, ash or fat content when comparing SC-3Q1 and SC-0T7 suggesting that harvest of the cane stalk in the sugar storage (SC-3Q1) or plant growth phase (SC-0T7) had no effect on proximate composition. Processing conditions had the greatest effect on proximate composition. The use of shearing forces (SC-8B3) compared to ripping saws (SC-NK) was associated with significant increases in protein (p = 0.0004) and ash (p = 0.0112), and a significant

decrease in fat (p = 0.0306). Compared to sugarcane bagasse, SC products contained similar levels of moisture (3.24 – 5.93%) and protein (0.83 – 0.96%) while containing higher amounts of ash (0.78 – 1.59%) and fat (0.15 – 0.30%)(Sangeetha et al., 2011). Ash content is a cumulative measure of inorganic material that can be used to quality of food products with respect to the presence of mineral micronutrients while fat content is representative of the hydrophobic components of plants including waxes and oils.

3.1.2 Dietary fibre and carbohydrate composition

There were no significant differences in TDF, IDF, SDF, or lignin content across all five SC samples (**Table 2**). SC was found to be entirely insoluble dietary fibre (IDF) since no soluble dietary fibre (SDF) was detected. The IDF content of sugarcane fibre (73 – 86%) was higher than steamed, acid or alkali treated sugarcane bagasse (72 – 75%) (Sangeetha et al., 2011) suggesting that SC is a better source of IDF than bagasse and could potentially fill a niche role since most dietary fibres are SDF. The absence of SDF in SC suggests that the xylose was present its insoluble form with implications in reducing digestibility, increasing water holding capacity, and the absorption of dietary components thereby enhancing the physiological effects of insoluble dietary fibres (Kumar et al., 2012).

In general, our results were in agreement with previous analyses of sugarcane bagasse that found similar amounts of lignin and TDF (Masarin et al., 2011). Differences in monosaccharide composition were observed between the SCs, indicating that strain, harvest period, and processing conditions had an influence on the plant cell wall components with the greatest variations observed in xylose content. Overall, xylose was lower in strain $Q208^{A}$ (SC-8B3) compared to $Q252^{A}$ (SC-954, p = 0.0002) and $Q240^{A}$ (SC-3Q1, p = 0.0003). Within the same strain ($Q240^{A}$, SC-3Q1 and SC-OT7), less xylose was present in the growth phase compared to the storage phase of the plant. Decreases in monosaccharide composition due to harvest period were also seen in arabinose, mannose, glucose, and galactose though not to the extent of xylose. Since xylose is a major

component of the hemicelluloses, a class of compounds that involved in plant structural integrity (Gibson, 2012), it is unsurprising that xylose content was higher in the mature plant.

	SC-NK	SC-8B3	SC-954	SC-3Q1	SC-OT7	Psyllium Husk	Wheat Dextrin
Moisture	4.19 ± 0.25^{a}	5.58 ± 0.65^{a,b}	5.93 ± 0.07^{b}	6.12 ± 0.46^{b}	4.72 ± 0.94^{a,b}	10.53 ± 0.10^{c}	6.45 ± 0.01^{d}
Nitrogen	0.08 ± 0.01^{a}	0.15 ± 0.01^{b}	0.13 ± 0.14^{b}	0.21 ± 0.22^{c,d}	0.19 ± 0.19^{c}	0.24 ± 0.01^{d}	ND
Protein	0.54 ± 0.04^{a}	0.94 ± 0.01^{b}	0.84 ± 0.04^{b}	1.38 ± 0.09^{c,d}	1.22 ± 0.04^{c}	1.50 ± 0.09^{d}	ND
Fat	1.17 ± 0.09^{a}	0.79 ± 0.13^{b}	0.96 ± 0.06^{a,b}	0.89 ± 0.18^{a,b}	1.16 ± 0.17^{a}	0.46 ± 0.15^{c}	0.02 ± 0.04^{d}
Ash	1.92 ± 0.06^{a}	2.74 ± 0.36^{b}	2.13 ± 0.11^{a,b}	2.34 ± 0.13^{a,b}	2.42 ± 0.12^{a,b}	2.34 ± 0.14^{a,b}	ND
TDF	83.94 ± 0.60^{a}	82.12 ± 0.64^{a}	86.97 ± 0.57^{a}	84.46 ± 0.41^{a}	84.62 ± 0.46^{a}	77.24 ± 1.18$_{b}$	10.31 ± 1.19^{c}
IDF	86.65 ± 1.95^{a}	86.08 ± 7.48^{a}	86.06 ± 1.49^{a}	73.43 ± 5.54^{a}	85.78 ± 2.52^{a}	71.74 ± 0.87^{a}	ND
SDF	ND	ND	ND	ND	ND	ND	5.64 ± 1.14
Lignin	20.23 ± 1.08^{a}	18.66 ± 0.16^{a}	19.73 ± 0.64^{a}	19.72 ± 0.76^{a}	19.85 ± 0.94^{a}	4.69 ± 0.21^{b}	ND
Rhamnose	ND	ND	ND	ND	ND	2.39 ± 0.03	ND
Arabinose	5.83 ± 0.45^{a}	6.30 ± 0.54^{b}	6.93 ± 0.57^{b}	7.18 ± 0.62^{b}	5.37 ± 0.53^{a}	46.8 ± 0.49^{c}	1.27 ± 0.2^{d}
Xylose	31.8 ± 2.47^{a}	34.1 ± 2.90^{a}	40.4 ± 3.38^{b}	40.2 ± 3.44^{b}	31.9 ± 3.12^{a}	24.1 ± 0.25^{c}	0.19 ± 0.01^{d}
Mannose	1.54 ± 0.12^{a}	1.59 ± 0.14^{a}	1.56 ± 0.13^{a}	1.89 ± 0.16^{b}	1.35 ± 0.13^{a}	4.24 ± 0.04^{c}	21.3 ± 0.22^{d}
Glucose	17.3 ± 1.34^{a}	18.7 ± 1.59^{a}	18.3 ±1.51^{a}	17.5 ± 1.50^{a}	14.9 ± 1.46^{b}	11.2 ± 0.12^{c}	74.5 ± 0.78^{d}
Galactose	0.74 ± 0.06^{a}	0.73 ± 0.06^{a}	0.82 ± 0.07^{a}	0.95 ± 0.08^{a}	0.65 ± 0.06^{a}	2.09 ± 0.16^{b}	ND
Extractive	9.08 ± 0.12^{a}	10.6 ± 0.56^{b}	6.77 ± 0.43^{c}	7.87 ± 0.41^{d}	9.01 ± 0.53^{a}	0.95 ± 0.08^{d}	N/D
WHC*	8.19 ± 0.98^{a}	11.14 ± 007^{a}	8.11 ±0.67^{a}	8.79 ± 0.42^{a}	9.27 ± 0.03^{a}	45.03 ± 4.00^{b}	N/A

Table 2 Chemical composition, extractive content, and water holding capacity of sugarcane fibres (SCs), psyllium husk, and wheat dextrin. Values are expressed g/100g total weight unless not detected (ND) and represent the mean ± the standard deviation (n = 3). ^{a,b,c,d}Values in the same row with different letters are significantly different from each other as determined by one-way ANOVA and tukeys testing ($p < 0.05$). *Water Holding Capacity represents the amount of water absorbed by 1 gram of dry sample.

3.1.3 Ethanol extract

Comparison of SC-NK with SC-8B3, which were prepared from the same strain of sugarcane ($Q208^{A}$) and harvested at the same time (storage phase), showed that transition from ripping saws (SC-NK) to shearing forces (SC-8B3) as the initial size reducing step was associated with a significant increase in extractive content (p = 0.0032) due to processing conditions (Table 1, Extractive). The transition to shearing forces, which are considered milder than ripping saws, may have kept the plant cell walls more intact thereby keeping the extractive fraction within the fibre. Comparison between different strains found significant variation in extractive content due to genotypic differences. This is consistent with the accumulation of polyphenols in sugarcane varieties contributing to increased resistance to diseases such as smut, a parasitic fungal disease (De Armas et al., 2007).

The highest extractive content was found in SC-8B3 (strain $Q208^{A}$), while SC-954 (strain $Q252^{A}$), contained the least. Strain $Q208^{A}$ is a recently developed high-yielding variety of sugarcane grown in the Burdekin region of Australia and is known for its high resistance to diseases including smut, leaf scald, chlorotic streak, orange and brown rust, red rot, and yellow spot. Strain $Q240^{A}$ (SC-3Q1) was produced from parents QN81-289 and SP78-3137 from Brazil and has high resistance to both leaf scald, smut, orange rust, and red rot but is susceptible to brown rust and yellow spot. Strain $Q252^{A}$ is related to $Q208^{A}$ and was produced from breeding between the $Q208^{A}$ and Q96 varieties and has intermediate resistance to smut and high resistance to leaf scald, chlorotic streak, orange and brown rust, red rot, and yellow spot. Varieties with high resistance to a broad range of diseases ($Q208^{A}$ and $Q240^{A}$), were found to contain higher extractive content than the less resistant variety ($Q252^{A}$) suggesting that compounds within the ethanol extract play a role in resistance against the disease. Our findings suggest that processing conditions and strain are the major contributors to extractive content. These findings are also in agreement with Rocha et al. (2015) that showed the greatest variation in extractive content between strains and milling procedures.

3.1.4 Mineral content

All SCs contained a range of minerals including chromium, copper, iron, manganese, and zinc, which are considered essential trace minerals (Table 3). Strain was associated with variations in mineral content depending on the element. Strain Q208^{A} (SC-8B3) contained the highest amounts of potassium (161 mg/100g), sodium (43.4 mg/100g), chromium (2.54 mg/100g), and iron (35.2 mg/100g), while strain Q240^{A} (SC-3Q1) contained the highest amounts of magnesium (48.7 mg/100g) and manganese (2.28 mg/100g). Processing condition had the most significant effect on mineral content; size reduction by ripping saws (SC-NK) resulted in a significant decrease in all minerals except magnesium and manganese, in comparison to processing by shear forces (SC-8B3). As a milder processing condition, shearing forces appeared to retain the micronutrient content of the cane stalk by means of keeping the plant cell walls intact. Harvest period had no observable effect on mineral content as seen by comparing SC-3Q1 and SC-OT7 that were not significantly different in any of the minerals detected.

	SC-NK	SC-8B3	SC-954	SC-3Q1	SC-OT7	Psyllium Husk	Wheat Dextrin
Calcium (Ca)	34.4 ± 0.2^{a}	43.1 ± 0.2^{b}	$41.5 \pm 0.7^{a,b}$	$42.5 \pm 0.2^{a,b}$	$42.3 \pm 0.3^{a,b}$	104 ± 0.9^{c}	1.9 ± 0.8^{d}
Potassium (K)	69.9 ± 0.8^{a}	161 ± 6.5^{b}	132 ± 1.8^{c}	104 ± 1.2^{d}	105 ± 1.4^{d}	805 ± 2.3^{e}	ND
Magnesium (Mg)	45.2 ± 1.4^{a}	45.8 ± 1.3^{a}	40.3 ± 0.9^{b}	48.7 ± 1.5^{a}	48.7 ± 0.9^{a}	19.7 ± 3.7^{c}	0.6 ± 0.1^{d}
Sodium (Na)	24.5 ± 0.9^{a}	43.4 ± 3.2^{b}	31.5 ± 1.9^{c}	26.0 ± 1.3^{a}	27.7 ± 1.9^{a}	62.3 ± 2.7^{d}	2.1 ± 0.7^{e}
Chromium (Cr)	0.67 ± 0.07^{a}	2.54 ± 0.08^{b}	1.75 ± 0.32^{c}	2.15 ± 0.19^{d}	$2.03 \pm 0.10^{c,d}$	0.28 ± 0.06^{e}	ND
Copper (Cu)	0.21 ± 0.01^{a}	0.71 ± 0.06^{b}	0.95 ± 0.04^{c}	0.71 ± 0.13^{b}	$0.59 \pm 0.02^{b,d}$	0.36 ± 0.26^{d}	ND
Iron (Fe)	12.7 ± 0.5^{a}	35.2 ± 0.4^{b}	29.6 ± 0.1^{c}	32.9 ± 0.8^{d}	32.8 ± 0.7^{d}	9.3 ± 0.2^{e}	ND
Manganese (Mn)	1.07 ± 0.04^{a}	1.31 ± 0.04^{b}	1.15 ± 0.17^{a}	2.28 ± 0.12^{c}	2.34 ± 0.14^{c}	1.15 ± 0.07^{a}	ND
Zinc (Zn)	0.64 ± 0.12^{a}	1.29 ± 0.16^{b}	1.43 ± 0.42^{b}	1.36 ± 0.21^{b}	1.45 ± 0.31^{b}	ND	ND

Table 3. Total mineral content of samples. Values are expressed as mg per 100g and represent the mean ± the standard deviation (n=3). ^{a,b,c,d}Values in the same row with different letters are significantly different from each other by one-way ANOVA and tukeys test ($p < 0.05$).

3.2 Chemical comparison of sugarcane fibre, psyllium husk and wheat dextrin

Having determined the effect of different conditions on the chemical composition of SC, WD, and PH were characterised to compare the chemical composition of SC with other dietary fibres.

3.2.1 Proximate analysis, particle morphology and solubility

Proximate analysis found significant differences in the composition of SC, PH, and WD. SC contained less moisture and protein, and more total fat, TDF and lignin than PH (Table 2). Wheat dextrin contained more moisture, and less TDF, protein, ash and lignin. Based on the images obtained by SEM (Supplementary Figure 1), SC particles were the most heterogeneous in terms of particle size, shape, and texture. This was likely due to the inclusion of different parts of the sugarcane stalk since similarities were seen in the morphology of particles described in a previous study (Wong Sak Hoi and Martincigh, 2013). In comparison, PH and WD particles were morphologically uniform; PH had the largest particle size (> 500 µm) that appeared as smooth sheets of organised cells (Supplementary Figure 1, d), while WD particles were small (100 – 250 µm) and shared similar characteristics to acid hydrolysed starch granules (Utrilla-Coello et al., 2014). In terms of water solubility, WD was completely soluble in water, SC was completely insoluble, and PH formed a thick gel. PH possessed an extremely high-water holding capacity (45g water per g fibre) compared to SC (9 -11g water per g fibre) that was non-gel forming (Table 2). Water holding capacity (WHC) is defined as the amount of water that a given food can hold. These variations between SC, PH, and WD indicate significant differences in mechanical and physiological function.

3.2.2 Dietary fibre and carbohydrate composition

The most evident differences between SC, WD and PH were seen in the lignin and carbohydrate content (Table 2). Lignin was a defining feature of SC, constituting 18 – 20%, compared to 5% in PH, and none in WD. By weight, lignin contributed to approximately 25% of the IDF content of SC. In conjunction with cellulose and hemicellulose, lignin is an integral part of the plant cell wall

structure forming a natural barrier against pathogenic attack, providing structural support and resistance to chemical degradation (Gibson, 2012). High lignin content has also been associated with increased resistance to digestion (Knudsen, 1997). The effect of lignin content on the physicochemical properties of fibres has been shown by the chemical removal of lignin that results in an increase in water holding and oil binding capacity (Sangnark and Noomhorm, 2003). The biosynthesis of lignin in the plant cell wall has been linked to the production of polyphenolic secondary metabolites (Ferrer et al., 2008), such as the cinnamic acids and flavonoids, and may explain the presence of these compounds in SC.

Total carbohydrate content was lower in SC compared to PH and WD. Monosaccharide analysis found characteristic differences between the three samples; SC was predominantly comprised of xylose (31.8 – 40.4 g/100g) and glucose (13.9 – 18.7 g/100g), PH of arabinose (46.8 g/100g) and xylose (24.1 g/100g), and WD of mannose (21.3 g/100g) and glucose (74.5 g/100g). The presence of arabinose and xylose in PH was in agreement with previous work that showed the gel forming component of the husk was a highly branched arabinoxylan (Fischer et al., 2004). Most of the physiological effects of PH, such as feelings of satiety, increased faecal bulking, and control of blood glucose and cholesterol levels have already been attributed to its gel forming properties and high water holding capacity (McRorie, 2015). As a completely soluble dietary fibre, WD is readily fermented in the colon, increasing the production of microbial metabolites, such as short chain fatty acids, that are absorbed and influence host metabolism by regulating serum concentrations of glucose, insulin, and cholesterol (Slavin et al., 2009). While SC was not water soluble, the moderate water holding capacity of SC may assist in the relief of constipation, an activity already observed in fibre derived from bagasse (Sangeetha et al., 2011). As an insoluble dietary fibre, it is possible that SC may exhibit anti-nutritive activity by binding compounds such as minerals and bile salts that may be useful in treating cardiovascular disease through the reduction of dietary sodium (Schedle et al., 2008).

3.2.3 Mineral content

There were significant differences in the mineral content of SC, PH and WD. Compared to SC, PH contained significantly higher amounts of potassium (805 mg/100 g), calcium (104 mg/100 g) and sodium (62.4 mg/100 g) (Table 3). There was approximately half as much magnesium in PH compared to SC and significantly lower levels of chromium (0.28 mg/100 g), copper (0.36 mg/100 g), and iron (9.3 mg/100 g). In contrast, WD contained significantly lower amounts of calcium (1.93 mg/100 g), magnesium (0.6 mg/100 g), and sodium (2.1 mg/100 g). Other minerals were not detected. The presence of chromium in SC is significant due to its scarcity in most food products and involvement in glucose metabolism and homeostasis (Cefalu and Hu, 2004). SC-8B3 supplies 25.4 µg of chromium in a single gram of powder providing most of the daily dietary recommended intake (30 – 35 µg/day for males, 24 – 25 µg/day for females). All nine minerals detected are considered essential components of the human diet and support proper metabolism by functioning as electrolytes, cofactors, structural components in bone, and catalytic centres of enzymes (Raab et al., 2016). In particular, chromium, copper, iron, magnesium, manganese, and zinc, have been found to be deficient in patients with type 2 diabetes (Kaur and Henry, 2014). These results demonstrate that if consumed on a regular basis, both SC and PH supply at least 10% of the daily recommended intake of these minerals and may be considered minor dietary sources.

3.3 Bioaccessibility of minerals in sugarcane powder

Mineral availability has been shown to decrease due to physical entrapment and interaction with dietary fibre components (Macagnan et al., 2016). To test this, we performed water extractions and an in vitro digestion to measure the bioaccessibility of minerals in the solid dietary fibres. Mineral content of PH was found to be completely inaccessible to both water extraction and *in vitro* digestion and no change in mineral content was observed. This was unsurprising due to the high WHC and gel forming behaviour of PH that has previously been associated with the suppression of mineral absorption (Yu et al., 2008).

Mineral content of SC-8B3 untreated (total), following water extraction, and following *in vitro* digestion, is shown in Figure 1. Water extraction decreased the amount of potassium (43%), magnesium (10%), sodium (90%), iron (20%), chromium (33%), and zinc (55%), within the fibre indicating some degree of water solubility of these minerals. Following *in vitro* digestion, a decrease in potassium (56%), magnesium (27%), iron (12%), chromium (33%), zinc (55%) in the fibre was observed. Since less potassium was present in the sample, digestion of SC-8B3 appeared to improve potassium availability compared to water extraction. Calcium, copper, and manganese exhibited minimal bioaccessibility with both water and *in vitro* digestion suggesting that these minerals are largely inaccessible. Surprisingly, the amount of sodium in the SC sample increased by approximately 2000% (Figure 1) following *in vitro* digestion. This increase in sodium content was thought to indicate binding of bile salts within the bile extract to the SC matrix since it was a possible source of external sodium. To further investigate the high sodium content and potential bile salt binding activity, a series of *in vitro* digestions were performed with increasing concentrations of bovine bile extract (0.5%, 1.0%, 2.0%, 2.5% w/v). The residues were washed with ultrapure water, then the sodium content was determined and compared against untreated SC (Figure 2). A dose dependent increase in sodium was observed in residues digested with increasing concentrations of bile extract and the highest sodium content was observed in the residue that was digested with 2.5% (w/v) bile extract. Our results suggest that SC may bind sodium bile salts. A previous study has shown that lignin content in fruits and vegetables was positively correlated with bile acid binding activity (Hamauzu and Mizuno, 2011). While these results suggest that SC may be useful in reducing sodium availability, this activity of SC requires further investigation.

3.4 Antioxidant activity and phenolic compound profile of the sugarcane fibre ethanol extract

While dietary fibre is most commonly defined as the structural components of plants, it has been suggested that polyphenolic compounds are closely associated with plant cell wall components and should be included in the analysis of dietary fibre (Macagnan et al., 2016). Polyphenolic

compounds are of interest in the food industry due to their potent antioxidant activity. The antioxidant activity of the ethanol extract was thus measured using the DPPH and FRAP assays. The DPPH assay is used extensively for screening for antioxidant activity due to its sensitivity and ease of use (Musa et al., 2013). As shown in Figure 3, all SCs exhibited DPPH radical scavenging activity measured in Ascorbic acid equivalents (mg AAe100g), with SC-8B3 being the most active (255.1 ± 2.6 mg AAe/100g) and SC-954 the least (196.4 ± 11 mg AAe100g). The ferric reducing antioxidant power (FRAP) assay measures reducing ability by the formation of a 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) coordination complex with an Fe^{3+} ion at its core (Benzie and Strain, 1996). FRAP was measured by spectrophotometry against a standard curve of trolox, a water-soluble vitamin E analog. FRAP activity was expressed as trolox equivalents (mg TXe/100g) with the highest in SC-NK (262.2 ± 39 mg TXe/100g) and lowest with SC-954 (182.6 ± 26 mg TXe/100g). FRAP activity followed a similar trend as DPPH activity with SC-954 being the least active while SC-NK and SC-8B3 were the most active depending on the assay. PH possessed significantly less FRAP and DPPH activity when compared against the SCs while wheat dextrin showed no activity as detected by FRAP or DPPH assays.

Total phenolic content (TPC) of the extractive fraction was between 258.7 ± 3.9 mg gallic acid equivalents (GAe) /100g (SC-954) and 318.1 ± 11 mg GAe/100g (SC-NK) as shown in Figure 3. This result was lower than previous reports of TPC (783 mg GAe/100g) in sugarcane bagasse (Zheng et al., 2017). Phenolic content was similar or lower than common fruits with similar TPC to apples (296.3 ± 6.4 mg GAe/100g) and white guava (247.3 ± 4.5 mg GAe/100g), but less than blueberry (261 + 585 mg GAe/100g) and cranberry (527.2 ± 22 mg GAe/100g) (Balasundram et al., 2006). Phenolics are the secondary metabolites of plants which contribute to the coloring shades in fruits and vegetables and the protection of plants from pathogens, parasites, and predators (Zheng et al., 2017).

To further characterise the ethanol extract of SC, a dried sample was resuspended in water then partitioned between organic solvents giving the aqueous (AQ), petroleum ether (PE), ethyl acetate (EA), and butanol (BU) fractions, which were tested with the DPPH assay to identify the most active fraction (Supplementary Figure 2). For all SCs, EA was the most active fraction followed by BU and AQ. No DPPH activity was detected in the PE fraction. The EA fraction was analysed by UHPLC-MS and compared against a mixture of nine polyphenolic standards that have commonly been found in sugarcane bagasse (Supplementary Figure 3, A). A compound with retention time 3.01 min exhibited a based peak at *m/z* 163.03941 $[M-H]^-$ and a fragment ion at *m/z* 119.04944 $[M-H]^-$ corresponding to the loss of a carbonyl group. In comparison with a calculated theoretical accurate mass and an external standard, the compound was identified as *p*-coumaric acid, a key intermediate in lignin biosynthesis that has previously been found in sugarcane (Duarte-Almeida et al., 2011; Ferrer et al., 2008; Zheng et al., 2017). Calibration curves were used to calculate the concentration of *p*-coumaric acid, which were 6.21 mg/g of dry SC-NK, 14.5 mg/g SC-8B3, 17.2 mg/g SC-954, 6.77 mg/g SC-3Q1, and 12.7 mg/g SC-OT7. The absence of kaempferol, quercetin, rutin hydrate, and caffeic, ferulic, gallic, and sinnapic acids was surprising since these have commonly been found in sugarcane bagasse (Zheng et al., 2017). However, most studies that have identified these polyphenolic compounds in sugarcane begin with dry bagasse and grinding is performed in the absence of water. The absent compounds may have been lost at an earlier stage of production when the ground fibres are steeped in water.

4. Conclusions

The results of this study demonstrate that the chemical composition of a dietary fibre derived from sugarcane stalk was influenced conditions such as strain, harvest period, and processing conditions. When compared with other fibre products such as WD and PH, which are both soluble dietary fibres, SC was found to be a rich source of insoluble dietary fibre, essential minerals such as chromium and manganese, and compounds with antioxidant activity. Lignin was major component constituting up to 20% by weight and may be contributing to sodium binding activity

potentially through bile salt binding. The use of sugarcane fibre alone or as part of functional food preparations may be of benefit to diet related diseases such as type 2 diabetes, inflammatory bowel syndrome, and cardiovascular disease due to the presence of significant quantities of dietary fibre, chromium, and sodium binding activity, which are dietary components or activities that have associated with positive health outcomes.

Acknowledgements

We thank Gratuk Technologies Pty. Ltd. for providing the sugarcane samples. This work was funded by the Australian Research Council Industrial Transformation Training Centre for Molecular Technologies in the Food Industry, Macquarie University, New South Wales, Australia.

Conflict of interest statement

Dr Malcolm Ball is a director of a company that produces health supplements that include whole sugarcane stalk as an active ingredient. All other authors declare no conflicts of interest.

Figure captions

Table 1. Samples IDs and descriptions of the different strains, harvest period, and processing conditions used to prepare samples included in this study

Table 2. Chemical composition, extractive content, and water holding capacity of sugarcane fibres (SCs), psyllium husk, and wheat dextrin. Values are expressed g/100g total weight unless not detected (ND) and represent the mean ± the standard deviation of $n = 3$ technical replicates. The sample with the highest amount in each in shown in bold. Values in the same row with different superscript letters are significantly different from each other as determined by one-way ANOVA ($p < 0.05$). *Water Holding Capacity represents the amount of water absorbed by 1 gram of dry sample.

Table 3. Total mineral content of samples as determined by MP-AES. Values are expressed as mg per 100g unless not detected (ND) and represent the mean ± the standard deviation of $n = 3$

technical replicates. Values in the same row with different superscript letters are significantly different from each other as determined by one-way ANOVA and tukeys testing ($p < 0.05$).

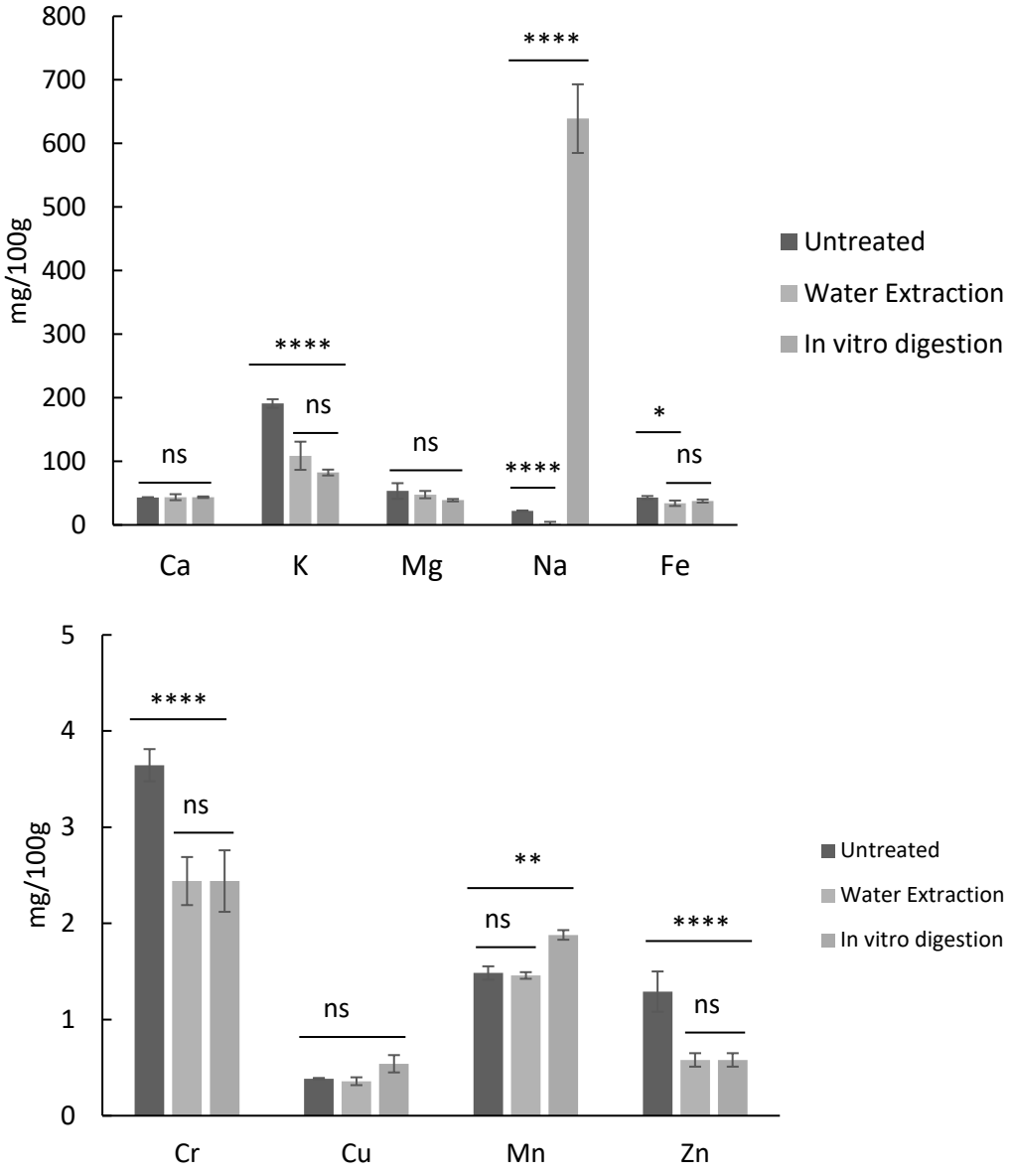


Figure 1. Mineral content of sample SC-8B3 untreated, following water extraction and *in vitro* digestion to determine bioaccessibility. A reduction in the amount of minerals in the sample after extraction was indicative of release from the fibre matrix and used to access bioaccessibility. Error bars are the standard deviation of $n = 3$ technical replicates. One-way ANOVA and tukeys test were used to determine statistically significant differences ($p > 0.05$)

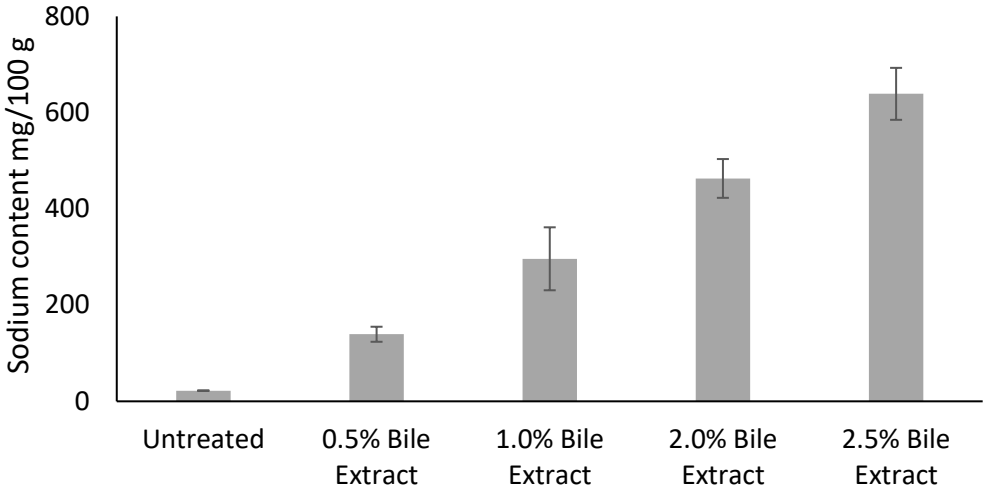


Figure 2. Sodium content of sample SCP-8B3 untreated and after *in vitro* digestion with increasing amounts of bile extract determined by MP-AES. A dose dependent increase in sodium in the residue was observed as the concentration of bile extract was increased. Error bars are the standard deviation of $n = 3$ technical replicates.

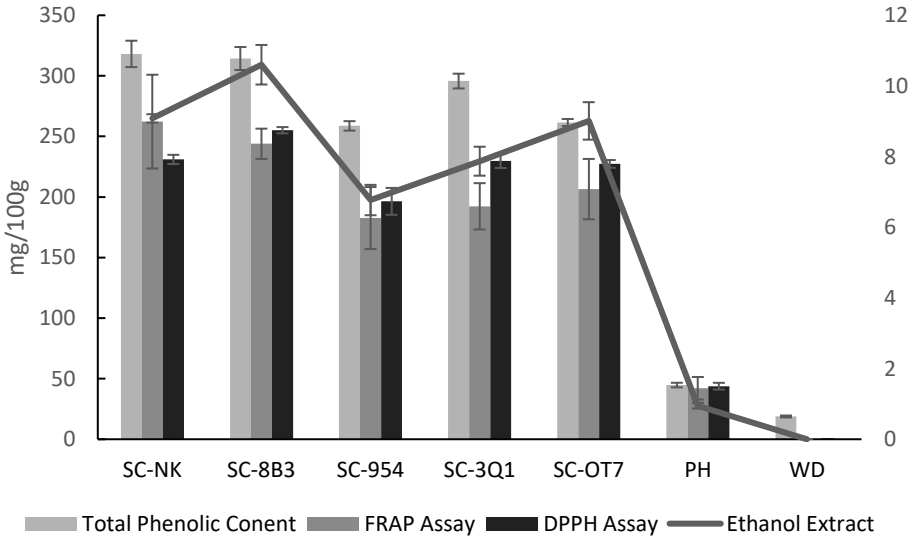
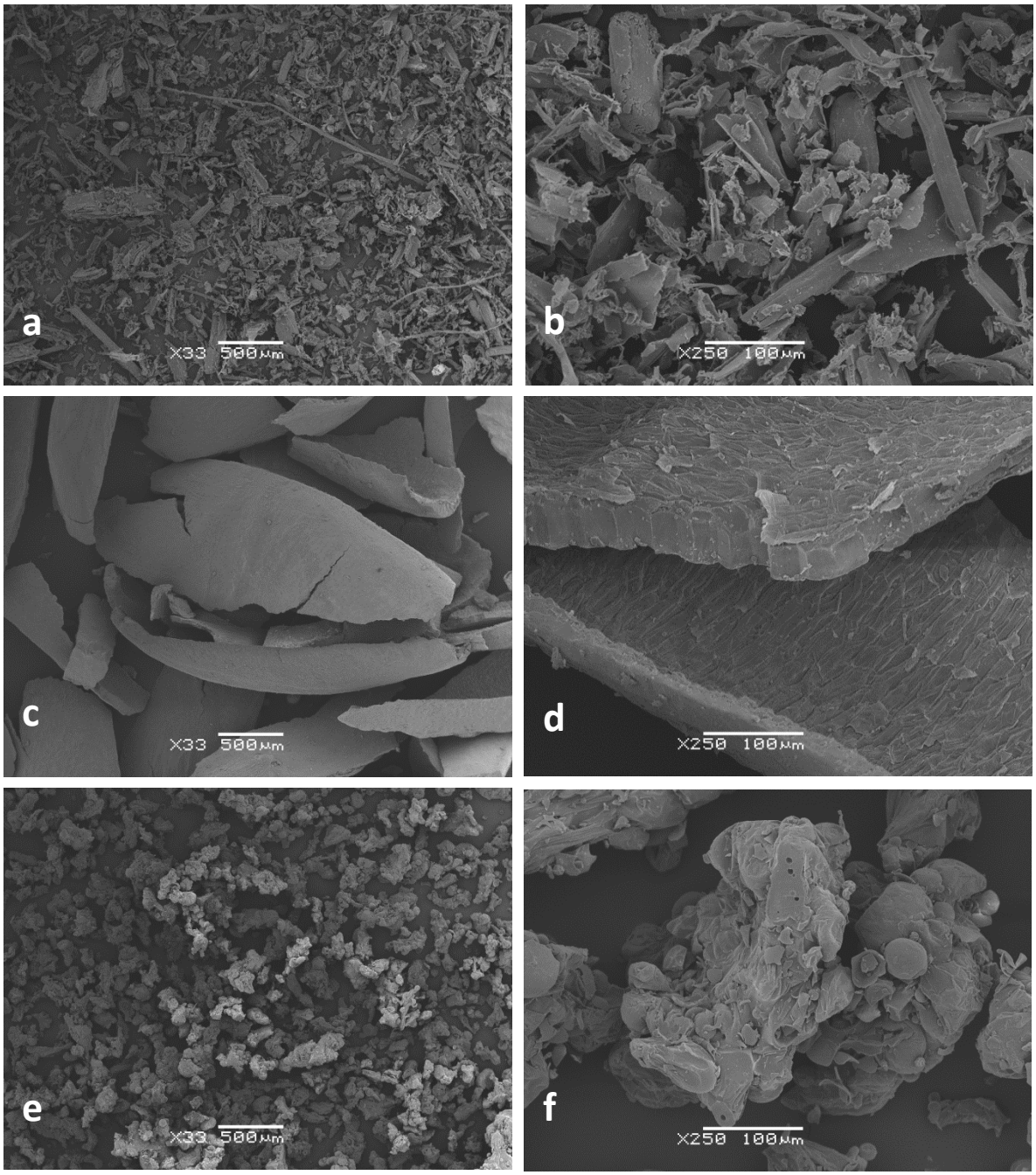
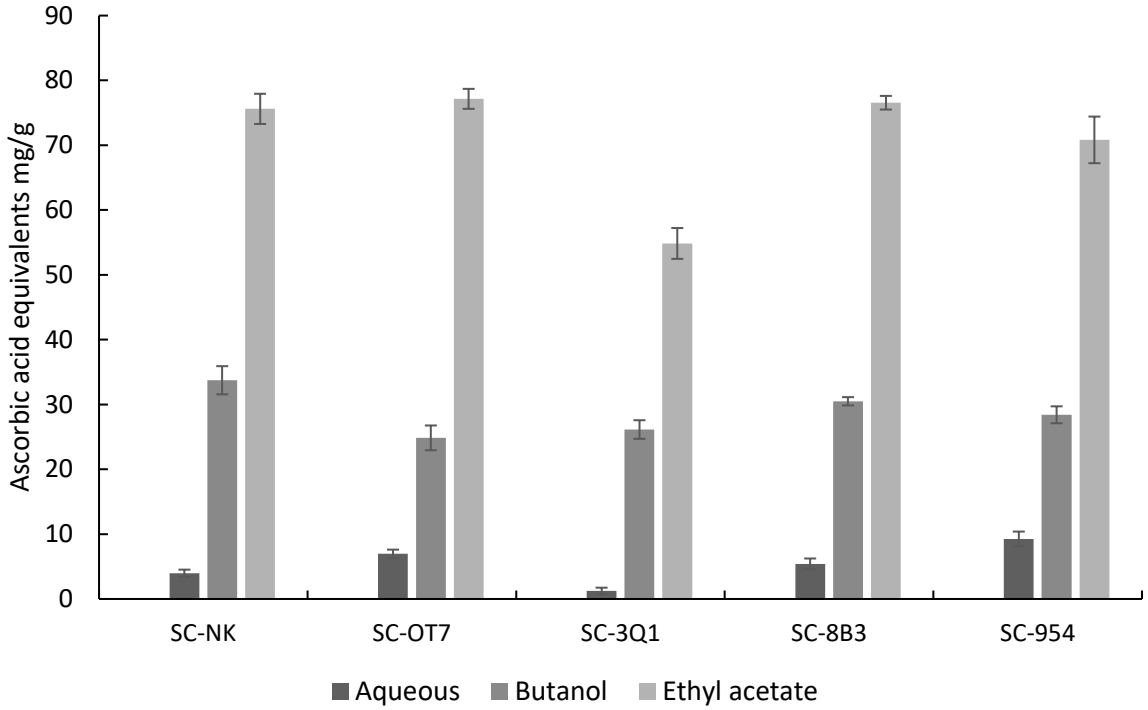


Figure 3. Total phenolic content and antioxidant activity of SC, PH, and WD samples compared against dry weight of the ethanol extract. The left y axis represents mg/100 g in equivalents of gallic acid, ascorbic acid, or trolox, for TPC, DPPH, and FRAP assays respectively. The right y axis represents dry weight of the ethanol extract in g/100 g. Error bars represent the standard deviation of $n = 3$ technical replicates. Values are provided in Supplementary Table 1.

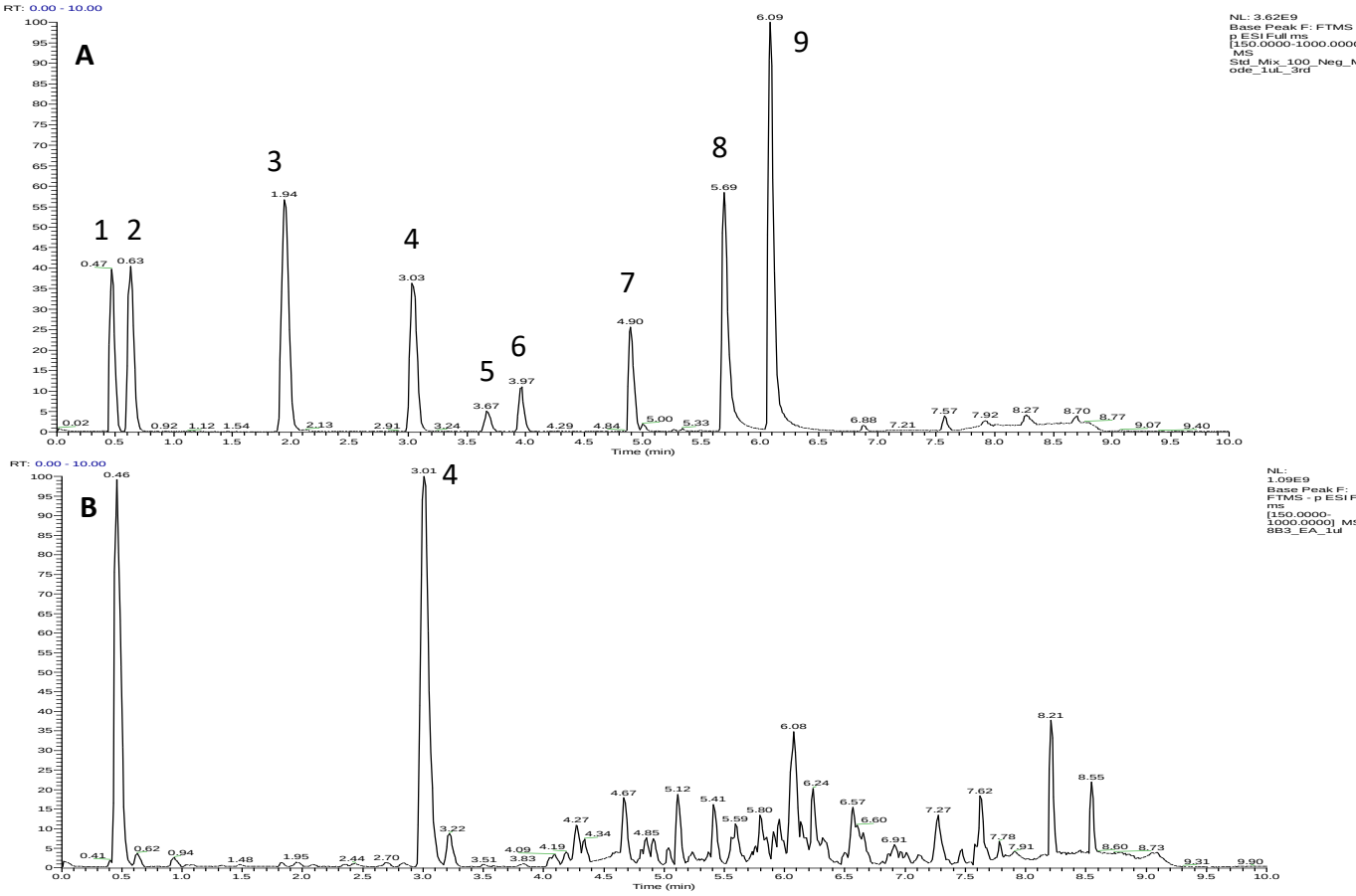
Supplementary Figures and Tables



Supplementary Figure 1. Scanning electron microscopy images of SC-NK (a,b), PH (c,d), and WD (e,f). Significant variation in particle size and morphology was observed between SC-NK, PH and WD. There was no variation between SC-NK and other SC samples.



Supplementary Figure 2. DPPH radical scavenging activity of aqueous (AQ), butanol (BU), and ethyl acetate (EA) fractions obtained from liquid partitioning of the dried ethanol extract. In all SC samples, the ethyl acetate fraction exhibited the highest antioxidant activity determined by DPPH radical scavenging activity. This was the rationale behind characterisation of the ethyl acetate fraction by UHPLC-MS. Values represent mg of ascorbic acid equivalents per gram of dried fraction and error bars are the standard deviation of $n = 3$ technical replicates.



Supplementary Figure 3. UHPLC-MS chromatogram of (A) a mixture of standards and (B) the EA fraction of SCP-8B3 ethanolic extract. Compounds are 1: ascorbic acid, 2: gallic acid, 3: caffeic acid, 4: p-coumaric acid, 5: ferulic acid, 6: sinapic acid, 7: rutin hydrate, 8: quercetin, 9: kaempferol, at a concentration of 1 mg/mL. The compound eluting at 3.01 min in (B) was identified as p-coumaric acid by retention time and spectral matching against the external standard in (A).

Supplementary Table 1. Values for dry weight of the ethanol extract and mg/100 g equivalents for TPC, DPPH, and FRAP assays, as shown in Figure 3.

	SC-NK	SC-8B3	SC-954	SC-3Q1	SC-OT7	PH	WD
Ethanol extract (g/100g)	9.08 ± 0.12	10.6 ± 0.56	6.77 ± 0.43	7.87 ± 0.41	9.01 ± 0.53	0.95 ± 0.08	ND
TPC (mg GAe /100g)	318.1 ± 10.9	314.3 ± 9.5	258.7 ± 3.9	295.7 ± 6.1	261.5 ± 2.9	44.73 ± 1.9	18.79 ± 0.8
FRAP (mg TXe /100g)	262.2 ± 38.7	243.9 ± 12.5	182.6 ± 25.6	192.3 ± 19.1	206.5 ± 24.9	42.1 ± 9.3	ND
DPPH (mg AAe /100g)	231.0 ± 3.8	255.1 ± 2.6	196.4 ± 11.1	229.8 ± 5.9	227.3 ± 3.2	43.8 ± 2.8	ND

2.3 Chapter summary

To begin the investigation of the effect of dietary fibre on intestinal health, we characterised the chemical composition of NutriKane in comparison with Benefiber (wheat dextrin) and psyllium husk, two commercially available dietary fibres. NutriKane is the first example of a dietary fibre to be prepared from while fresh sugarcane stalk rather than bagasse and was shown to contain valuable components including significant quantities of insoluble dietary fibre, rare minerals and compounds with antioxidant activity. Following comparison with Benefiber and psyllium husk, the unique carbohydrate content and monosaccharide composition of each fibre was shown to contribute to their vastly different physical properties. These three dietary fibres became the focus of further investigation into their effect on intestinal health, and their differences allowed correlations to be made between chemical composition and physiological activity. In the next chapter, we used an *in vitro* gut mimicking model to submit NutriKane, Benefiber and psyllium husk to simulated digestion before incubating them with human faecal samples to determine the effect of dietary fibre on the production of short chain fatty acids by the gut microbiota. In chapter four, we used NutriKane and Benefiber as diet interventions in a mouse high fat diet feeding study to determine the influence of dietary fibre on intestinal health in an *in vivo* setting.

Chapter 3: Dietary fibres alter short chain fatty acid production *in vitro*

3.1 Introduction

It is well established that the gut microbiota plays a key role in the maintenance of gastrointestinal health (Clemente et al., 2012; Tremaroli and Bäckhed, 2012). The main mechanism by which the gut microbiota interacts with the host is through the production of metabolites (Macfarlane and Macfarlane, 1997) and accumulating evidence suggests that the short chain fatty acids (SCFAs), a class of bacterial metabolites, are important to the health of the gastrointestinal tract and the wider host (Hamer et al., 2008; Koh et al., 2016; Mortensen and Clausen, 1996). The major SCFAs, acetate, propionate, and butyrate, are produced by the fermentation of dietary polysaccharides in the colon (Cummings, 1981). Since they are largely comprised of plant polysaccharides, dietary fibres are the main substrate for microbial fermentation in the colon due to their indigestibility in the human digestive system (Cummings, 1981; Miller and Wolin, 1996; Ríos-Covián et al., 2016).

The physiological activity of SCFAs range from local effects in the gastrointestinal tract, to wider effects on host metabolism (Mortensen and Clausen, 1996; Tan et al., 2014). Local effects include energy provision and trophic activity for the epithelial cells of the colonic mucosa (Clausen and Mortensen, 1995; Mortensen et al., 1991), modulation of the gut microbiota composition (Macfarlane and Macfarlane, 2012) regulation of inflammatory processes (Vinolo et al., 2011), and the inhibition of carcinogenesis (Scheppach et al., 1995). Wider effects of SCFAs include energy salvage from undigested dietary components for host utilisation (Bergman, 1990; Inoue et al., 2014), acting as a substrate for glucose, lipid, and cholesterol biosynthesis (den Besten et al., 2013a), and regulating the secretion of hormones such as GLP-1 and PPAR-γ (den Besten et al., 2013b; Ríos-Covián et al., 2016).

Due to their physiological effects and the means by which they are produced, the abundance of SCFAs can be used as a biomarker of individual health status and specific diet regimes have been

shown to exhibit characteristic amounts and ratios of faecal SCFAs (Ríos-Covián et al., 2016). Specifically, in individuals who consume diets high in dietary fibre and low in fat, there are higher amounts of SCFAs in their gut and stool (Cuervo et al., 2013; Ou et al., 2013); while diets that are low in fibre and high in fat exhibited lower quantities of SCFAs, particularly butyrate (Jakobsdottir et al., 2013). Through the gut microbiota and the production of SCFAs, dietary fibre performs an interesting role in the promotion and maintenance of intestinal health and it is important to understand how the gut microbiota responds to dietary fibres. However, the gastrointestinal tract is difficult to examine due to the dynamic nature of the environment and the invasiveness of procedures. Alternatively, the use of *in vitro* systems that simulate parts of or the entire digestive process provides numerous advantages over purely observational studies. These i*n vitro* systems are often rapid, highly reproducible, easily controlled, and are not restricted by ethical limitations associated with human or animal studies (Minekus et al., 2014).

To investigate the effect of dietary fibres on the composition and metabolic output of the human gut microbiota, we collaborated with Hasinika K. A. H. Gamage, an associate PhD candidate from the Australian Research Council's Industrial Transformation Training Centre (ARC ITTC) for Molecular Technology in the Food Industry, to use an *in vitro* gut mimicking system to simulate the digestion of various dietary fibre products and their subsequent fermentation in the colon. Two separate studies were performed. The first study examined the incubation of three dietary fibre products: 1) Nutrikane, a dietary fibre derived from sugarcane, 2) psyllium husk, a seed husk from the *Plantago ovata* plant, or 3) Benefibre, a modified wheat dextrin, with the faecal microbiota obtained from human adults. The second study examined the incubation of four cereal fibre products prepared from wheat, sorghum, rice, or oats, with the faecal microbiome obtained from human infants. The aim of these studies was to determine the effect of dietary fibres on the composition of the human gut microbiota and measure changes in SCFA production under *in vitro* conditions. Changes in gut microbiota composition were measured by 16S rRNA sequencing (Gamage) and were correlated with changes in SCFA production (Chong) determined using a gas

chromatography (GC) flame ionisation detection (FID) method that was optimised by the candidate described below.

3.2 Method development

3.2.1 The *in vitro* gut mimicking model

To simulate the conditions of the human colon, the fermentative digestion of fibres was performed using an *in vitro* gut mimicking model according to the specifications of Minekus et al. (2014). To mimic the digestive process, incubations were performed at 37°C. Gastrointestinal fluids and enzyme solutions were prepared as close as possible to physiological conditions specific for each phase of digestion. The salivary phase involved the incubation of fibre or controls solutions with salivary α-amylase (75 UmL^{-1}) for 2 min at pH 7. The gastric phase involved digestion with porcine pepsin (2000 UmL^{-1}) for 2 hours at pH 3. The small intestinal phase was performed for 2 hours at pH 7 with the addition of the following: porcine trypsin (100 UmL^{-1}), bovine chymotrypsin (25 UmL^{-1}), porcine pancreatic lipase (2,000 UmL^{-1}), porcine pancreatic colipase (2:1 colipase to lipase molar excess) and bile salts (10 mM). Following digestion, samples were frozen at -80°C and freeze dried.

Following digestion, freeze dried fibre samples were incubated with a basal media designed to simulate the human large intestine and optimise microbial fermentation. The basal medium was prepared under strict anaerobic conditions to the following composition: Peptone 0.5 g, yeast extract 0.5 g, $NaHCO_3$ 6 g, Hemin solution (0.05% (w/v) Hemin, and 0.2% (w/v) NaOH) 1 mL, L-cysteine HCl 0.5 g, Bile salts 0.5 g, Tween 80 2 mL, Resazurin solution [0.1% (w/v)] 1 mL, Vitamin stock 1 mL (Scheifinger et al., 1975), K_2HPO_4 0.228 g, KH_2PO_4 0.228 g, $(NH_4)_2SO_4$ 0.228 g, NaCl 0.456 g, $MgSO_4$ 0.0456 g, $CaCl_2.2H_2O$ 0.0608 g, and 1 mL trace mineral solution (Balch et al., 1979) with additional $NiSO_4.6H_2O$ (0.1 g/L), Na_2SeO_4 (0.19 g/L) and $Na_2WO_2.2H_2O$ (0.1 g/L). The pH of the medium was adjusted to 7.0 ± 0.2.

Each incubation was prepared to a final fibre product concentration of 1% (w/v) which was then inoculated with a filtered faecal homogenate to obtain a final concentration of 2% (w/v). Culture vials were incubated anaerobically at 37° C with agitation (100 rpm) for 48 hours. Prior to sample collection, cultures were left without agitation to allow solid to settle and aliquots were taken from the top liquid fraction at 0, 24, and 48 hours intervals. Following incubation, samples were centrifuged (100 x g, 5 – 15 mins) to obtain a solid fraction, containing undigested fibres and bacteria in the pellet, and a liquid supernatant, containing the combined digestive fluids and bacterial metabolites.

The major drawback of static *in vitro* models is that digestion products are not removed as they would be within *in vivo* systems through the absorption and exchange across the gastrointestinal tract. The main implication is that digestion products may inhibit the activity of digestive enzymes resulting in incomplete digestion. Product inhibition can be overcome by using low substrate concentrations in a dilute system and we have fulfilled this by using a final fermentation concentration of 2% (w/v) (Minekus et al., 2014). Additionally, static systems lacking the removal of digestion products via absorption may provide additional fermentable material than compared to an *in vivo* system. While static models may lack the simulation of the realistic factors such as enzyme substrate ratios, pH profiles, transit times and removal of digestion products, they excel in the studying the digestion of single substrate or simple meals under specific conditions, such as the fibre solutions used herein (Hur et al., 2011; Minekus et al., 2014).

3.2.2 Extraction and derivatisation of SCFAs for GC analysis

The analysis of SCFAs is typically performed using gas chromatography (Cummings et al., 1987). SCFAs exist as sodium salts at physiological pH and must first be protonated to free acids to become volatile for GC analysis. However, once in their acidic form they become incompatible with polysiloxane stationary phases that are most commonly used. To overcome these issues,

SCFAs are often extracted into non-polar organic solvents and derivatised to non-polar volatile esters (Henningsson et al., 2002; Weaver et al., 1988).

Initially, we attempted to extract SCFAs from 0.5 mL of *in vitro* liquid supernatants by acidifying with 0.5 mL formic acid (0.2 M) followed by phase partitioning with ethyl acetate (Deda et al., 2015). The supernatants were spiked with acetate, propionate, butyrate, and 4-methyl valeric acid as an internal standard at a concentration of 100 ppm. Following extraction into ethyl acetate, derivatisation of the SCFAs was attempted using *N*,*N*-Dimethylformamide dibutyl acetal (DMF-dba) at 5% sample volume (50 µL into 1 mL) by heating to 100° C for 30 minutes. Upon addition of DMF-dba, an insoluble bubble of liquid was formed indicating the production of an aqueous by-product that had to be removed before GC-MS analysis.

Derivatisation mixtures were dried by passing over anhydrous sodium sulfate prior to analysis by GC-MS to remove aqueous by-products. Following analysis by GC-MS, no masses corresponding to butylated SCFAs were detected indicating either unsuccessful derivatisation or incomplete extraction into the ethyl acetate phase. To test the effect of acidification, 1 µL of the acidified ethyl acetate layer without standards or real samples was injected at 280 °C onto a DB-5 5% polysiloxane column (30 m x 0.25 mm i.d., x 0.5 µm film thickness) using the following temperature gradient: 1 min at 40 °C, linear increase at 40 °C/min to 60 °C, held for 3 min at 60 °C, linear increase at 25 °C/min to 210 °C, linear increase at 40 °C/min to 315 °C, and held for 3 min at 315 °C, Helium was used as carrier gas at a constant flow rate of 1.20 mL/min and the ionisation source was kept at 280 °C. Ions were detected in the negative mode using SIM.

Acetic acid and ethanol were detected suggesting that in the presence of acid, ethyl acetate can undergo hydrolysis to produce acetic acid and ethanol. Extraction into diethyl either or the inclusion of pyridine (1:1 ratio with the extraction solvent) did not improve the extraction or derivatisation of SCFAs. From our experiments, the process of extraction and derivatisation was extremely sensitive to water, prone to sample loss, and was difficult to accomplish in a

reproducible manner. Considering the success of other studies using alternative stationary phases (Zhao et al., 2006), we abandoned the approach of extraction of the SCFAs from the aqueous liquid supernatant and the necessary derivatisation reactions to ensure compatibility with polysiloxane stationary phases. Instead, we adopted the approach of direct injection of the liquid supernatant onto the GC using an aqueous compatible stationary phase.

3.2.3 Direct injection of SCFAs for GC analysis

Using a Hewlett-Packard INNOWax column (30 m x 0.25 mm i.d. x 0.5 µm film thickness, Agilent) with a polyethylene glycol (PEG) based stationary phase we were able to optimise a method that was compatible with aqueous mixtures allowing direct injection of liquid supernatants without the need for prior extraction of SCFAs. Gas chromatography was coupled with flame ionisation detection (FID), which is the gold standard in GC-coupled quantification of SCFAs.

3.2.4 Pre-treatment optimisation with SCFA standards

While SCFAs no longer required extraction from the aqueous layer by this method, they still required acidification to become volatile acids. However, without the extraction of SCFAs into organic solvent, inorganic minerals acids such as HCl could not be used as an acidifying agent since they can cause permanent damage to GC columns and greatly reduce column lifetime. Initially, we used formic acid, an organic and volatile acid, to acidify the SCFAs prior to direct injection. Acidification of a standard mixture of acetic (Ace), propionic (Pro), butyric (But), and 4-methyl valeric acid (IS) prepared at 100 ppm of each, with formic acid (10% v/v final concentration) produced irregular peak shapes when separated on the INNOWAX PEG column (**Figure 3-1**, **a**). Since the FID response is proportional to the carbon count of each analyte, we expected to see peaks increasing in size from Ace to IS. The small area of the IS peak suggested incomplete conversion to the free volatile acid, and thus a smaller response compared to Ace, indicating that a stronger acid was required. Subsequently, we used trifluoroacetic acid (TFA), a much stronger acid than formic acid, that is also organic and volatile, to acidify the SCFAs prior

to direct injection. Using TFA (0.1% v/v final concentration) and a standard mixture of SCFAs, we were able to produce sharp peaks with high resolution (**Figure 3-1**, **b**). The increasing size of each SCFA standard peak was proportional to the carbon count of the analytes and consistent with the expected FID response.

3.2.4.1 Pre-treatment of the liquid supernatant for SCFA analysis

Due to the µmolar concentrations of SCFAs and complex nature of the *in vitro* culture liquid supernatants, samples were diluted by a factor of 10 with a solution of ethanol and TFA (0.1% v/v) to within the range of the calibration standards (100 ppm). For each sample, 500 µL of liquid supernatant was mixed with 10 µL of internal standard (10 000 ppm) and diluted to 5 mL with a solution of ethanol and TFA (0.1% v/v). Dilution with ethanol was also thought to sterilise samples and assist with the precipitation of proteins and other potentially non-volatile compounds from the liquid supernatants. To ensure that precipitates or particulates were not injected on the GC, diluted liquid supernatants were filtered by passing through 0.45 µm PTFE membrane syringe filters prior to GC analysis.

3.2.5 GC Injection parameters and gradient optimisation

Considering the potentially high concentrations of SCFAs reported in previous *in vitro* studies (Beards et al., 2010; Kaur et al., 2011; Pylkas et al., 2005; Zampa et al., 2004), a split injection mode (1/10) and injection volume of 0.5 µL were selected to prevent column overload. Since the boiling points of acetic acid and 4-methyl valeric acid range between 118.1°C and 119-201°C respectively, the GC inlet temperature was required to be at least 200°C to allow complete vaporisation of samples during injection. Three inlet temperatures (210, 230 and 250 °C) were evaluated. At the lower inlet temperature of 210 °C, all SCFAs standards were retained and eluted. At higher inlet temperatures of 230 and 250 °C, there was an increase in contaminating peaks but no increase in SCFA recovery.

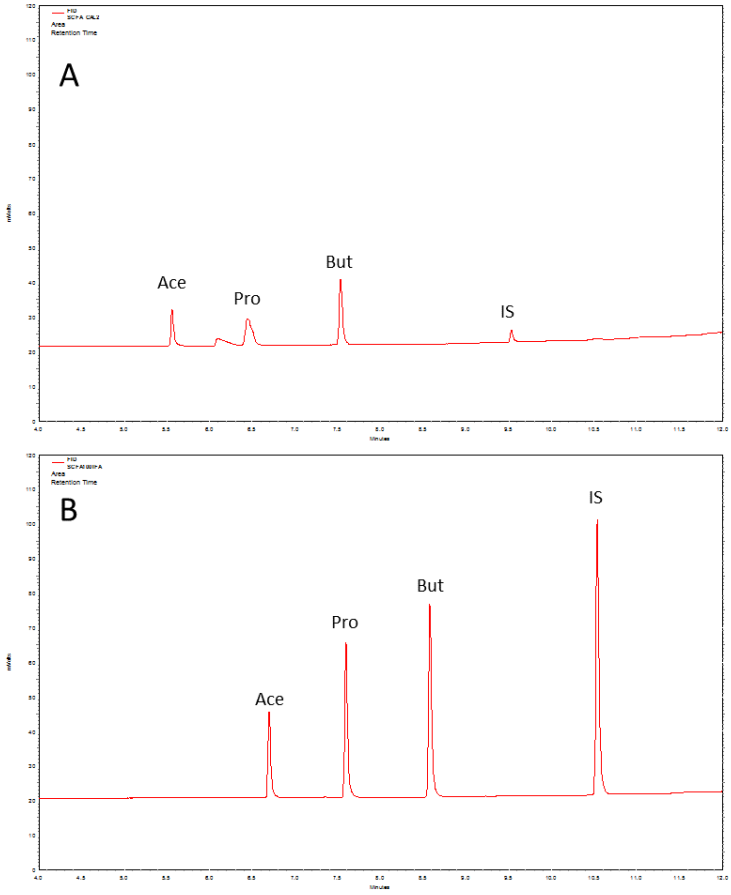


Figure 3-1. GC-FID chromatogram of a standard mixture of acetic (Ace), propionic (Pro), butyric acids (But), and the internal standard 4-methyl valeric acid (IS) prepared in different acids. Each standard was prepared to a concentration of 100 ppm in **a)** 10% (v/v) formic acid and 90% ethanol, and **b)** 0.1% (v/v) trifluoroacetic acid and ethanol. Peak identities were confirmed by direct injection of individual standards and retention time matching.

Hence, the inlet temperature of 210 °C was chosen for further studies. The inlet port of a gas chromatograph is typically filled with a deactivated glass liner that influences the vaporisation, dispersal, and elution of samples during injection. A liner filled with glass wool was selected to assist in the dispersal of samples during injection and provided the added benefit of cleaning the tip of the injection needle as it entered the wool plug. Due to the complex nature of the culture supernatants and the relatively low inlet temperature of 210°C, a build-up of contaminants was observed that manifested as contaminating peaks present in subsequent injections and a general broadening of peaks. Changing the liner was required after approximately 250 injections.

During the preliminary testing for column temperature programming, the initial column temperatures were set at 60, 70 and 80°C. At 60°C, the first peak (Ace) eluted at 6.5 mins while

at 80°C this was reduced to 4.5 minutes and produced sharper peaks (data not shown). Multiple column temperature gradients rates were evaluated (10 – 30 °C/min) and found to have minimal impact on SCFA resolution. In all gradients, resolution between acetic, propionic, butyric acid and the internal standard was greater than 2 and were clearly separated from each other. Since the boiling point of the internal standard was 199-201°C, the final column temperature was set to 200°C. The final column temperature program was 80°C for 1 min, a slope of 25°C/min for 2 mins then a slope for 10°C/min until 200°C with a total run time of 12 mins.

3.2.6 Calibration and quantification of SCFAs

By spiking samples with 4-methyl valeric as an internal standard (100 ppm final concentration), quantification was possible by internal calibration. A standard mixture of acetic, propionic, butyric, and the internal standard, each prepared to 100 ppm was run separately to determine response factors relative to the internal standard. Once peak areas were obtained by integration of chromatographic peaks, quantification was achieved using the following equation:

$$Cx = \left(\frac{A\,.\,x}{A\,.\,IS}\right) \times \left(\frac{C\,.\,IS}{rf}\right)$$

A = area under curve, x = analyte, *IS* = internal standard, *C* = concentration (ppm), *rf* = response factor.

3.3 Results and Discussion

Using the *in vitro* gut mimicking system, approximately 1 gram of each dietary fibre or cereal fibre was subjected to *in vitro* digestion mimicking digestion in the oral cavity, stomach, and small intestine (Gamage et al., 2018). Digested fibres were then incubated with 1g of faeces obtained from human volunteers (for Study 1 refer to Section 3.3.1, for Study 2 refer to Section 3.3.2) to mimic the conditions of the colon. At 0, 24, and 48 hours after incubation at 37°C, 2 mL aliquots were removed and centrifuged at 20,238 × g for 15 min to obtain solid and liquid fractions. Solid pelleted fractions were used to determine changes in microbial composition while the liquid fraction was used to quantify SCFA production. The concentration of SCFAs in *in vitro* culture supernatants was determined by GC-FID using the method optimised in Section 3.2 (**Figure 3-2**).

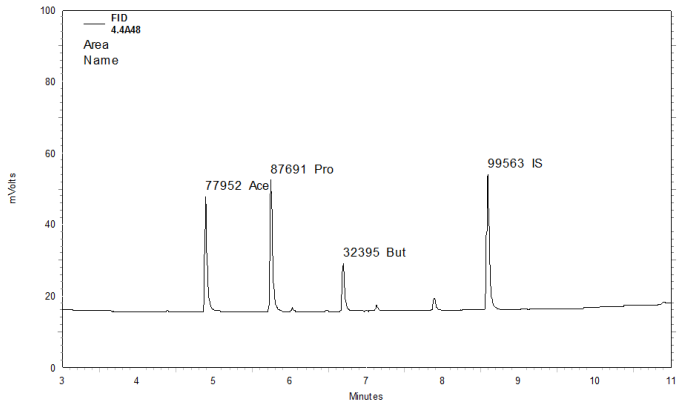


Figure 3-2. Representative chromatogram of an *in vitro* culture supernatant sample. Clear separation between all SCFAs and the internal standard was observed. The integrated area under the curve is shown next to each peak. Ace: acetic acid; Pro: propionic acid; But: butyric acid; IS: internal standard, 4-methyl valeric acid.

3.3.1 Study 1: Effect of Nutrikane, Benefibre, and psyllium husk on SCFA production by the adult gut microbiota in vitro

To investigate the effect of dietary fibre products on the adult gut microbiota, four dietary fibre products: 1) NutriKane, a dietary fibre derived from sugarcane; 2) autoclaved Nutrikane; 3) Benefibre (BF), a commercially available wheat dextrin; and 4) psyllium husk (PH), the seed husk from the *Plantago ovata* plant, were digested using an *in vitro* gut mimicking system then co-incubated with faecal slurry to determine changes in SCFA production by the gut microbiota.

Faecal samples were obtained from six different adults between the ages of 20 and 60. After incubating each faecal sample with NutriKane, autoclaved NutriKane, BF, or PH, for 0, 24, and 48 hours, approximately 2 mL of *in vitro* culture liquid supernatant was removed for SCFA determination. SCFA concentrations in the liquid supernatants were determined by GC-FID.

The production of the three major SCFAs, acetic (Ace), propionic (Pro), and butyric (But) acids in the *in vitro* gut mimicking system was different for the three fibres. Incubation with autoclaved NutriKane produced an almost identical response to non-Autoclaved NutriKane suggesting that endogenous microbes found on NutriKane (unpublished data) did not contribute to the fermentation profile. Incubation of the faecal inoculum with NutriKane, BF, and PH resulted in a statistically significant increase in the production of total SCFAs compared with the faecal slurry control (**Figure 3-3**). BF produced the sharpest increase in total SCFAs at 24 hours, followed by PH, then Nutrikane, indicating that Benefibre was the most readily fermented dietary fibre. Between 24 and 48 hours of incubation, the addition of BF and PH but not NutriKane increased total SCFA production.

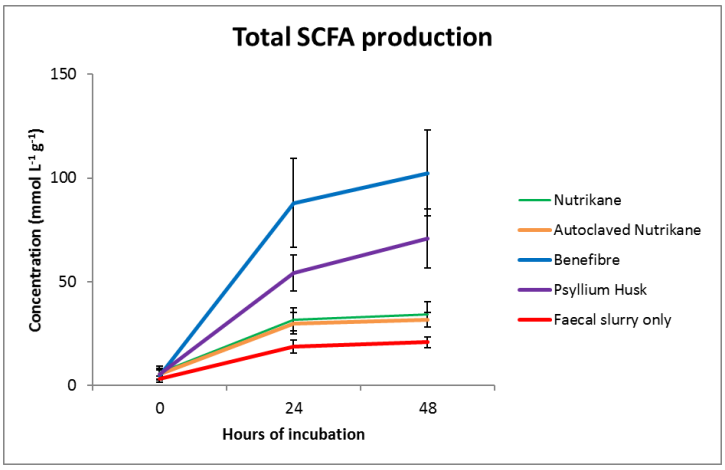


Figure 3-3. Total SCFA production following incubation of the adult faecal microbiota with NutriKane, autoclaved NutriKane, BF, and PH. Data are mean ± standard deviation values calculated from six biological replicates and three technical replicates.

Each fibre product produced a different fermentation profile in terms of the quantity and proportions of the SCFAs Ace, Pro, and But. Overall, Ace was the most abundant SCFA produced in each incubation regardless of dietary fibre followed by Pro then But (**Figure 3-4**).

Table 3-1. SCFA concentrations following incubation of the faecal slurry with dietary fibres.

	Hours		
SCFA (mmol L^{-1} g^{-1})	0	24	48
Faecal control			
Acetic acid	2.40 ± 0.8	13.9 ± 1.9[a]	15.5 ± 1.5[a]
Propionic acid	0.34 ± 0.3	2.76 ± 0.6[a]	3.11 ± 0.6[a]
Butyric acid	0.41 ± 0.4	1.98 ± 0.6[a]	2.24 ± 0.5[a]
Nutrikane			
Acetic acid	5.19 ± 2.2	23.9 ± 3.2[b]	24.5 ± 3.0[b]
Propionic acid	0.47 ± 0.4	4.50 ± 1.0[a]	5.44 ± 1.7[a]
Butyric acid	0.61 ± 0.6	3.41 ± 1.3[a]	4.24 ± 1.3[a]
Autoclaved Nutrikane			
Acetic acid	4.22 ± 1.9	22.5 ± 3.0[b]	23.5 ± 1.7[b]
Propionic acid	0.42 ± 0.3	4.25 ± 0.9[a]	4.76 ± 1.1[a]
Butyric acid	0.61 ± 0.5	3.13 ± 1.2[a]	3.48 ± 0.8[a]
Benefibre			
Acetic acid	3.55 ± 2.2	56.8 ± 10.0[c]	64.8 ± 10.8[c]
Propionic acid	0.42 ± 0.4	22.2 ± 7.7[b]	27.9 ± 8.3[b]
Butyric acid	0.54 ± 0.5	9.05 ± 3.8[b]	9.68 ± 1.7[b]
Psyllium husk			
Acetic acid	4.40 ± 1.7	34.7 ± 3.9[d]	42.7 ± 5.9[d]
Propionic acid	0.32 ± 0.3	14.6 ± 2.6[c]	20.9 ± 3.9[c]
Butyric acid	0.56 ± 0.5	4.90 ± 2.2[c]	7.14 ± 4.6[c]

Data are mean ± standard deviation values calculated from six biological replicates
[a,b,c,d] Values within the same SCFA and column with different superscript letters are significantly different from each other using two-way ANOVA and Tukeys testing ($p < 0.05$). Differences in SCFA profiles at 0 hours are due to experimental variation.

Incubation with BF produced the highest amounts of Ace, Pro, and But at 0, 24 and 48 hours. At 24 hours of incubation, Ace, Pro, and But concentrations were 216%, 1.6, and 1.7 times higher for NutriKane, 1.6, 1.5, and 1.6 times higher for Autoclaved NutriKane, 4.1, 8.0, and 4.6 times higher for BF, and 2.5, 5.3, and 2.5 times higher for psyllium husk, than the faecal control respectively. These values indicate that fermentability was highest in BF followed by PH then NutriKane and Autoclaved NutriKane. A study by Timm et al. (2010) involving the same dietary fibres reported that after 24 hours of incubation with PH, the concentrations of Ace, Pro, and But increased by 2.7, 1.5, and 1.8 times higher than the control, respectively. Similarly, 24 hours of incubation with

wheat dextrin, which is chemically similar to BF, increased the concentrations of Ace, Pro, and But by 3.2, 2.0, and 1.6 times higher than the control, respectively. In comparison, our incubation resulted in similar relative changes in Ace concentrations and more pronounced relative changes in Pro and But concentrations. These differences are likely due to the high SCFA concentrations in the control of the study by Timm et al. (2010) that was indicative of residual fibre in the faecal inoculum. The variations in SCFA production seen in this study and other fermentation studies could be due to a combination of differences in the microbial composition of faecal inoculate and the chemical composition and fermentability of dietary fibre products.

After 48 hours of incubation, Ace, Pro, and But concentrations were 1.6, 1.7, and 1.9 times higher for NutriKane, 1.5, 1.5, and 1.6 times higher for Autoclaved NutriKane, 4.4, 9.0, and 4.3 times higher for BF, and 2.8, 6.7, and 3.2 times higher for PH, than the faecal control respectively. Between 24 and 48 hours, Ace, Pro, and But concentrations increased by 2.5%, 20.9%, and 24.3% for NutriKane, 4.4%, 12.0%, and 11.2% for Autoclaved NutriKane, 19.4%, 25.7%, and 7.0% for BF, and 23.1%, 43.2%, and 45.7% for PH. These values appear to suggest that between 24 and 48 hours, the production of Pro and But is higher in NutriKane than Autoclaved, however, they are still within experimental error.

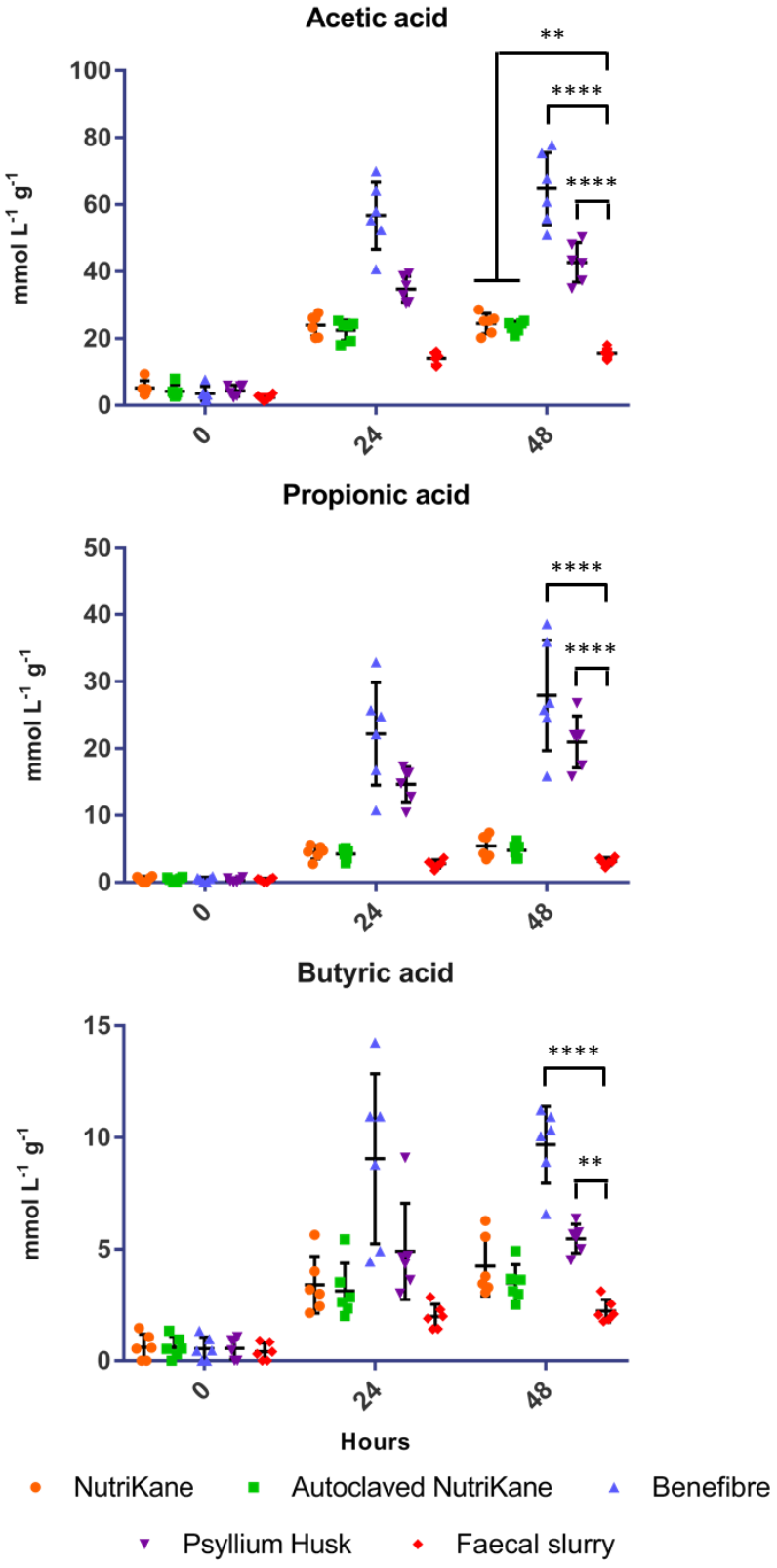


Figure 3-4. Concentration of acetic, propionic, and butyric acid over time in response to the addition of NutriKane, autoclaved NutriKane, BF, or PH, to the *in vitro* gut mimicking system. Error bars represent the mean ± the SD, each point represents a different biological sample. Significant differences were determined by two-way ANOVA and Tukeys testing (**$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

All three dietary fibres included in this study contained significant amounts of carbohydrate and distinctly different monosaccharide compositions that would contribute to variations in the fermentability of dietary fibres. As confirmed in Chapter 2, BF, which is a type of wheat dextrin, is primarily composed of glucose (Pasman et al., 2006) while the major polysaccharide in PH is an arabinoxylan (Fischer et al., 2004). Both of these types of polysaccharides are known to be readily fermented (Timm et al., 2010). The difference in fermentation between BF and is most likely due to the linkage, type and structural orientation of the carbohydrate component in each fibre (Salvador et al., 1993). Linear molecules, such as those found in BF (Noack et al., 2013), are more readily fermented than branched arrangements like those found in PH (Fischer et al., 2004). In contrast, NutriKane has been shown to contain significant quantities of lignin, a non-carbohydrate structural polymer, as well as glucose and xylose (as shown in Chapter 2) that are the main components of cellulose and hemicellulose that are known to be resistant to fermentation by the gut microbiota (Campbell et al., 1997).

The differences in SCFA production by the addition of the three different dietary fibres may also be due to the unique particle morphology of each dietary fibre. BF possesses a small, uniform particle size and is completely soluble in water making it highly accessible for microbial fermentation (Slavin et al., 2009). This was consistent with the high production of SCFAs found in this study and has also been reported elsewhere (Pylkas et al., 2005; Stewart et al., 2009; Timm et al., 2010). PH possesses much larger particles than either BF or NutriKane, exhibits gel forming ability and an extremely high water holding capacity, which may be the most likely explanation for the delayed fermentation and production of SCFAs due to the resultant increases in viscosity of the culture supernatants that is typical of soluble fibres (Kaur et al., 2011). The significantly lower SCFA concentrations produced from incubation with Nutrikane compared to BF and PH after 24 hours indicated significantly lower fermentability. This is probably due to the insoluble nature of this dietary fibre and the presence of significant amounts of cellulose and xylose that are resistant to fermentation (Barry et al., 1995; Campbell et al., 1997). Our SCFA results are included

in the following publication that was prepared in collaboration with Hasinika K. A. H. Gamage *et al.* along with the chemical composition, antioxidant potential and polyphenolic profiles of each dietary fibre, which were found to be highly variable. The changes in SCFA production are discussed in conjunction with the fibre-dependent changes in microbiota structure and composition in response to dietary fibre incubations with the gut mimicking *in vitro* system.

3.3.1.1 The effect of dietary fibres on the human adult gut microbiota (Paper 2)

Accepted Manuscript: Hasinika K. Gamage, Sasha G. Tetu, **Raymond W. Chong**, Daniel Bucio-Noble, Carly P. Rosewarne, Liisa Kautto, Malcolm S. Ball, Mark Molloy, Nicolle H. Packer, Ian T. Paulsen. (2018). *Fibre Supplements Derived From Sugarcane Stem, Wheat Dextrin And Psyllium Husk Have Different In Vitro Effects On The Human Gut Microbiota*. Frontiers in Microbiology, *9*, p.1618

The following paper includes the changes in gut microbiota composition, as determined by DNA sequencing, together with the changes in SCFA production in response to the addition of each dietary fibre using the *in vitro* gut mimicking model. The *in vitro* digestion of dietary fibres, incubation of the faecal slurry with digested fibres, and 16S rRNA sequencing was performed by Hasinika K. A. H. Gamage. Quantitative SCFA measurements were performed by me, Raymond W. Chong using the method optimised and described in this chapter. Raymond W. Chong also contributed the chemical composition of fibre supplements used in this study. Daniel Bucio-Noble determined the antioxidant potential and polyphenol content. Hasinika K. A. H. Gamage, Raymond W. Chong, Daniel B. Noble, Carly P. Rosewarne, Liisa Kautto, Malcolm S. Ball, Mark Molloy, Nicolle H. Packer and Ian T. Paulsen were involved in the experimental design and interpretation of data. This paper has been published in the Journal of Frontiers in Microbiology (2018) in the section for Food Microbiology.

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Fiber Supplements Derived From Sugarcane Stem, Wheat Dextrin and Psyllium Husk Have Different *In Vitro* Effects on the Human Gut Microbiota

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There is growing public interest in the use of fiber supplements as a way of increasing dietary fiber intake and potentially improving the gut microbiota composition and digestive health. However, currently there is limited research into the effects of commercially available fiber supplements on the gut microbiota. Here we used an *in vitro* human digestive and gut microbiota model system to investigate the effect of three commercial fiber products; NutriKane™, Benefiber® and Psyllium husk (Macro) on the adult gut microbiota. The 16S rRNA gene amplicon sequencing results showed dramatic fiber-dependent changes in the gut microbiota structure and composition. Specific bacterial OTUs within the families *Bacteroidaceae, Porphyromonadaceae, Ruminococcaceae, Lachnospiraceae,* and *Bifidobacteriaceae* showed an increase in the relative abundances in the presence of one or more fiber product(s), while *Enterobacteriaceae* and *Pseudomonadaceae* showed a reduction in the relative abundances upon addition of all fiber treatments compared to the no added fiber control. Fiber-specific increases in SCFA concentrations showed correlation with the relative abundance of potential SCFA-producing gut bacteria. The chemical composition, antioxidant potential and polyphenolic content profiles of each fiber product were determined and found to be highly variable. Observed product-specific variations could be linked to differences in the chemical composition of the fiber products. The general nature of the fiber-dependent impact was relatively consistent across the individuals, which may demonstrate the potential of the products to alter the gut microbiota in a similar, and predictable direction, despite variability in the starting composition of the individual gut microbiota.

Keywords: gut microbiota, dietary fiber supplementation, *in vitro* gut models, 16S rRNA gene, short chain fatty acids, polyphenols

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INTRODUCTION

Trillions of microorganisms reside in the human large intestine, which is collectively referred to as the gut microbiota (Cani et al., 2016). The gut microbial composition is shaped by exogenous and endogenous factors, and interacts with the host metabolism and physiology (Kovatcheva-Datchary et al., 2015). The adult gut microbiota is usually dominated by the phyla *Firmicutes* and *Bacteroidetes* (Bäckhed et al, 2005; Arumugam et al., 2011) while *Actinobacteria, Proteobacteria* and *Verrucomicrobia* constitute minor proportions of the bacterial populations (Eckburg et al., 2005; Lozupone et al., 2012). Compositional and functional alterations of the gut microbiota have been associated with various inflammatory and metabolic diseases such as obesity (Turnbaugh et al., 2009), type 2 diabetes (T2D) (Karlsson et al., 2013), type 1 diabetes (T1D) (Knip and Siljander, 2016), and inflammatory bowel disease (IBD) (Frank et al., 2007).

Diet has been shown to impact the composition and activities of the gut microbiota (Conlon and Bird, 2015; Fontana and Partridge, 2015). The overall structure of the gut microbiota has been reported to respond within a day to short-term consumption of entirely animal or plant based diets (David et al., 2014). There is also evidence that individual dietary preferences correlate to some degree with longer-term gut microbiota composition (Wu et al., 2011). Previous studies have shown that bacteria in the genus *Bacteroides* are more dominant in the gut microbiota of people consuming high levels of protein and animal fat, while *Prevotella* are dominant in the gut microbiota of frequent fiber and carbohydrate consumers (Wu et al., 2011). Similar observations have been made in a number of studies that looked at different communities of people who consume diets rich in fiber in comparison to diets low in fiber (De Filippo et al., 2010; Yatsunenko et al., 2012; Lin et al., 2013; Schnorr et al., 2014; O'Keefe et al., 2015).

Modulation of the gut microbiota using dietary components is potentially therapeutically useful (Doré and Blottiere, 2015; Wu et al., 2015). Prebiotics are generally non-digestible by humans, but are fermented by the gut microbiota to yield energy and metabolic end products of microbial fermentation, such as short chain fatty acids (SCFAs) (Tremaroli and Backhed, 2012; Slavin, 2013; Janssen and Kersten, 2015). SCFAs, mainly acetate, propionate and butyrate have established roles in host physiology. These compounds provide an energy source that accounts for up to 10% of daily caloric value (Sonnenburg and Backhed, 2016). They act as modulators of autophagy in colonocytes; as precursors and regulators of cholesterol, fatty acids and glucose; as well as activators of anti-inflammatory effects, tumor suppression and production of the hormone leptin (Koppel et al, 2016).

The most commonly studied prebiotics are dietary fiber, which include carbohydrates such as cellulose, xylan, resistant starch, pectin, inulin and mannan (Slavin, 2013). Fermentation of dietary fiber by the gut microbiota and concomitant effects on human health has been investigated in the context of conditions such as IBD, T2D and obesity (Rastall and Gibson, 2015). For example, studies have shown an increase in the abundance of bifidobacteria upon consumption of inulin, short chain fructooligosaccharides (FOS) or galactooligosaccharides (GOS) (Yasmin et al., 2015) and the ability of these dietary fibers to reduce inflammatory markers associated with obesity and T2D (Vulevic et al., 2013; Dehghan et al., 2014). *In vivo* studies conducted using inulin and various oligofructoses have shown an inhibition of animal and human pathogenic bacterial groups and increase in bifidobacteria and SCFAs (Saad et al., 2013). Several recent studies have also employed *in vitro* models to compare the effects of introducing pectin, inulin (Johnson et al., 2015; Chung et al., 2016), and wheat dextrin (Hobden et al., 2013) on the gut microbiota. These studies have demonstrated the potential of the fiber additions to enrich specific members of the genus *Bacteroides* and phylum *Firmicutes* (Hobden et al., 2013; Johnson et al., 2015; Chung et al., 2016).

Dietary polyphenols and antioxidants have also been studied for their ability to beneficially change gut microbial composition and functions (Hervert-Hernández and Goñi, 2011; Cheng et al., 2016) and therefore also have prebiotic potential. Polyphenols from various fruits and tea have been shown to inhibit the growth of pathogens and maintain the growth of *Lactobacillus, Bifidobacterium, Lachnospiraceae,* and *Eubacterium rectale* (Hervert-Hernández and Goñi, 2011; Sheflin et al., 2016). Several other studies have indicated the ability of the antioxidant action to be delivered to the gut epithelia resulting in a reduction of inflammation (González-Gallego et al., 2010; González et al., 2011; Bonaccio et al., 2016) and improvement in tissue recovery in IBD patients (Shapiro et al., 2007).

Utilization of *in vitro* models of the human gut microbiota to investigate the impact of dietary interventions on the microorganisms provide powerful information for proof of concept studies prior to *in vivo* validation (Payne et al., 2012; Williams et al., 2015). *In vitro* models facilitate frequent sampling, increase the reproducibility and provide a simplified model to focus on the gut microbiota without issues such as host variability, ethical approval and volunteer compliance (McDonald et al., 2013). Various *in vitro* models of the gut microbiota have been used to examine the effects of prebiotics (Bussolo de Souza et al., 2014; Chung et al., 2016), probiotics (Cordonnier et al., 2015), diet (Condezo-Hoyos et al., 2014), and dietary modulations (Aguirre et al., 2016) on the gut microbiota and its metabolites.

The recommended daily individual intake of dietary fiber in many countries ranges from 25 to 30 g/day (McRorie, 2015), however, increasing amounts of data show that this requirement is poorly met, especially in many western countries (Cordain et al., 2005). Many commercially available fiber supplements are marketed as a means of bridging this gap in dietary fiber intake. However, to date only a few studies have directly examined commercially available dietary fiber products for prebiotic potential (Hobden et al., 2013; Grimaldi et al., 2016).

In this study, we investigated the effect of three commercially available fiber products in the Australian market, namely, NutriKane™, Benefiber® and Psyllium husk (Macro) on the human gut microbiota using an *in vitro* model system. Alterations in microbial community composition, as well as the production of metabolites such as SCFAs were examined. The

chemical composition, antioxidant potential and polyphenolic content of the products were also determined.

MATERIALS AND METHODS

Compositional Analysis of Fiber Products

Fiber products used in this experiment are derived from dried whole sugarcane and pectin from apple and citrus fruits (NutriKane), wheat dextrin (Benefiber) and psyllium husk (Macro Organic Psyllium husk).

The chemical composition of each fiber product was determined using the following protocols. Total Nitrogen content was measured by the Dumas method with a Series II CHNS/O Analyzer 2400 (Perkin Elmer, Australia). Protein content was calculated by multiplying the nitrogen content by a factor of 6.25 (Ulsemer et al., 2016). Fat content was determined by Soxhlet extraction according to the AOAC Method 945.16. Dietary fiber was determined enzymatically according to the AOAC method 985.29. Insoluble and soluble dietary fiber content was determined according to AOAC 991.43. Acid insoluble lignin was measured gravimetrically following acid hydrolysis (Willför et al., 2009). Monosaccharides were quantified using acetylated samples on a Shimadzu 17A gas chromatograph with flame ionization detection (GC-FID). Quantitation was performed by acetylation of a mixture of monosaccharide standards and 2-deoxy-D-glucose as an internal standard, which was added to all samples at 100 ppm concentration to allow calculation of response factors (full chemical composition and ingredient list provided in **Table 1**).

TABLE 1 | The chemical composition and nutritional profile of fiber products **(A)** The chemical composition of each fiber product determined as described in the Methods section. Values are expressed as g/100 g total weight, unless not detected (ND). The mean ± SD is presented for each compound (n = 3), and **(B)** ingredients and nutritional profile of each product according to the information on the packaging.

Compound	NutriKane	Benefiber	Psyllium husk
(A)			
Nitrogen	0.08 ± 0.01	ND	0.24 ± 0.01
Protein	0.54 ± 0.04	ND	1.50 ± 0.09
Fat	1.17 ± 0.09	0.02 ± 0.04	0.46 ± 0.15
Total dietary fiber	83.94 ± 0.60	10.31 ± 1.19	77.24 ± 1.18
Insoluble dietary fiber	86.65 ± 1.95	ND	71.74 ± 0.87
Soluble dietary fiber	ND	5.64 ± 1.14	ND
Lignin	20.23 ± 1.08	ND	4.69 ± 0.21
Rhamnose	ND	ND	2.39 ± 0.03
Arabinose	5.83 ± 0.45	1.27 ± 0.2	46.8 ± 0.49
Xylose	31.8 ± 2.47	0.19 ± 0.01	24.1 ± 0.25
Mannose	1.54 ± 0.12	21.3 ± 0.22	4.24 ± 0.04
Glucose	17.3 ± 1.34	74.5 ± 0.78	11.2 ± 0.12
Galactose	0.74 ± 0.06	ND	2.09 ± 0.16
Fiber supplement	**NutriKane**	**Benefiber**	**Psyllium husk**
(B)			
Ingredients	Sugarcane (sucrose removed)	100% wheat dextrin (derived from wheat)	100% organic psyllium husk
	Pectin (from apple and citrus fruits)		
Dietary fiber content per 100 g	55.2 g	83 g (soluble fiber)	90 g
Nutritional information (average quantity per 100 g)	Energy 784 kJ	Energy 913 kJ	Energy 759 kJ
	Protein 0.8 g	Protein < 1 g	Protein 1.3 g
	Fat total 0.1 g	Fat total < 1 g	Fat total < 1 g
	Saturated 0.1 g	Saturated < 1 g	Saturated < 1 g
	Carbohydrate 6.6 g	Carbohydrate 14.2 g	Carbohydrate < 1 g
	Sugars 4.5 g	Sugars < 1 g	Sugars < 1 g
	Dietary fiber 55.2 g	Dietary fiber (total) 83 g	Dietary fiber 90.1 g
	Sodium 15 mg	Sodium < 5 mg	Sodium 17 mg
	Gluten ND		
	Chromium 391 μg		
	Potassium 5.7 g		

In Vitro Digestion of Fiber Supplements

All enzymes and reagents were purchased from Sigma Aldrich, Australia, unless otherwise stated. Gratuk Technologies Pty Ltd, Australia, provided NutriKane. Benefiber and Psyllium husk (Macro) were purchased from a local Australian supermarket.

Each of the three fiber products and a sterile water (Milli-Q, Millipore, Australia) sample as the no added fiber control were processed by a simulated oral, gastric and small intestinal digestion as described previously (Minekus et al., 2014). According to this protocol all enzymatic treatments were performed at 37°C, samples were first incubated with human salivary α-amylase (75 UmL^{-1}) for 2 min at pH 7, followed by porcine pepsin (2,000 UmL^{-1}) for 2 h at pH 3. The small intestine digestion was performed for another 2 h with the following enzymes: porcine trypsin (100 UmL^{-1}), bovine chymotrypsin (25 UmL^{-1}), porcine pancreatic lipase (2,000 UmL^{-1}), porcine pancreatic colipase (2:1 colipase to lipase molar excess) and bile salts (10 mM) at pH 7. Samples were frozen at −80°C and freeze dried, following digestion.

Preparation of the Basal Medium

A basal media was designed to simulate human large intestine conditions (Gamage et al., 2017). The composition of the basal medium per liter was: Peptone 0.5 g, yeast extract 0.5 g, $NaHCO_3$ 6 g, Hemin solution (0.05% (w/v) Hemin, and 0.2% (w/v) NaOH) 1 mL, L-cysteine HCl 0.5 g, Bile salts 0.5 g, Tween 80 2 mL, Resazurin solution [0.1% (w/v)] 1 mL, Vitamin stock (Scheifinger et al., 1974) 1 mL, K_2HPO_4 0.228 g, KH_2PO_4 0.228 g, $(NH_4)_2SO_4$ 0.228 g, NaCl 0.456 g, $MgSO_4$ 0.0456 g, $CaCl_2.2H_2O$ 0.0608 g, and 1 mL trace mineral solution (Balch et al., 1979) with additional $NiSO_4.6H_2O$ (0.1 g/L), Na_2SeO_4 (0.19 g/L) and $Na_2WO_2.2H_2O$ (0.1 g/L). The pH of the medium was adjusted to 7.0 ± 0.2.

Preparation of the basal medium and subsequent culturing was conducted under strict anaerobic conditions using a 5% hydrogen and 95% carbon dioxide anaerobic chamber (COY Lab products, Australia). The anaerobic basal medium was aliquoted into airtight glass vials with rubber stoppers and aluminum lids prior to sterilization.

Collection and Preparation of Fecal Inocula

This study was carried out in accordance with the recommendations of the "National Statement on Ethical Conduct in Human Research 2014" (National Health and Medical Research Council of Australia). The protocol and experimental procedures were reviewed and approved by Macquarie University Human Research Ethics Committee (Medical Sciences, Reference number 5201400595). All participants were provided with a participant information and consent form. To secure anonymity written consent was not obtained, and participation was voluntary.

One fecal sample each was collected from six healthy volunteers (3 male and 3 female) aged 20–60 years, who had not taken antibiotics in at least 3 months, had no history of gastrointestinal diseases and were on a nonspecific omnivorous diet (metadata provided in Supplementary Table S1).

Fresh fecal samples were collected in a sterile container and immediately placed in an anaerobic jar (Anaero jar, Oxoid Limited, UK) with an Anaerogen sachet (Oxoid, UK) and an Oxoid anaerobic indicator (BR0055B, Oxoid, UK). Samples were transported to the laboratory anaerobically and processing occurred within 2 h of collection. Fecal slurries were prepared from individual samples by homogenizing in anaerobic sterile basal medium and filtering through a sterile Nylon mesh cloth (985 μm). This was conducted under strict anaerobic conditions as used for basal media preparation.

In Vitro Fermentation of Fiber Supplements

In vitro digested and freeze-dried samples of NutriKane, Benefiber and Psyllium husk were added into separate vials with sterile anaerobic basal medium, the final fiber product concentration was maintained at 1% (w/v). A control sample was run in parallel with no added fiber. Each of these vials was then inoculated with filtered fecal homogenate to obtain a final concentration of 2% (w/v) in a final volume of 50 mL (1.0 g feces per vial). Experiments were performed in triplicate for each of the six fecal samples (details of the experiment design are provided in Supplementary Figure S1).

Culture vials were incubated anaerobically at 37°C with agitation (100 rpm). Cultures were left without agitation for 5–10 min, to allow the solids to settle, prior to collecting 2 mL aliquots from the top liquid fraction at 0, 24, and 48 h. Harvested samples were stored at −80°C immediately prior to further analysis. The pH of the cultures at 48 h was measured using pH indicator strips universal pH 0–14 and 4.5–10 (Dosatest, VWR, Australia).

After collecting the liquid fraction samples at 48 h, insoluble fiber biomass (fiber fraction) was separated from each vial with fiber products by centrifugation at 100 × g for 5–15 min. Separated fiber fraction samples were resuspended in Phosphate-buffered saline (PBS) prior to dissociation of tightly adherent microorganisms as previously described (Rosewarne et al., 2011). According to this protocol the insoluble fiber fraction was mixed with a 1:2 (w/v) acid butanol solution [0.1% (v/v) Tween 80, 1% (v/v) methanol and 1% (v/v) tert-butanol, at pH 2.0]. Harvested microbial cells were stored in PBS at −20°C prior to DNA extraction.

Analysis of the Microbial Composition

Microbial cells from the liquid fraction samples were harvested by centrifugation at 20,238 × g for 15 min. Harvested cells from the liquid and fiber fraction samples were used for bacterial DNA extraction using a FastDNA spin kit (MP Biomedicals, Australia) according to the manufacturer's instructions. The lysing matrix in the kit was replaced by matrix E (MP Biomedicals, Australia) according to previously published protocols (Gillings, 2014). The V4 region of 16S rRNA gene was amplified using a Five prime hot master mix (5 prime, VWR, Australia) with a final primer concentration at 0.2 μM in a final volume of 25 μL. The PCR was performed with 30 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s using 515 forward (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806 reverse (5′-GGACTACHVGGGTWTCTAAT-3′) primers with custom barcodes for Illumina MiSeq sequencing (Caporaso et al., 2011, 2012). Fiber and liquid fraction samples were randomly allocated to libraries. The resulting amplicons were quantified using Quant-iT™ PicoGreen® (Invitrogen, Australia) and equal molar amounts of barcoded amplicons from each sample were pooled, gel purified (Wizard® SV gel and PCR clean up system, Promega, Australia) and sequenced on an Illumina MiSeq platform (2 x 250 bp paired-end sequencing) at the Ramaciotti Centre for Genomics, Australia.

Raw sequence data was processed using Quantitative Insights Into Microbial Ecology (QIIME) software (version 1.9.0) (Caporaso et al., 2010). Reads with full length and high quality (-q 19 and with other default parameters) were used to pick Operational Taxonomic Units (OTUs) at 97% similarity using an open reference protocol against the Greengenes (version 13_8) database (DeSantis et al., 2006).

Liquid fraction samples ($n = 216$, 3 fiber products and a no added fiber control × 6 biological samples × 3 time-points × 3 technical replicates) resulted in a total of 21,052,381 reads (mean 97, 464 ± 25,865) prior to filtering out the OTUs with < 0.005% reads. Reads per sample was rarefied to 38,249 reads (four samples failed to meet this requirement and therefore, were eliminated from further analyses, at least two technical replicates remained for each condition) prior to further statistical analyses.

Fiber fraction samples ($n = 54$, 3 fiber products × 6 biological samples × 3 technical replicates) were analyzed with liquid fraction samples ($n = 54$) at 48 h and this analysis resulted in 9,276,308 reads (mean 85,891 ± 32,494). Reads per sample was rarefied to 12,864 reads prior to statistical analysis, following filtering out OTUs with < 0.005% reads (two samples failed to meet this requirement and therefore were eliminated from further analyses, however at least two technical replicates remained for each condition).

Rarefied and filtered OTUs abundances (Log (x+1) transformed) were used for statistical analyses using the PRIMER-7 software package (Clarke and Gorley, 2015), default parameters on PRIMER-7 were used unless otherwise stated. Permutational Multivariate Analysis of Variance (PERMANOVA) and pairwise tests were conducted using PERMANOVA+ (Clarke et al., 2014) to investigate differences in the microbial community structure in each sample. Type III sums of squares with 9999 permutations were used to determine the *P*-values. Non-metric multi-dimensional scale (nMDS) plots were constructed to visualize the differences in the community structure in each biological sample based on Bray-Curtis similarity of Log (x+1) transformed values of the abundance of the OTUs. The Shannon diversity index, Simpson's evenness index and Chao1 index per liquid fraction sample ($n = 212$) was also determined using PRIMER-7.

Similarity Percentages (SIMPER) analyses with a 5% cut off for low contributions was used to determine the OTUs with significant differences in each treatment using PRIMER-7. Distinct phylotypes (bacterial families and OTUs) between each fiber product and no added fiber control at 48 h were identified using LEfSe analyses (online Galaxy version 1.0) (Segata et al., 2011). LEfSe analysis was conducted with treatment conditions as subject (no subclasses) and with all other default parameters. The significantly differentially abundant OTUs between the fiber adherent and liquid fraction microbiota were also determined using LEfSe analyses. Analyses were conducted with fractions in each product as subject (no subclasses) and with all other default parameters.

Quantification of SCFAs

The supernatants of the liquid fraction samples (500 μL) collected at 0, 24, and 48 h were spiked with an internal standard (4-methyl valeric acid). This was further diluted in a 70% (v/v) ethanol and 0.1% (v/v) trifluroacetic acid (TFA) solution to obtain a final concentration of the internal standard at 100 ppm. The solution was vortexed then filtered through a 0.2 μm membrane filter (Millipore, Australia). Analysis was performed using a GC-FID (Shimadzu GC-17A). Samples were separated on a 30 m × 0.25 × 0.5 μm i.d. HP-INNOWax fused silica column (Hewlett-Packard, Australia) as per the manufacturer's instructions. GC-FID analysis for each sample was performed in three technical replicates ($n = 636$). The concentrations of SCFAs are reported in $mmolL^{-1}$ per gram of feces.

Quantification of Antioxidant Potential and Polyphenol Content

Total Polyphenolic Content (TPC) was determined as previously described (Singleton and Rossi, 1965). Briefly, 20 μL of sample was mixed with 1.58 mL of water and 100 μL of the Folin-Ciocalteu reagent. After 6 min of incubation, the solution was mixed with 300 μL of 7.5% (w/v) Na_2CO_3 and left to stand for 2 h. Gallic acid standards ranged from 25 to 500 mg/L. Absorbance was read at 765 nm and results were reported in mg of Gallic acid per liter.

Ferric reducing antioxidant power (FRAP) was performed as previously described (Benzie and Strain, 1996). The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine solution and 20 mM $FeCl_3$ in a 10:1:1 ratio. 20 μL of sample was mixed with 0.2 mL of water and 1.8 mL of FRAP reagent and incubated at 37°C for 10 min. Ferrous sulfate standards ranged from 125 to 2,500 μM. The absorbance was read at 593 nm and results reported in millimolar ferric ions converted to the ferrous form per liter.

Statistical Analysis

D'Agostino-Pearson omnibus normality tests were performed and Kruskal-Wallis test with Dunn's multiple comparisons test or Tukey's multiple comparison test was used where appropriate to determine statistically significant differences. Significant differences in the Log (x+1) transformed abundance of the OTUs identified from the SIMPER analysis, Shannon diversity indices, Simpson's evenness indices, pH measurements, concentration of SCFAs, TPC and FRAP measurements were determined between each fiber product and no added fiber control at 24 and 48 h using GraphPad Prism (version 7) software (GraphPad Software, La Jolla California, USA). Biological samples were analyzed independently. The correlations between the relative abundance of the bacterial families, SCFA concentrations, TPC and FRAP measurements were determined using Spearman's correlation analyses (two-tailed test) using GraphPad Prism (version 7) software.

RESULTS

Samples of three commercially available fiber products; NutriKane, Benefiber and Psyllium husk (Macro), with varying chemical composition (**Table 1**), were chosen to investigate the impact of fiber supplementation on the human gut microbiota *in vitro*. Each fiber product was subjected to a series of pH-controlled enzyme treatments to simulate human digestion, and the effect of each on the human gut microbiota was examined in an *in vitro* system with an anaerobic basal medium, which simulates conditions in the human large intestine. Fecal material obtained from six healthy adults as independent biological samples were inoculated separately into the basal medium (metadata provided in Supplementary Table S1). For each biological sample, four treatments were applied, this included three fiber products and one "no added fiber" control (details of the experiment design are provided in Supplementary Figure S1).

The anaerobic cultures for all tested fiber products with each fecal inoculum produced visually detectable gas by 24 h, indicating that the microbiota was metabolically active. At 48 h the pH of the culture vials with Benefiber significantly reduced ($P < 0.001$) compared to the samples with NutriKane, Psyllium husk or no added fiber control, which maintained the pH at 7.0 ± 0.5 in the buffered media (Supplementary Figure S2).

Samples were collected at 0, 24, and 48 h from the liquid fraction, and at 48 h the insoluble fiber fraction was additionally sampled. The 16S rRNA gene amplicons were sequenced from each sample. A total of 21,052,381 reads were generated for the liquid fraction samples, and after filtering and rarefaction a total of 8,261,784 reads were

used for further analyses. A total of 4,400,597 reads were generated for the fiber fraction samples, and after filtering and rarefaction a total of 681,792 reads were used for further analyses.

Effects of Fiber Addition on Microbial Community Structure and Diversity

To determine the impact of different fiber products on the microbiota in the liquid fraction, statistical analyses were performed to compare the bacterial community structure of samples over time and between treatment conditions. We observed fiber-dependent changes in the bacterial community structure over time based on Bray-Curtis similarity nMDS plots for each biological sample (**Figure 1**) and PERMANOVA tests. Fiber product-mediated shifts in the gut microbiota structure showed very similar trends upon each treatment at 24 and 48 h across biological samples (**Figure 1** and Supplementary Figure S3). For all individuals, supplementation with NutriKane resulted in a significantly different community structure at 24 h compared to at 0 h ($P < 0.05$). At 48 h this shift was more pronounced ($P < 0.005$, comparing 0 and 48 h). Clear shifts in the microbial community structure were observed in the nMDS plots upon addition of NutriKane compared to the no added fiber samples at both 24 and 48 h (**Figure 1**), however, these differences were not statistically significant based on PERMANOVA tests. Addition of Benefiber and Psyllium husk resulted in very dramatic changes in the community structures. Both the products resulted in significant differences ($P < 0.001$) in the community structure at 24 and 48 h compared to that of the no added fiber control and community at 0 h ($P < 0.001$).

Ordination of the gut microbiota of all samples ($n = 212$) showed significant fiber addition-mediated changes in the community structure common across the biological samples (Supplementary Figure S3). At 0 h all samples grouped according to the individual fecal inoculum, rather than the treatment condition, indicative of the individual variation in the gut microbial composition of the volunteers.

The microbial diversity, evenness and richness of each sample were determined using a Shannon diversity index, Simpson's evenness index and Chao1 index, respectively (**Figure 2** and Supplementary Figure S4). Shannon diversity indices of samples with Benefiber and Psyllium husk reduced to 3.2 ± 0.5 and 2.4 ± 0.4, respectively, at 48 h, while samples with NutriKane showed no significant loss of diversity (3.8 ± 0.4) compared to the no added fiber control at 48 h (3.7 ± 0.2) and all samples at 0 h (4.0 ± 0.2). A similar trend was observed for the microbial evenness. Simpson's evenness indices for samples with Psyllium husk were significantly lower (0.71 ± 0.01) compared to the no added fiber control at 48 h (0.93 ± 0.04), while the microbial evenness of samples with NutriKane (0.92 ± 0.07) and Benefiber (0.89 ± 0.06) showed no significant loss of evenness. Microbial richness determined using a Chao1 index demonstrated no significant change in any of the treatments over time.

Effects of Fiber Addition on Microbiota Composition

For all individuals the starting fecal microbiota communities (0 h) were dominated by the phyla *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria*, and *Verrucomicrobia*. However, the relative abundances of these phyla differed substantially between biological samples. Similar individual-specific variations were observed at a family and genus level, and supplementation with each fiber product differentially altered the microbiota composition at 24 and 48 h in each of the six biological samples (Supplementary Figure S5).

The OTUs that contributed most to these product-specific changes in the microbial community composition were identified using SIMPER analyses, and changes in the relative abundance of this set of OTUs in each treatment and time point were analyzed for each biological sample (Supplementary Figure S6 and Supplementary Table S2). The OTUs in the genus *Bacteroides* showed a higher relative abundance in all samples supplemented with fiber, however, the specific OTUs varied between fiber products. In five out of six biological samples, the relative abundance of *Bacteroides* OTU589071 was significantly higher upon addition of Benefiber. While in at least four biological samples, the relative abundance of three OTUs (OTU364179, OTU535375 and OTU583117) in the genus *Bacteroides* were significantly higher with addition of Psyllium husk. In all biological samples, the relative abundance of two OTUs (OTU585914 and OTU180082) in the genus *Parabacteroides* were significantly higher upon addition of Benefiber. In three out of six biological samples, the relative abundance of *Coprococcus* (OTU362501) was significantly higher in samples with NutriKane compared to the no added fiber control, samples with Benefiber and Psyllium husk showed a reduction in the relative abundance of this OTU.

The complete set of bacterial families and OTUs showing significant differences in the relative abundance between each fiber addition and the no added fiber control was identified with LEfSe analyses. A family level LEfSe analysis showed 2, 17, and 15 differentially abundant families in NutriKane, Benefiber and Psyllium husk, respectively, compared to the no added fiber control (**Figure 3**). For NutriKane, the relative abundance of *Bifidobacteriaceae* and *Pseudomonadaceae* were shown to be significantly different. Benefiber and Psyllium husk supplementation resulted in an increase in the relative abundance of *Bacteroidaceae* compared to the no added fiber control. The relative abundance of the family *Porphyromonadaceae* significantly increased with addition of Benefiber. A decrease in the relative abundance of *Lachnospiraceae, Ruminococcaceae, Enterobacteriaceae* and *Bifidobacteriaceae* was observed upon supplementation with Benefiber and Psyllium husk.

The LEfSe analyses at the OTU level showed 72, 259, and 203 OTUs with significantly altered abundances in response to supplementation with NutriKane, Benefiber and Psyllium husk, respectively (Supplementary Table S3). While most of these trends were commonly observed across biological samples, the degree of changes varied between individuals

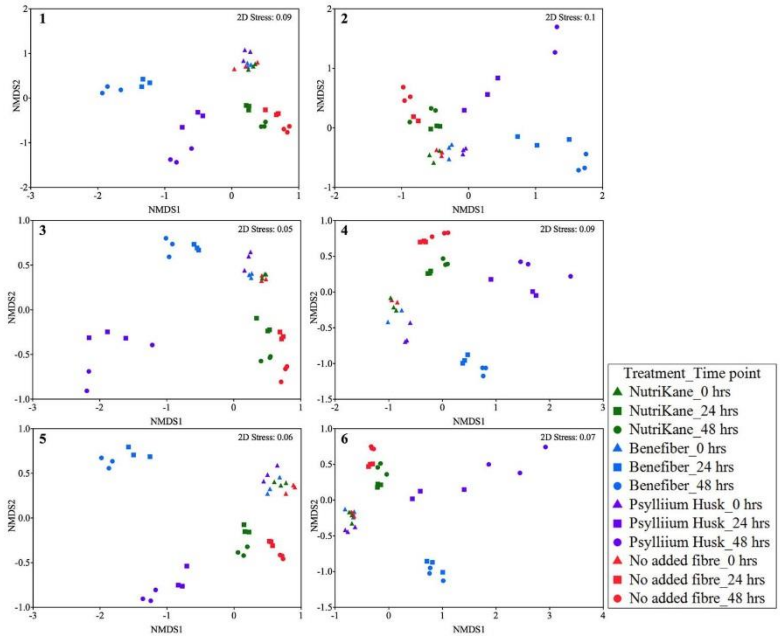

FIGURE 1 | Ordination of the gut microbiota in each biological sample (sample 1-6) at 0, 24, and 48 h. Data is shown as Bray-Curtis similarity of Log (X+1) relative abundance based nMDS plots. At 0 h (triangles) all samples group together. Fiber-dependent shifts were observed at 24 (squares) and 48 (circles) hours in all the treatments. NutriKane (green), Benefiber (blue) and Psyllium husk (purple) had different communities to the no added fiber control (red) while samples with Benefiber and Psyllium husk showed the most dramatic shifts compared to the no added fiber control and other treatments.

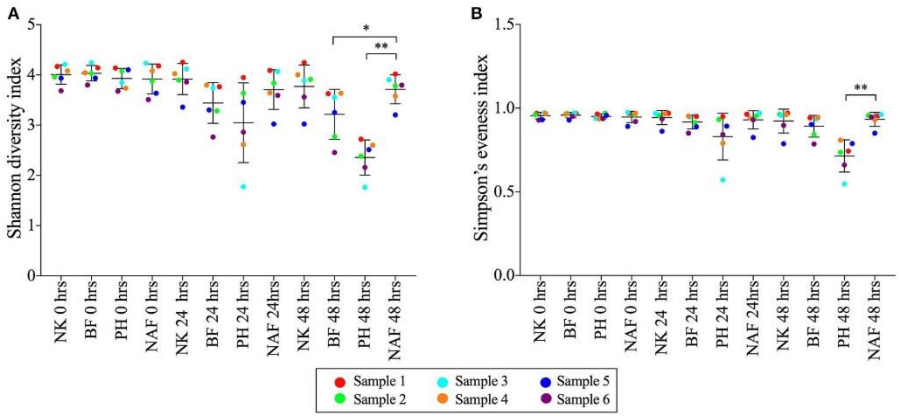

FIGURE 2 | The **(A)** Shannon diversity indices and **(B)** Simpson's evenness indices of microbial communities from each treatment at 0, 24, and 48 h. Data is shown as mean ± SD for samples with NutriKane (NK), Benefiber (BF), Psyllium husk (PH) and no added fiber control (NAF) at 0, 24, and 48 h. Biological samples (sample 1–6) are indicted by color-coded dots as shown in the key. Significance was determined using Kruskal-Wallis test with Dunn's multiple comparisons (*$P < 0.05$, **$P < 0.01$).

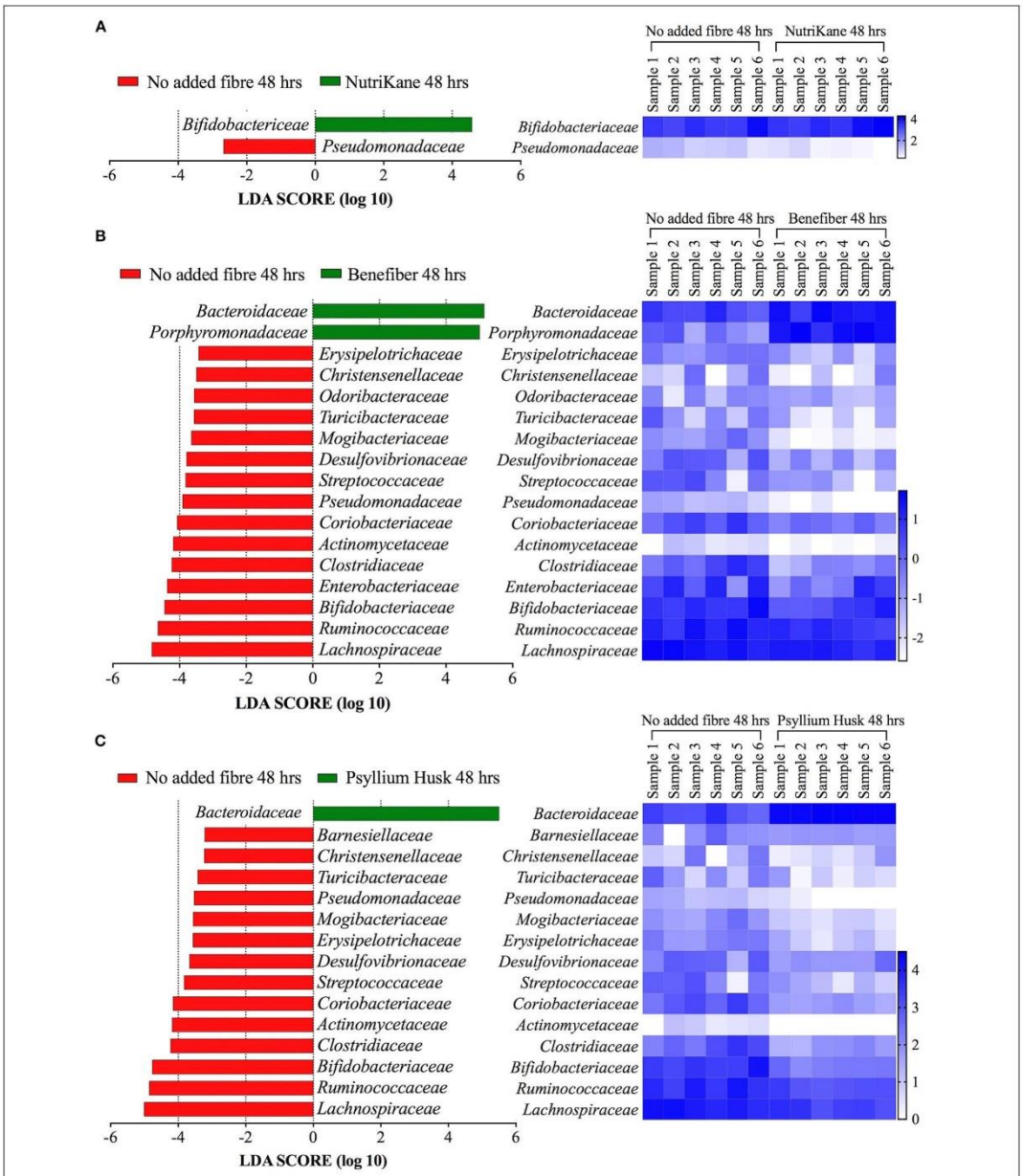

FIGURE 3 | Key gut microbiota bacterial families that respond to fiber supplementation at 48 h. Data was obtained using LEfSe analyses between **(A)** NutriKane vs. no added fiber control, **(B)** Benefiber vs. no added fiber control and **(C)** Psyllium husk vs. no added fiber control. The left histogram shows the LDA scores computed for each bacterial family and the right heat map shows the relative abundance (Log_{10} transformation) of the families in each of the six biological samples. In the heat map, rows correspond to bacterial families and columns correspond to an individual (Sample 1–6). Blue and white denote the highest and lowest relative abundance, respectively, as shown in the key.

(Supplementary Table S4). The relative abundance of many specific OTUs within the *Bacteroidaceae* were significantly higher in samples with each of the three fiber products compared to the no added fiber control (15, 35, and 33 OTUs in NutriKane, Benefiber and Psyllium husk, respectively, Supplementary Table S3). Among the fiber specific changes, Benefiber addition resulted in higher relative abundance of 5 and 15 OTUs in *Faecalibacterium prausnitzii* and *Parabacteroides* (of these, 8 OTUs were identified as *Parabacteroides distasonis*), respectively, a change not observed for the other products. NutriKane supplementation promoted high relative abundance of an OTU (OTU723) within the *Bifidobacteriaceae*, whereas supplementation with Benefiber and Psyllium husk decreased the relative abundance of 2 and 11 *Bifidobacteriaceae* family OTUs, respectively.

The relative abundance of OTUs in the *Enterobacteriaceae* decreased upon addition of all fiber products (2, 5, and 2 OTUs in NutriKane, Benefiber and Psyllium husk, respectively). The OTU646549 in the family *Pseudomonadaceae* also showed a lower abundance upon addition of each of the three fiber products. The relative abundance of many *Lachnospiraceae* OTUs decreased upon Benefiber and Psyllium husk supplementation (65 and 58 OTUs, respectively), whereas for NutriKane the relative abundance of 3 OTUs in this family decreased, while the abundance of 27 OTUs increased. Similarly, in the family *Ruminococcaceae* the relative abundance of 41 and 30 OTUs decreased in samples with Benefiber and Psyllium husk, while for NutriKane, the abundance of 11 OTUs in this family increased.

Variation in the Response of the Biological Samples to Fiber Supplementation

The addition of fiber products resulted in several common changes observed across most of the biological samples; including changes in the relative abundance of specific OTUs in the families *Bacteroidaceae, Porphyromonadaceae, Bifidobacteriaceae, Lachnospiraceae, Ruminococcaceae, Pseudomonadaceae,* and *Enterobacteriaceae* (**Figure 3**, Supplementary Figures S5, S6).

In addition to these, we also observed individual-specific changes in some bacterial groups (Supplementary Figure S5). Most notably, the relative abundance of the genus *Megamonas* increased dramatically only in biological sample 1 and 2 in the presence of Benefiber. In biological sample 2, *Butyricimonas* was highly abundant in the presence of all fiber products. The relative abundance of *Prevotella* showed dramatic changes only in biological samples 2 and 5 in the presence of Benefiber and Psyllium husk, respectively. The increase in the relative abundance of *Bifidobacterium* observed upon addition of NutriKane was substantially higher for biological sample 5 than for other biological samples. The abundance of *Bacteroidales* S24-7 was higher only in biological samples 1 and 6 following Psyllium husk and NutriKane treatments. The family *Comamonadaceae* was highly abundant upon addition of fiber products only in biological sample 4. In biological sample 5, the relative abundance of *Enterobacteriaceae* increased at 24 h upon addition of fiber products.

Comparison of the Fiber-Adherent and Liquid Fraction Microbiota

To investigate possible differences in the microbial communities adhered to the fiber relative to the liquid fraction, we examined the microbiota detached from insoluble material in the cultures at 48 h. While the community structure was observed to be similar between fiber and liquid fractions (Supplementary Figure S7), some differences in the composition were observed (Supplementary Figure S8). Analysis of the fiber adherent microbial community relative to the liquid fraction at the OTU level was performed using LEfSe analyses (Supplementary Table S5). The relative abundance of 13, 19, and 31 OTUs were higher in the fiber fraction of samples with NutriKane, Benefiber and Psyllium husk, respectively, most of these OTUs were in the families *Turicibacteraceae, Lachnospiraceae* and *Ruminococcaceae*. The relative abundance of 45, 44, and 24 OTUs were higher in the liquid fraction of NutriKane, Benefiber and Psyllium husk, respectively, most of these OTUs were in the families *Bacteroidaceae, Lachnospiraceae* and *Ruminococcaceae.* While most of these trends were commonly observed across biological samples, we again observed some individual-specific differences (Supplementary Table S6).

Fiber Additions Increased the Production of SCFAs

To examine the impact of the fiber products on microbial production of SCFAs, the concentrations of acetate, propionate and butyrate were measured in the liquid fraction of the samples using a gas chromatograph with flame ionization detection (GC-FID, **Figure 4**). Addition of NutriKane, Benefiber and Psyllium husk resulted in significantly higher ($P < 0.05$) concentrations of all three SCFAs at 48 h, compared to the same treatments at 0 h. In comparison to the no added fiber control at 48 h, Benefiber and Psyllium husk supplementation showed significantly higher ($P < 0.005$) concentrations of all three SCFAs. The increase in concentration of all three SCFAs was the highest upon addition of Benefiber, followed by Psyllium husk and NutriKane. All fiber additions resulted in a significant increase in the concentrations of acetate, followed by propionate and butyrate. Samples with NutriKane had similar concentrations of propionate and butyrate at 48 h, while samples with Benefiber and Psyllium husk had two and three-fold higher concentrations of propionate relative to butyrate, respectively.

Changes in the relative abundance of the *Parabacteroides* correlated with the concentrations of all three SCFAs (Spearman's $r > 0.33$, $P < 0.0001$). Changes in the abundance of *Bacteroides* correlated with the concentration of propionate (Spearman's $r = 0.43$, $P < 0.0001$). While all biological samples showed similar trends with the specific fiber additions, we observed individual-dependent differences in the concentrations of each SCFA (Supplementary Table S7).

Comparison of the Polyphenol Content and Antioxidant Potential of Fiber Products

The polyphenolic content and antioxidant potential of each fiber product at time 0, 24, and 48 h were determined using total

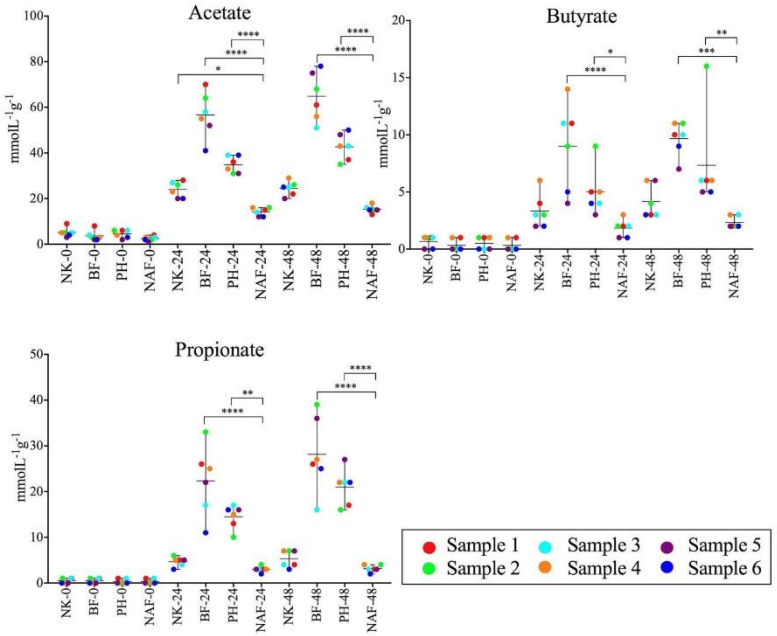

FIGURE 4 | Average concentrations of acetate, butyrate and propionate in each sample at 0, 24, and 48 h. Concentrations of all the SCFAs increased upon the fiber additions (NK- NutriKane, BF- Benefiber, PH- Psyllium husk) at 24 and 48 h compared to the no added fiber control (NAF). The mean ± SD concentrations per treatment at 0, 24, and 48 h are shown. The mean concentration for each biological sample (sample 1–6) is indicated by color-coded dots as shown in the key. Significance was determined using Tukeys's multiple comparison tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). The measured SCFA concentrations are provided in Supplementary Table S7.

polyphenolic content (TPC) and Ferric reducing antioxidant power (FRAP) techniques respectively (**Figure 5**, Supplementary Table S8). NutriKane showed significantly higher ($P < 0.0001$) antioxidant potential and polyphenolic content compared to Psyllium husk at 0 h. The antioxidant potential of NutriKane was significantly higher ($P < 0.05$) compared with Benefiber at 0 h. In all fiber-supplemented samples polyphenolic content decreased across the full incubation while antioxidant potential decreased over the first 24 h, but no further decrease was observed at 48 h.

DISCUSSION

This research examined the effect of three commercially available fiber products on the human gut microbiota from healthy individuals *in vitro*. Our findings demonstrated fiber product-induced strong shifts in gut microbiota community structure and composition at 24 h, which were further pronounced at 48 h. These changes in the relative abundance of microbial families and OTUs following fiber additions are largely consistent with selection for the abundance of bacteria capable of polysaccharide digestion. Amongst the most highly stimulated families were groups known to produce high numbers of Carbohydrate-active enzymes (CAZymes) that contribute to the digestion of polysaccharides by the gut microbiota. This includes specific members of the phyla *Bacteroidetes* such as *Bacteroidaceae* and *Porphyromonadaceae*, reported to encode the highest number of CAZymes, or members of the *Firmicutes* (*Lachnospiraceae* and *Ruminococcaceae*) and *Actinobacteria* (*Bifidobacteriaceae*), which are also CAZyme-producing (El Kaoutari et al., 2013).

Tested Fiber Products Have Distinct Chemical Compositions

NutriKane is primarily derived from dried whole sugarcane stem and pectin, while Benefiber and Macro Psyllium husk is produced using wheat dextrin and psyllium husk, respectively. Composition analysis of the three fiber products demonstrated distinct chemical profiles in dietary fiber, carbohydrates, protein and fat content for each product. The amount of lignin and xylose was highest in NutriKane, while Benefiber had the highest amounts of mannose and glucose, and Psyllium husk contained the highest amount of arabinose. Previous studies have reported sugarcane to be rich in cellulose, hemicellulose and lignin (Ouensanga, 1989; Hoang et al., 2016), and contain a range of

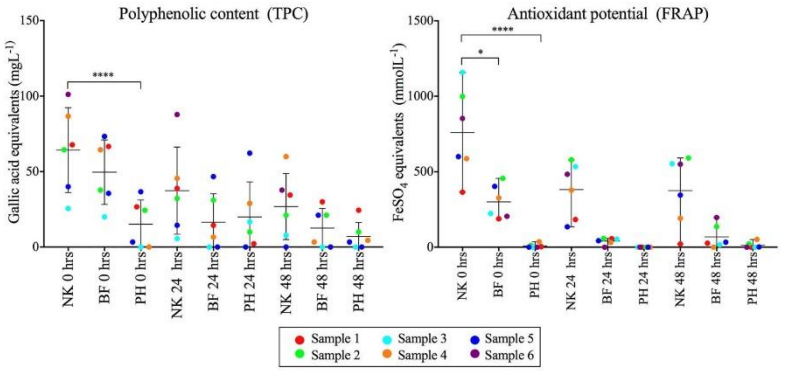

FIGURE 5 | Comparison of the polyphenol content (mgL^{-1}) and antioxidant potential ($mmolL^{-1}$) of the fiber products (NK- NutriKane, BF- Benefiber, PH- Psyllium husk) in each sample at 0, 24 and 48 h. Measurements for each fiber addition in each biological sample have been normalized against the no added fiber control at equivalent time points. Data is shown as mean ± SD for each treatment at 0, 24, and 48 h. Biological samples are indicted by color-coded dots as shown in the key. Significance was determined using Kruskal-Wallis test with Dunn's multiple comparisons (*$P < 0.05$, ****$P < 0.0001$). The measured concentrations are provided in Supplementary Table S8.

β-1, 4, and α-1, 4 linkages between glucose, xyloglucans, xylans, glucomannan, arabinoxylan, glucuronoxylan and D-galacturonic acid (Sun et al., 2003, 2004; Scheller et al., 2010; Hemsworth et al., 2016). Wheat dextrin contains typical starch glucosidic bonds (α-1, 4, and α-1, 6) and bonds atypical of starch (α-1, 2, and α-1, 3) between D-glucose subunits (Noack et al., 2013; McRorie, 2015). Psyllium husk typically contains viscous fiber such as certain hemicelluloses and arabinoxylans, and has been reported to consist of densely substituted main chains of β-1, 4 linked D-xylopyranosyl residues (Fischer et al., 2004). According to our results, NutriKane had the highest amount of total dietary fiber, followed by Psyllium husk and Benefiber, this observation is different from the information provided on the packaging. However, the testing applied here followed a standard protocol, which is less effective for lower molecular weight products and therefore may have potentially underestimated the soluble dietary fiber fraction for Benefiber in particular. As there is currently no universally applied protocol required for determining package labeling nutritional information for Australian dietary fiber products, the protocols used to generate the packaging information are not known and may not have been consistent across products, highlighting the need for standardized protocols.

Each Fiber Product Resulted in Distinct Alterations to the Microbiota Composition

Fiber-specific microbial community changes were observed, which are potentially linked to the chemical composition of each tested product. The abundance of OTUs in *Bacteroidaceae* (genus *Bacteroides*) and *Porphyromonadaceae* (genus *Parabacteroides*) were significantly higher upon addition of Benefiber and Psyllium husk, whilst OTUs in *Lachnospiraceae* and *Ruminococcaceae* were highly abundant in the presence of NutriKane. The members in the genus *Parabacteroides* (*P. distasonis* in particular) have been shown to digest chemically modified starch, while members in *Bacteroides, Ruminococcaceae* and *Lachnospiraceae* digest starch as well as more complex polysaccharides such as cellulose, hemicellulose and pectin (Martínez et al., 2010; Cockburn and Koropatkin, 2016). An increase in the abundance of *Bifidobacterium, Lactobacilli, Roseburia* and *Clostridium* cluster XIVa upon addition of wheat dextrin has been previously reported (Hobden et al., 2013; Noack et al., 2013; Carlson et al., 2015), whilst an increase in the relative abundance of *Bacteroides* and *Parabacteroides* has not been previously reported. Previous studies have also investigated the effect of specific purified dietary fibers on the gut microbiota and have shown that different types of resistant starch (Martínez et al., 2010), pectin (Licht et al., 2010), hemicellulose (Sanchez et al., 2009), cellulose (Chassard et al., 2010) and inulin (Van de Wiele et al., 2007) have different effects on the gut microbiota, likely due to variations in the chemical composition of different dietary fibers (Hamaker and Tuncil, 2014). In addition to differences in the dietary fiber and carbohydrate profiles between NutriKane, Benefiber and Psyllium husk, differences in the protein and fat content could also have contributed to observed fiber product-specific gut microbiota changes. The relative abundance of the OTUs in the genera *Bacteroides* was significantly higher in samples with Psyllium husk. This product had the highest amount of protein, consistent with the ability of the members of the genus *Bacteroides* to metabolize dietary protein (Portune et al., 2016).

Significant increases in the relative abundance of *Bifidobacteriaceae* (genus *Bifidobacterium*) were observed solely for NutriKane. NutriKane contained higher levels of xylose, and *Bifidobacteriaceae* has been shown to cross-feed on xylan (Cockburn and Koropatkin, 2016). Increased relative abundance of *Bifidobacterium* has been linked to potential prebiotic effects

and has been shown to increase in IBD patients upon remission (Morgan et al., 2012; Papa et al., 2012; Delzenne et al., 2013; Hamaker and Tuncil, 2014). *Faecalibacterium prausnitzii* showed higher relative abundance following supplementation with Benefiber. This is potentially linked to the ability of this group to digest smaller carbohydrates such as glucose (Cockburn and Koropatkin, 2016), which are highly available in this product. Increases in this species have been observed to have potential anti-inflammatory effects on patients with Crohn's disease (Sokol et al., 2008).

The OTUs in the families *Enterobacteriaceae* and *Pseudomonadaceae* showed the highest relative abundance in samples with no added fiber. Most of the members of these families belong to the normal microbiota, while some are associated with inflammation (Morgan et al., 2012; Shin et al., 2015). Observed reductions in the relative abundance of *Enterobacteriaceae* and *Pseudomonadaceae* upon supplementation with these fiber products might indicate the potential of the products to improve or maintain host health.

Analysis of the diversity and evenness of each community after fiber addition indicated varied capacities of the fiber products to maintain gut microbiota diversity and evenness *in vitro*. Of the tested fibers, only NutriKane treatment resulted in maintenance of the microbial diversity, while supplementation with Benefiber and Psyllium husk resulted in significant reductions. The Simpson evenness indices reflected the same observation whereby the microbial evenness was significantly lower in samples with Psyllium husk. These are likely explained by the dramatic increases in fiber-digesting groups such as *Parabacteroides*, which constituted 30.5% of the total bacteria at 48 h in Benefiber, and the *Bacteroides*, which constituted 68.9 and 24.0% of the total bacteria at 48 h in the samples with Psyllium husk and Benefiber, respectively. Whilst reduced gut microbiota diversity has been shown in individuals with obesity, T2D and IBD (Ott, 2004; Le Chatelier et al., 2013), we believe such dramatic increases in specific groups would likely be ameliorated by host factors, the presence of other fiber and food components and phage controls *in vivo*. Hence the reduction in diversity and evenness observed in the *in vitro* system is unlikely to be observed *in vivo*.

Microbiota Composition Differed Between Fiber-Adherent and Liquid Fractions

The relative abundance of bacterial groups attached to the insoluble fiber fraction, and therefore potential primary degraders, varied between each tested fiber product. The relative abundance of members of the phyla *Firmicutes* (*Lachnospiraceae* and *Ruminococcaceae*), and *Verrucomicrobia* (*Turicibacteraceae*) were higher in the fiber fraction compared to the liquid fraction. Bacterial groups in the phyla *Firmicutes*, *Actinobacteria* and *Verrucomicrobia* are known to be more nutritionally specialized and initiate the degradation of complex carbohydrates undigested by human (Flint et al., 2012) and, as primary degraders, may be preferentially found attached directly to the complex polysaccharides in the tested products. Previous studies have also demonstrated that the microbial communities attached closely to the insoluble material in human fecal samples are different to the liquid fraction communities, potentially due to the ability of the communities adhered to the insoluble material to act as primary degraders (Walker et al., 2008; White et al., 2014).

Fiber Additions Stimulated Production of Acetate, Propionate, and Butyrate

The concentration of measured SCFAs was significantly higher in all fiber-supplemented samples. Acetate, propionate and butyrate are all major bacterial fermentation products, each of which is likely to contribute to host health (Koh et al., 2016). Butyrate is generally used as an energy source in the colonic epithelial cells, while acetate and propionate have been shown to reach the liver and other peripheral organs and contribute in regulating gluconeogenesis and lipogenesis (Tremaroli and Backhed, 2012). The degree of stimulation of SCFA production was fiber product dependent and was more pronounced following addition of Benefiber and Psyllium husk compared to NutriKane. High levels of SCFA production in the presence of Benefiber and Psyllium husk correlated with the higher relative abundance of *Parabacteroides* and *Bacteroides* following addition of these fiber products. This is potentially linked to the ability of the members of these bacterial genera to digest highly available dietary fiber in the fiber products, and produce SCFAs (Kelder et al., 2014).

While production of all three SCFAs was higher with fiber additions, the pH levels significantly reduced only in samples with Benefiber. The significantly higher SCFA production in Benefiber supplemented samples may have surpassed the buffering capacity of the medium, while buffering was maintained in other samples where SCFA production was lower. Metabolic activities of SCFA-producing bacteria have been previously reported to reduce the pH of the large intestine, and lower intestine pH levels has also been linked to inhibit the growth of pathogenic *Escherichia coli* (Duncan et al., 2009).

Polyphenol and Antioxidant Availability Differed Between Fiber Products

We observed significant differences in the availability of polyphenols and antioxidants in the tested fiber products. Such differences may contribute to the product-specific changes observed in the gut microbiota. Dietary polyphenols have the potential to be used by the gut microbiota, and therefore, alter the microbial composition both *in vitro* (Tzounis et al., 2008; Condezo-Hoyos et al., 2014) and *in vivo* (Tzounis et al., 2011). Of the tested fiber products, NutriKane showed the highest availability of polyphenols and antioxidant potential. This could potentially have contributed to the higher relative abundance of the family *Bifidobacteriaceae* in samples with this particular fiber, as previous literature has shown an increase in the abundance this family upon addition of various polyphenol extracts and polyphenol rich foods both *in vitro* and *in vivo* (Hervert-Hernández and Goñi, 2011; Tzounis et al., 2011). The observed reduction of polyphenols and antioxidant potential of the fiber products over the time of incubation is likely due to metabolism

of these compounds by the gut microbiota (Dueñas et al., 2015; Ozdal et al., 2016).

Fiber Supplementation-Induced Biological Sample-Specific Microbial Community Shifts

In addition to common microbiota changes observed across biological samples, several sample-specific alterations were also observed, especially with bacterial groups such as *Megamonas*, *Butyricimonas*, *Bifidobacterium*, *Bacteroidales* S24-7, *Comamonadaceae* and *Prevotella*, which showed comparatively high relative abundances in some biological samples at 48 h while other biological samples did not show substantial differences. Of these, *Megamonas*, *Bacteroidales* S24-7, *Comamonadaceae* and *Prevotella* were not present or present at a very low relative abundance in all other biological samples except the specific samples that showed an increase in the relative abundance of these groups at 48 h. This may indicate that individual-specific differences are likely linked to the differences in the initial gut microbiota composition between the samples. Analysis of a larger number of biological samples, ideally with greater information on normal diet and host health, might be beneficial in determining possible reasons for this variability.

CONCLUSIONS

As dietary supplementation grows in popularity it is important to examine how commercial fiber products impact the human gut microbial communities and host health, and the degree to which this varies between products. We tested the *in vitro* impact of three different fiber supplements using fecal microbial communities sourced from six healthy individuals. For each specific fiber a broad pattern was observed across the biological samples with respect to changes in the microbial community composition and concentrations of SCFAs. Underlying this, in specific individual samples there were variations in the precise nature of the fiber-induced microbiota community shifts likely linked to differences in the starting microbial communities. This suggests that each of the tested fiber products may alter the gut microbiota in a generally similar, and rather predictable manner, despite variability in the starting composition of the individual gut microbiota.

The three different fiber products tested in this study all showed clear and distinct impacts on the structure and composition of the microbiota derived from healthy individuals. Differences in the impact on the microbiota could be linked to the composition of the dietary fiber and its associated micronutrients, for example the antioxidant and polyphenol content in each fiber product. The observed differences in microbial community composition upon fiber supplementation may also explain the observed fiber-specific differences in acetate, propionate and butyrate production.

Utilization of an *in vitro* gut mimicking model system in the present study facilitated frequent sampling without host interference and provided proof of concept information on how dietary fiber supplementation may influence the microbiota composition and function. To follow up, *in vivo* experiments could be conducted to gain further insight into the long-term effect of fiber products on the gut microbiota and how long the benefits last after consumption, while also taking differences in health, normal diet and colonic transit time between individuals into account (Verspreet et al., 2016).

AVAILABILITY OF DATA AND MATERIAL

The 16S rRNA gene sequence data generated and analyzed for this study can be found in the GenBank Sequence Read Archive (SRA) database under accession number SRP090829.

AUTHOR CONTRIBUTIONS

HG, ST, IP, and CR designed the study. HG conducted *in vitro* digestion, culturing, DNA extraction, bioinformatics and all statistical analyses. RC quantified SCFA concentrations and determined the chemical composition of fiber products. DB-N performed TPC and FRAP quantifications. HG, ST, IP, LK, MB, MM, and NP interpreted the results. HG drafted the manuscript with contributions of DB-N, RC, ST, and IP. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01618/full#supplementary-material

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Conflict of Interest Statement: MB is an employee of Gratuk Technologies Pty Ltd, producer of NutriKane^{TM}.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.3.1.2 Supplementary material

Supplementary material

Fibre Supplements Derived From Sugarcane Stem, Wheat Dextrin And Psyllium Husk Have Different *In Vitro* Effects On The Human Gut Microbiota

Hasinika K.A.H. Gamage[1,2], Sasha G. Tetu[1*], Raymond W.W. Chong[1,2], Daniel Bucio-Noble[1,2], Carly P. Rosewarne[3,4], Liisa Kautto[1,2], Malcolm S. Ball[5], Mark P. Molloy[1,2], Nicolle H. Packer[1,2], Ian T. Paulsen[1,2*]

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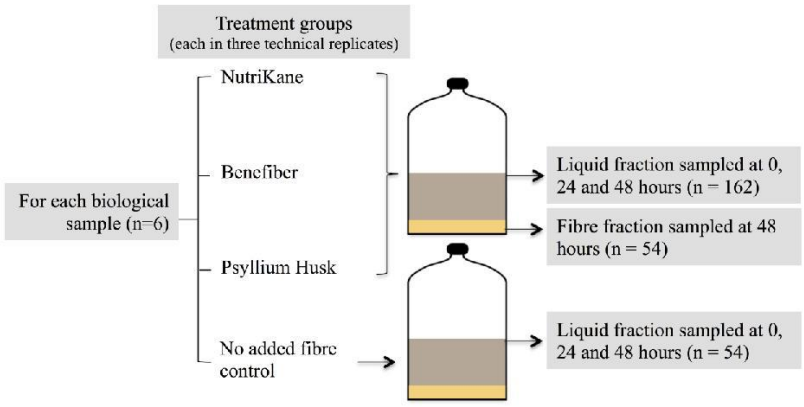
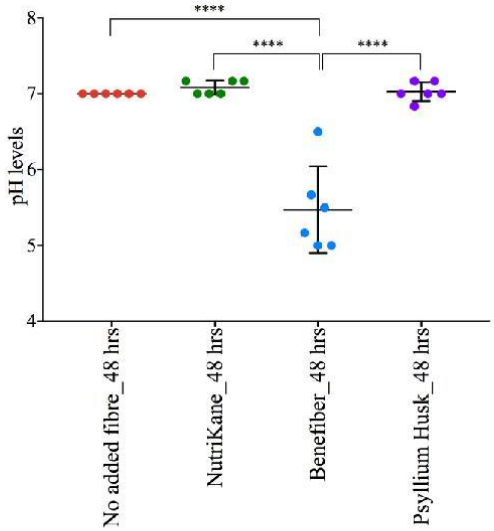
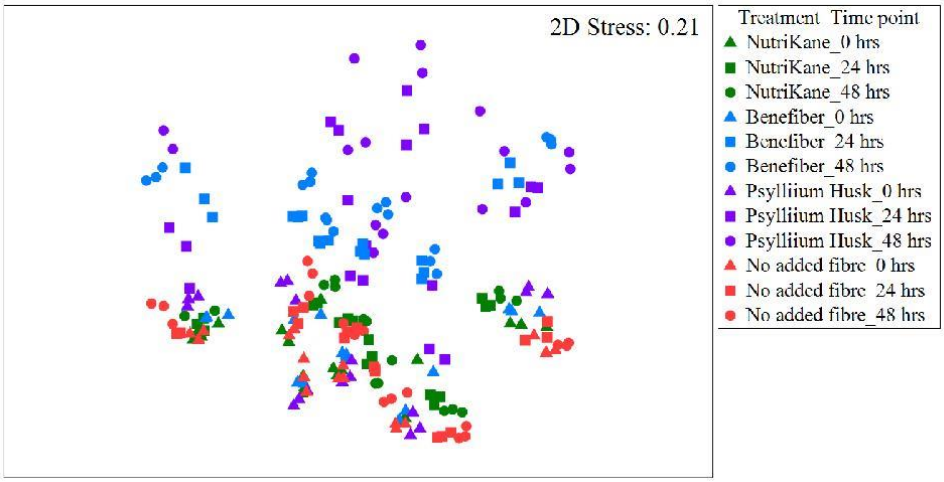
Supplementary Figures and table legends



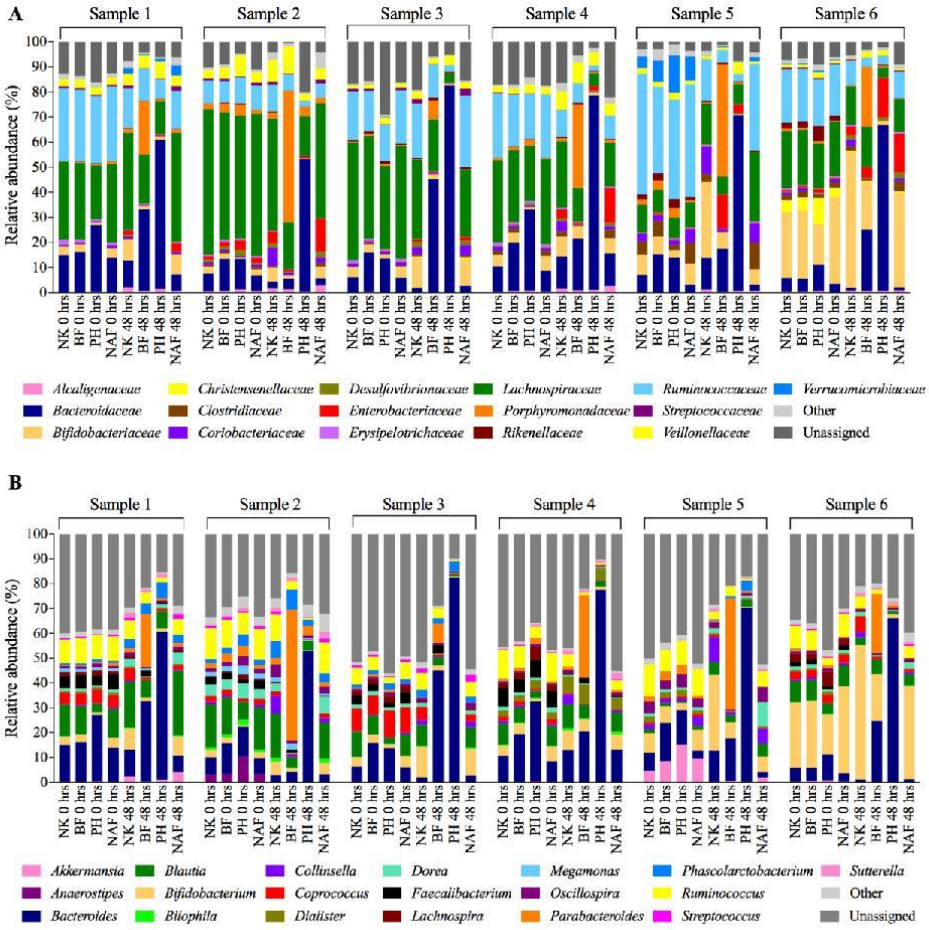
Supplementary Figure S1 Experimental design. Fecal material obtained from six healthy adults as independent biological samples (n=6) were inoculated separately into the basal medium. For each biological sample, four treatments were applied, this included three fibre products (NutriKane, Benefiber and Psyllium husk) and one 'no added fibre' control. Top liquid fraction of each culture was sampled at 0, 24 and 48 hours of incubation. At 48 hours the insoluble fibre fraction of cultures with the three fibre products were sampled separately. This resulted in a total of 216 samples from the liquid fraction and 54 samples from the fibre fraction.



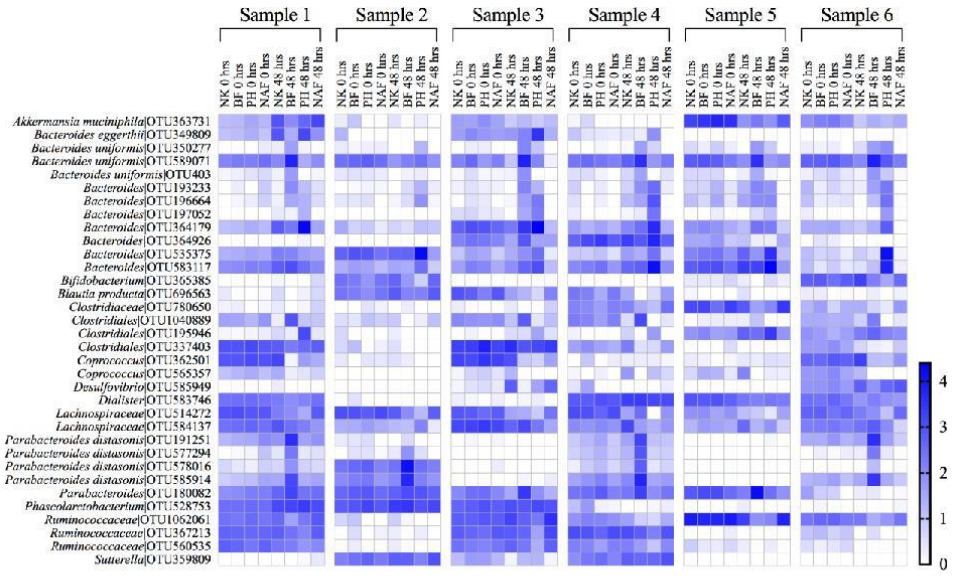
Supplementary Figure S2 Measurements of pH for all cultures at 48 hours. Mean pH values for each of the three technical replicates in each of the six biological samples are indicated (dots). Bars represent the mean pH levels with ± SD for each treatment. Significance was determined using ANOVA with Tukey's multiple comparisons test (**** $P < 0.0001$).



Supplementary Figure S3 Bray-Curtis similarity based nMDS plot indicating the ordination of the gut microbiota at 0, 24 and 48 hours for all six individuals. All biological samples showed similar fibre-dependent shifts in the microbial community structure. Samples at 0 hours were grouped together independent of fibre addition. Fibre-dependent shifts were observed at 24 and 48 hours.

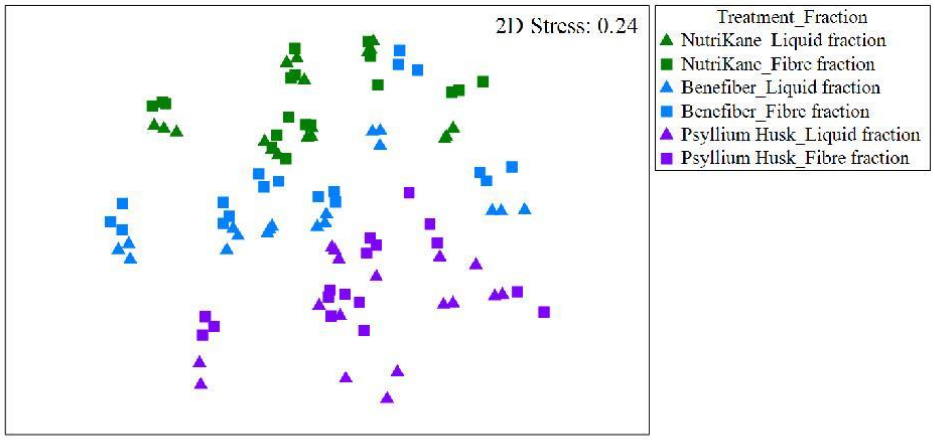


Supplementary Figure S4 (A) Family and **(B)** genus level taxonomic composition of the microbial communities across treatments at 0 and 48 hours for each biological sample (Sample 1-6). The relative abundance of 16S rRNA gene amplicons in the families and genera were determined using QIIME and graphed using GraphPad Prism (Version 7). Bacterial identifications that were not assigned to a family or genus are categorised as "Unassigned". Bacterial groups with a relative abundance < 2% in all the treatments at all time points are indicated as "Other". Column labels are abbreviated as, NK- NutriKane, BF- Benefiber, PH- Psyllium husk and NAF- No added fibre control at 0, 24 and 48 hours.

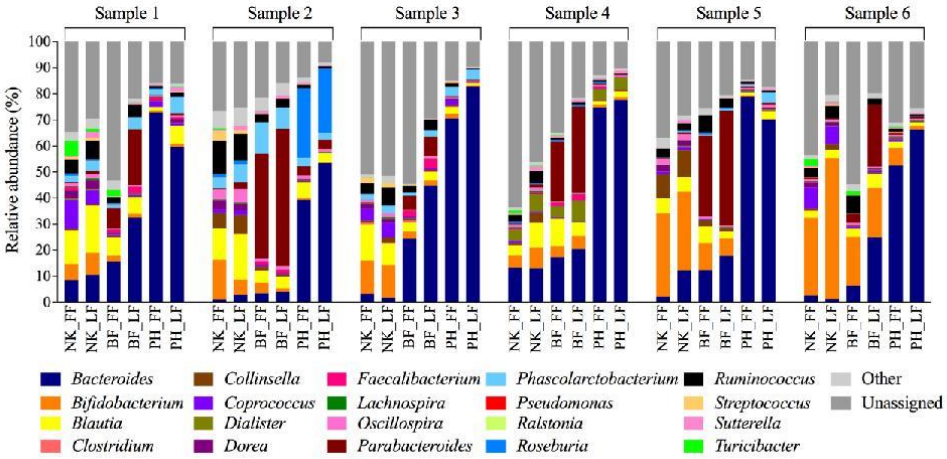


Supplementary Figure S5 Fibre-dependent changes in the relative abundance of differentially abundant OTUs in each treatment at 0 and 48 hours. This subset of OTUs were shown to have different relative abundances in each fibre treatment at 48 hours compared to the no added fibre control at equivalent time points, based on SIMPER analysis. Plotted data is Log_{10} transformed relative abundance of selected OTUs (rows) for each treatment condition per biological sample (Sample 1-6). The highest possible taxonomic identification is given before each OTU number. Blue and white denote highest and lowest relative abundance, respectively. The intensity of colours represents the level of the abundance as shown in the key. Columns are each fibre product treatment at 0 and 48 hours abbreviated as, NK- NutriKane, BF- Benefiber, PH- Psyllium husk and NAF- No added fibre control. The relative abundances and significance of these OTUs are provided in Table S2

Supplementary Figure S6 Key bacterial OTUs of the gut microbiota responding to fibre supplementations at 48 hours. Data was obtained using LEfSe analyses between **(A)** NutriKane vs no added fibre control, **(B)** Benefiber vs no added fibre control and **(C)** Psyllium husk vs no added fibre control. The histograms are based on the LDA scores computed for each bacterial OTU. The taxonomic identifications of these OTUs with the LDA scores are provided in Table S3. The relative abundance of all OTUs are provided in Table S4.



Supplementary Figure S7 Bray-Curtis similarity based nMDS plot indicating the ordination of the fibre-adherent and liquid gut microbiota fractions. The community of bacteria adhered to the fibre material was compared to that of the liquid fraction. The community structure between the fibre and liquid fraction were similar in all fibre products.



Supplementary Figure S8 Differences in the bacterial relative abundance between the fibre fraction (FF) and liquid fraction (LF) microbiota at the genus level. Data is shown for each biological sample (sample 1-6) with each fibre product (NK- NutriKane, BF- Benefiber, PH- Psyllium husk) at 48 hours. Bacterial identifications that were not assigned to a genus are categorised as "Unassigned". Bacterial groups with a relative abundance < 2% in all the treatments at all the time points are indicated as "Other".

Supplementary Figure S9 Significantly differentially abundant bacterial OTUs between the fibre (FF) and liquid fractions (LF) of the cultures at 48 hours. Data was obtained using LEfSe analyses between liquid and fibre fractions of **(A)** NutriKane, **(B)** Benefiber and **(C)** Psyllium husk. The histograms are based on the LDA scores computed for each bacterial OTU. The taxonomic identifications of these OTUs with the LDA scores are provided in Table S5. The relative abundance of OTUs are provided in Table S6.

Supplementary Table legends

Supplementary Table S1 Metadata of the six biological samples (sample 1-6). None of the volunteers had consumed antibiotics in at least three weeks prior to sample submission. All individuals consumed a non-specific omnivorous diet and had no existing medical conditions.

Supplementary Table S2 The relative abundance of specific OTUs that were found to contribute to fibre-specific microbiota alterations, based on SIMPER analysis. Mean ± SD for samples with NutriKane (NK), Benefiber (BF), Psyllium husk (PH) and no added fibre control (NAF) at 0 and 48 hours for each biological sample (sample 1-6) is shown. Significance was determined using Tukey's multiple comparisons tests with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ comparing fibre addition to the no added fibre control.

Supplementary Table S3 The OTUs that were significantly differentially abundant between each product and the no added fibre control at 48 hours. Data were obtained using LEfSe analysis between **(A)** NutriKane vs no added fibre control, **(B)** Benefiber vs no added fibre control and **(C)** Psyllium husk vs no added fibre control. The key OTUs with the taxonomic identifications and LDA scores are provided.

Supplementary Table S4 The abundance of the OTUs in cultures with each of the fibre additions (NutriKane (NK), Benefiber (BF) and Psyllium husk (PH)) and the no added fibre control (NAF) at 0, 24 and 48 hours (n=212). Data is shown for each biological sample (Sample 1-6).

Supplementary Table S5 The OTUs that were significantly differentially abundant between the fibre adherent and liquid fraction microbiota in cultures with each fibre product at 48 hours. Data were obtained using LEfSe analysis between the fibre adherent and liquid fraction microbiota of **(A)** NutriKane **(B)** Benefiber and **(C)** Psyllium husk. The key OTUs with the taxonomic identifications and LDA scores are provided.

Supplementary Table S6 The abundance of the OTUs in the fibre (FF) and liquid fraction (LF) microbiota of cultures with NutriKane (NK), Benefiber (BF) and Psyllium husk (PH) at 48 hours. Data is shown for each biological sample (Sample 1-6).

Supplementary Table S7 SCFA concentrations of each sample. Values are the average concentrations of the technical triplicates performed for each sample obtained across all the treatments, biological samples (Sample 1-6) and technical replicates. All values are expressed in $mmolL^{-1}g^{-1}$ with SD. ND = Not detected.

Supplementary Table S8 Antioxidant potential ($mmolL^{-1}$) and Polyphenolic content (mgL^{-1}) measurements for each biological sample (Sample 1-6). Measurements have been normalised against the no added fibre control. Mean values ± SD for technical replicates of NutriKane (NK), Benefiber (BF) and Psyllium husk (PH) at 0, 24 and 48 hours are provided.

Supplementary Table S1

Biological sample	Age (years)	Gender
Sample 1	20-30	Female
Sample 2	20-30	Male
Sample 3	45-60	Female
Sample 4	31-45	Male
Sample 5	20-30	Male
Sample 6	45-60	Female

3.3.2 Study 2: Effect of cereal products on SCFA production by the infant gut microbiota *in vitro*

To further explore the combination of the *in vitro* gut mimicking model, GC-FID analysis of SCFAs, and 16S rRNA sequencing as complimentary techniques to study the effect of diet on the gut microbiota, we participated in a secondary collaborative study with Hasinika Gamage. In this study, we investigated the effect of four cereal products derived from: 1) wheat; 2) sorghum; 3) rice; and 4) oats (**Table 3-3**), on the human infant gut microbiota. Each cereal product was digested using the *in vitro* gut mimicking system from the previous study then incubated with a faecal inoculum prepared from faeces obtained from six human infants of different ages, diets, and breast-feeding status (**Table 3-2**). As in the previous study, 2 mL samples of *in vitro* culture liquid supernatant were obtained at 0, 24, and 48 hours of incubation. SCFA concentrations in the liquid supernatants were determined by GC-FID.

Table 3-2. Metadata of infant faecal samples (Samples 1 – 6) (Gamage et al., 2017).

Sample	Age (months)	Frequency of breast feeding	Frequency of formula feeding	Types of solid food introduced	Medical conditions
1	5	Daily	None	Fruits, vegetables, grain, cereal, meat, eggs	None
2	5	None	Daily	Fruits, vegetables, grain, cereal,	None
3	5.5	Daily	None	Fruits, vegetables, grain, cereal, meat, eggs, dairy	None
4	7	Daily	Daily	Fruits, vegetables, grain, cereal, meat, eggs, dairy	Allergic to egg, spinach
5	9	Daily	Daily	Fruits, vegetables, grain, cereal, meat, eggs, dairy	None
6	11	None	Daily	Fruits, vegetables, grain, cereal, meat, eggs, dairy	None

Table 3-3. Ingredients and nutritional information of cereal products used in this study.

Cereal product	**Wheat**	**Sorghum**	**Rice**	**Oats**
Brand	Weet-Bix	Gluten free Weet-Bix	Bellamy's Organic baby rice cereal	Real good food Organic baby oat cereal
Ingredients	Wholegrain wheat (97%), Sugar, Salt, Barley malt extract, Vitamins (niacin, thiamine, riboflavin, folate), Mineral (iron)	Wholegrain sorghum (96%), Golden syrup, Salt Vitamins (E, niacin, thiamine, riboflavin, folate)	Organic rice (brown and white) (98.6%), Organic vegetable oil (1.2%), Mineral iron, Vitamin C, herb	Organic and biodynamic stoneground wholemeal oats (100%)
Dietary fibre per 100g	11.0 g	6.8 g	2.5 g	9.5 g
Nutritional information (average quantity per 100 g of dry cereal)	Energy 1480 kJ Protein, total 12.4 g Fat total 1.3 g (Saturated fat 0.3 g, polyunsaturated fat 0.8 g, monounsaturated fat 0.2 g), Carbohydrate, total 67 g Sugars 2.8 g Sodium 270 mg Iron 10 mg Potassium 340 mg Thiamine (Vitamin B1) 1.83 mg Riboflavin (Vitamin B2) 1.4 mg Niacin (Vitamin B3) 8.3 mg Folate 265 µg	Energy 1580 kJ Protein, total 12.3 g Fat total 3.6 g (Saturated fat 0.6 g, polyunsaturated fat 1.6 g, monounsaturated fat 1.4 g), Carbohydrate, total 69.7 g Sugars 2.2 g Sodium 235 mg Potassium 305 mg Thiamine (Vitamin B1) 1.83 mg Riboflavin (Vitamin B2) 1.43 mg Niacin (Vitamin B3) 8.3 mg Folate 265 µg Magnesium 107 mg Polyphenols total 221 Gallic acid equivalents	Energy 1679 kJ Protein 6.8 g Fat, total 4.0 g Carbohydrate total 87.2 g Sugars 0.3 g Sodium 22 mg Iron 21 mg Vitamin C 30 mg	Energy 1690 kJ Protein 10.6 g Fat, total 8.3 g Carbohydrate total 70.6 g Sugars 0.6 g Sodium 7 mg

Incubation with digested wheat, sorghum, rice, and oat cereal products significantly increased the production of total SCFAs in comparison with the faecal control (**Figure 3-5**). All cereal products produced a sharp increase in total SCFAs after 24 hours of incubation but no further increases in SCFA production were exhibited after 48 hours of incubation.

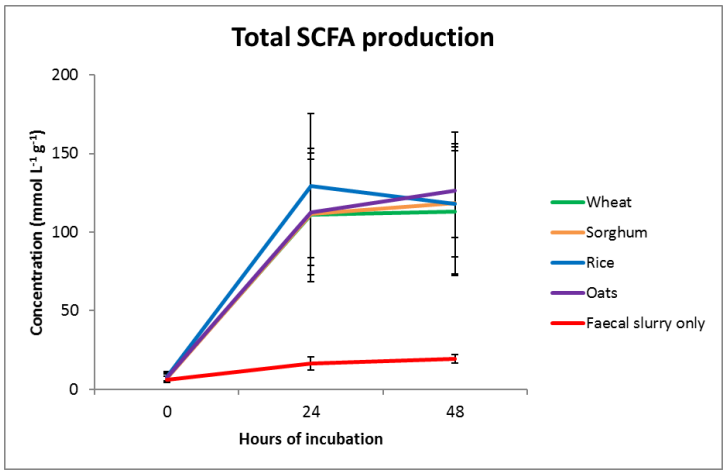


Figure 3-5. Total SCFA production following incubation of the infant faecal inoculum with cereal products. Data are mean ± standard deviation values calculated from six biological replicates.

All four cereal products produced almost identical fermentation profiles in both the quantity and proportions of SCFAs. Ace was the most abundant SCFA produced with concentrations increasing to 5.8 to 7.1 times higher than the faecal control after 24 hours of incubation (**Table 3-4**). Concentrations of Pro increased by 6.8 to 7.1 times the faecal control after 24 hours of incubation. Between 0 and 24 hours, concentrations of But increased by 3.5 and 6.6 times the faecal control. While concentrations of Ace and Pro increased significantly between 24 and 48 hours with oats, no increase was observed with wheat, sorghum or rice. By the end of the 48-hour incubation, Ace, Pro and But, were highest in oats, Ace and Pro were lowest in wheat, and But was lowest in Rice. The similarities in the proportions of Ace, Pro and But produced and total SCFA production suggest that the cereal products exhibited variable fermentabilities considering that each contained different quantities of dietary fibre (**Figure 3-6**). For example, rice, which was reported to contain 2.5g of dietary fibre, produced quantities of Ace and Pro that were not significantly different to oats despite having four times less dietary fibre.

Table 3-4. SCFA concentrations following incubation of the faecal slurry with cereal fibres

	Hours		
SCFA (mmol L^{-1} g^{-1})	0	24	48
Control			
Acetic acid	3.12 ± 1.1	8.41 ± 1.1[a]	9.33 ± 1.1[a]
Propionic acid	2.53 ± 0.9	6.82 ± 0.9[a]	7.56 ± 0.9[a]
Butyric acid	0.58 ± 0.1	1.71 ± 0.3[a]	2.44 ± 0.7[a]
Wheat			
Acetic acid	3.81 ± 1.7	55.5 ± 7.5[b]	55.6 ± 17[b]
Propionic acid	3.09 ± 1.4	44.9 ± 6.1[b]	45.1 ± 14[b]
Butyric acid	0.68 ± 0.3	7.96 ± 4.2[a]	12.6 ± 9.0[b]
Sorghum			
Acetic acid	3.63 ± 1.3	52.6 ± 7.4[b]	58.1 ± 19[b]
Propionic acid	2.94 ± 1.0	42.6 ± 6.0[b]	47.1 ± 15[b]
Butyric acid	0.63 ± 0.3	8.04 ± 4.3[b]	13.1 ± 10[b]
Rice			
Acetic acid	4.09 ± 1.5	59.4 ± 9.0[b]	59.6 ± 15[b]
Propionic acid	3.32 ± 1.2	48.2 ± 7.3[b]	48.3 ± 12[b]
Butyric acid	0.78 ± 0.2	6.11 ± 3.1[b]	10.1 ± 7.0[b]
Oats			
Acetic acid	3.87 ± 1.4	48.8 ± 4.7[d]	60.2 ± 13[b]
Propionic acid	3.14 ± 1.2	39.5 ± 3.8[c]	48.8 ± 11[b]
Butyric acid	0.69 ± 0.3	11.3 ± 3.2[c]	17.4 ± 6.0[c]

Data are mean ± standard deviation values calculated from six biological replicates
[a,b,c,d] Values within the same SCFA and column with different superscript letters are significantly different from each other using two-way ANOVA and Tukeys testing ($p < 0.05$). Differences in SCFA profiles at 0 hours are due to experimental variation.

Cereal fibres refer to fibres that originate from grains including barley, maize, millet, oats, rice, rye, sorghum, teff, triticale, and wheat varieties. Wheat (*Triticum aestivum* L.) is the most common grain used in bread, cakes and other baked goods and as such is a major source of dietary fibre depending on the use of white flour (2.5 – 4.5% dry wt.) or wholemeal (10 – 14% dry wt.). The main dietary fibre components of wheat are principally AX and β-glucan (Shewry et al., 2015). Sorghum (*Sorghum bicolor* L.) is an example of an ancient whole grain cereal that is more commonly used as an animal feed but has garnered recent interest as an alternative carbohydrate source (Stefoska-Needham et al., 2015). Dietary fibre content of sorghum ranges between 7.6 – 9.2% (dry wt.) and is comprised of cellulose, β-glucan, lignin, and insoluble glucuronoarabinoxylan (GAX), which are AXs substituted with glucuronic acid residues (Verbruggen et al., 1995).

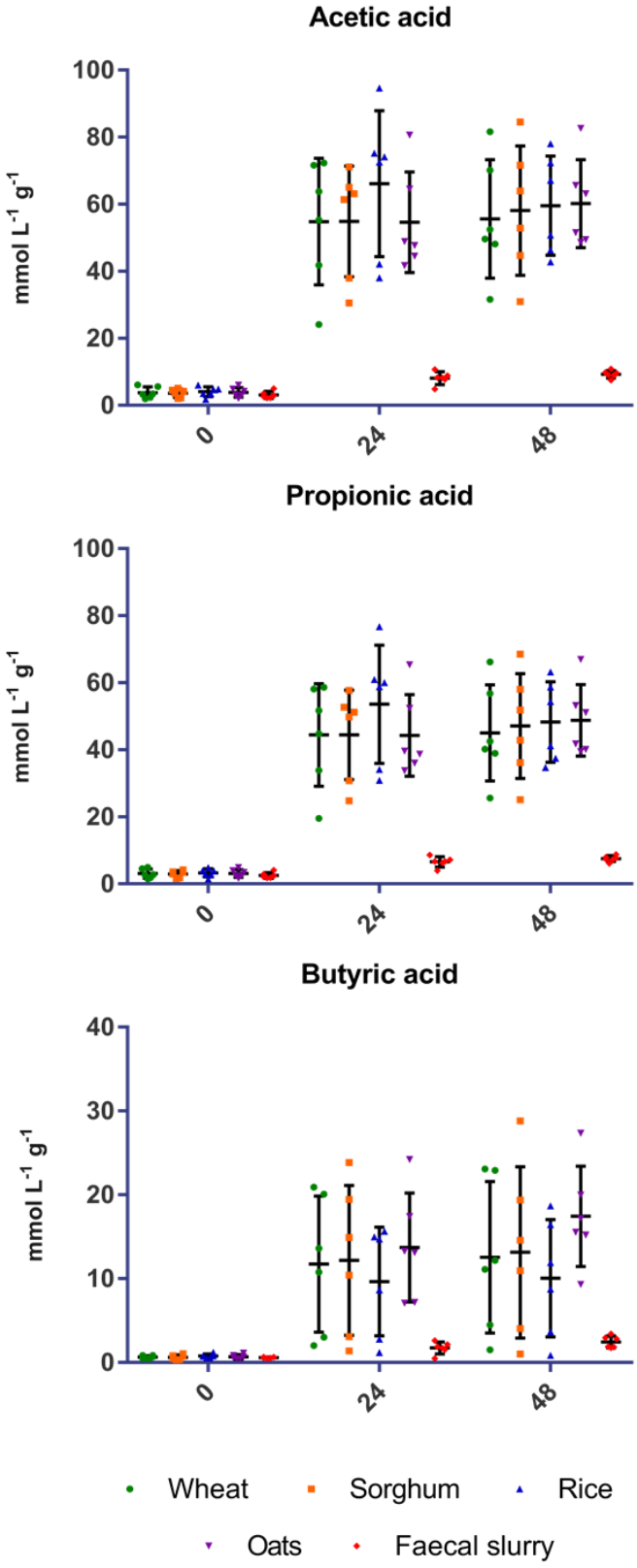


Figure 3-6. Concentration of acetic, propionic, and butyric acid over time in response to the addition of various cereal products to the in vitro gut mimicking system. Error bars represent the mean ± the SD, each point represents a different faecal sample. Significant differences were determined by two-way ANOVA and Tukeys testing (**$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Rice (*Oryza sativa* L.) is one of the oldest staple foods and is a major source of basic dietary nutrients. Rice bran consists of multiple layers of seed coat that contains mainly cellulose, lignin, and hemicellulose as dietary fibre components (Wang et al., 2016). Without the bran component, dietary fibre content of rice ranges widely between 0.28 – 3.49% (dry wt.) determined mainly by the breed and extent of processing (Sumczynski et al., 2015). Oats (*Avena sativa* L.) are another important staple food and are an important cereal crop in the developing world. Whole oats contain a significant amount of soluble (1-3)(1-4)β-glucan ranging between 2.3 – 8.5% (dry wt.) (Rasane et al., 2015).

Cereal grains are comprised of many different tissues and plant cell wall components thus different cereal species and processing conditions contribute to variations in dietary fibre content. The three main components of cereal products are starch, protein, and non-starch polysaccharides (NSP). Cereal NSPs include cellulose, arabinoxylan (AX), and β-glucan, which can vary significantly in proportion depending on the species of cereal and the part of the plant (Knudsen, 2014). While cellulose is generally less well fermented due to its insoluble nature, AX and β-glucan are both soluble linear polysaccharides that are readily fermented in the colon, (Salvador et al., 1993; Sirpa et al., 2000). The observed differences in SCFA production could be dependent on the compositional differences in dietary fibre components of each cereal fibre. The high total SCFA yielded from incubation with oats is supported by previous studies that have reported high quantities of soluble β-glucan in dietary fibre from oats (Belitz, 2009; Rose, 2014{Rasane, 2015 #1070)}. The high total SCFA yield of the comparatively lower fibre containing sorghum and rice products suggests a preference for plant cell wall components such as cellulose and hemicellulose since these components are shared between sorghum and rice and are less abundant in wheat and oats.

The similarities in SCFA production across all four cereal products was further surprising since each product exhibited significant variation in nutritional composition, particularly in protein, fat,

and carbohydrate content (**Table 3-3**). While the work of Blümmel et al. (1999) found no influence of protein or fat content on the stoichiometric relationship between SCFA production and gas production *in vitro*, the presence of these components can potentially impact SCFA production by the dilution of highly fermentable carbohydrates.

While mean SCFA production was almost identical in response to the four cereal product incubations, there was greater individual variation between the six faecal inoculates, as shown in **Figure 3-7**. Since each faecal sample was collected from an infant with different age, diet, and breast-feeding status, these results suggest that different microbiota compositions may be better adapted to ferment each cereal product resulting in the variation in individual SCFA profiles. Sequencing of 16S rRNA showed that each faecal sample comprised a unique initial microbial composition (Gamage et al., 2017), which is consistent with other studies that have found variations in microbial composition and consequently SCFA production as a result of differences in diet, breast feeding and formula feeding status (Bridgman et al., 2017; Fan et al., 2014). Our results demonstrate that cereal products derived from wheat, sorghum, oats, and rice induced identical increases in SCFA production when incubated with faecal samples obtained from human infants. The results of this study are included in the following publication that was prepared in collaboration with Hasinika K. A. H. Gamage and are discussed in conjunction with the changes in microbial community composition in response to incubation with cereal products.

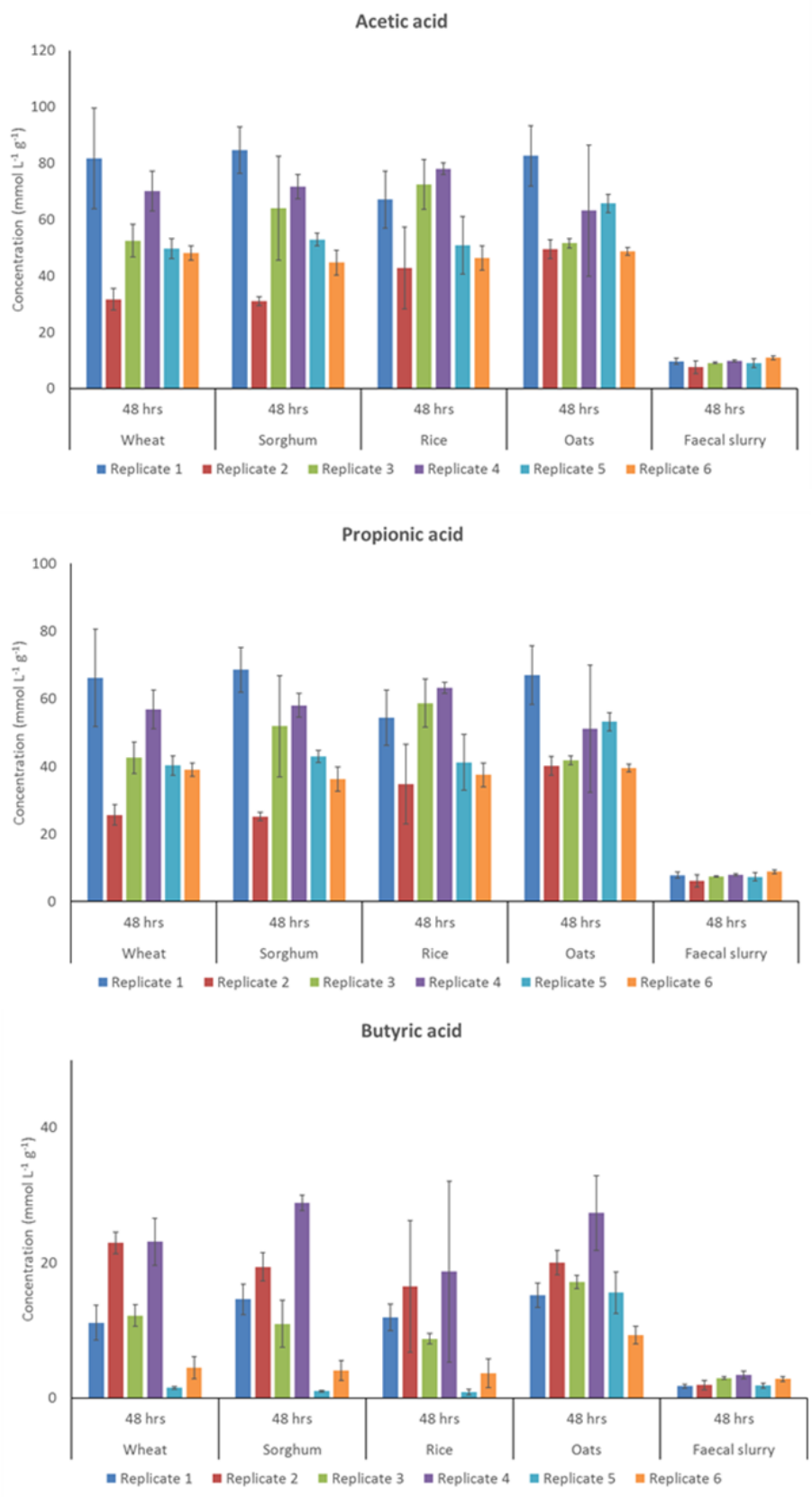


Figure 3-7. Concentration of acetic, propionic, and butyric acid after 48 hours of incubation with various cereal products. Each bar represents a different infant faecal sample. Error bars show the standard deviation of 9 technical replicates.

3.3.2.1 Effect of cereal products on the human infant gut microbiota (Paper 3)

Accepted Manuscript: Gamage, H.K.A.H., S.G. Tetu, **R.W.W. Chong**, J. Ashton, N.H. Packer, and I.T. Paulsen. (2017). *Cereal products derived from wheat, sorghum, rice and oats alter the infant gut microbiota in vitro.* Scientific Reports. 7:14312.

The following paper includes changes in gut microbiota composition, as determined by DNA sequencing, together with the changes in SCFAs produced in response to the addition of cereal products using the *in vitro* gut mimicking model. The *in vitro* digestion, culturing, 16S rRNA extraction, 16S rRNA sequencing, and bioinformatic analysis was performed by H. K. A. H. Gamage. Quantitative SCFA measurements were performed by me (R. W. W. Chong) using the method optimised in this chapter. H.K.A.H. Gamage, R.W.W. Chong, S.G. Tetu, J. Ashton, N. H. Packer and I. T. Paulsen were involved in the experimental design and interpretation of data. This paper has been published in Scientific Reports (2017) of the Nature Publishing Group.

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Cereal products derived from wheat, sorghum, rice and oats alter the infant gut microbiota *in vitro*

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The introduction of different nutrient and energy sources during weaning leads to significant changes in the infant gut microbiota. We used an *in vitro* infant digestive and gut microbiota model system to investigate the effect of four commercially available cereal products based on either wheat, sorghum, rice or oats, on the gut microbiota of six infants. Our results indicated cereal additions induced numerous changes in the gut microbiota composition. The relative abundance of bacterial families associated with fibre degradation, *Bacteroidaceae*, *Bifidobacteriaceae*, *Lactobacillaceae*, *Prevotellaceae*, *Ruminococcaceae* and *Veillonellaceae* increased, whilst the abundance of *Enterobacteriaceae* decreased with cereal additions. Corresponding changes in the production of SCFAs showed higher concentrations of acetate following all cereal additions, whilst, propionate and butyrate varied between specific cereal additions. These cereal-specific variations in the concentrations of SCFAs showed a moderate correlation with the relative abundance of potential SCFA-producing bacterial families. Overall, our results demonstrated clear shifts in the abundance of bacterial groups associated with weaning and an increase in the production of SCFAs following cereal additions.

The human gut microbiota co-develops with the host in early life[1]. Initial microbial colonisation of the gut depends on various maternal and postnatal factors such as *in utero* environment, mode of delivery (vaginal or caesarean-section), gestational age, environment, antibiotic treatments, host genetics and diet (breast milk, formula milk or solid food)[2–6]. These factors shift the composition and functions of infant gut microbiota towards an established adult-like status within the first three years of life[6]. The adult gut microbiota is relatively more stable, higher in species diversity and lower in inter-individual compositional and functional variations compared to infants[3,7]. Accumulating data suggest a link between early life gut microbial colonisation and development of diseases, such as obesity, diabetes (type 1 and 2), food allergies and inflammatory bowel disease[8–15]. Therefore, establishment of the gut microbiota during infancy and maintenance thereafter likely plays a critical role for human health[2].

The transition to solid food contributes significantly towards the infant gut microbiota development. This introduces infants to a larger range of plant and animal polysaccharides. Due to the lack of enzymes to digest most of these complex polysaccharides, infants largely depend on the gut microbiota to digest these otherwise non-digestible carbohydrates[16]. Therefore, weaning rapidly diversifies and alters the composition of the gut microbiota towards an adult-like composition, presumably to facilitate the metabolism of changing nutrients[5]. Exposure to new nutrients also leads to altered functions in the microbiota and production of different bacterial metabolites. For instance, introduction to solid food and more xenobiotics promote the growth of bacterial species associated with carbohydrate utilisation, vitamin biosynthesis and xenobiotic degradation[3,7]. Weaning is also linked with elevated levels of bacterial metabolic end products such as short chain fatty acids (SCFAs), possibly due to the high availability of non-digested dietary fibre[7,17].

A number of previous studies have examined changes in the infant gut microbiota in relation to transition to solid food. Weaning in general is associated with decreased proportions of *Bifidobacteria*, *Enterobacteria* and some groups of *Clostridium*, whilst it promotes the growth of *Bacteroidetes*[3,7,18,19]. The increase in *Bacteroidetes* could be due to their ability to digest a broad range of complex polysaccharides[7]. Very few *in vivo* studies have investigated the effect of specific dietary regimens on infant gut microbiota during the weaning phase. One such

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Biological sample	Age (months)	Frequency of breast feeding	Frequency of formula feeding	Types of solid food introduced	Medical conditions
Sample 1	5	Daily	None	Fruits, vegetables, grain, cereal, meat, eggs	None
Sample 2	5	None	Daily	Fruits, vegetables, grain, cereals	None
Sample 3	5.5	Daily	None	Fruits, vegetables	None
Sample 4	7	Daily	Daily	Fruits, vegetables, grain, cereals, meat, eggs, dairy	Food allergies*
Sample 5	9	Daily	Daily	Fruits, vegetables, grain, cereals, meat, eggs, dairy	None
Sample 6	11	None	Daily	Fruits, vegetables, grain, cereals, meat, eggs, dairy	None

Table 1. Metadata of the six biological samples (Sample 1–6). None of the infants were given antibiotics in at least three months prior to fecal sample submission. *Egg and spinach allergies.

study reported the effects of feeding infants with commercially available pureed meat, iron- and zinc-fortified cereals or iron-only fortified cereals on the gut microbiota[20]. Infants fed pureed meat demonstrated enriched *Clostridium* group XIVa, whilst feeding iron-only fortified cereals resulted in decreasing the abundance of *Lactobacilli* and *Bifidobacterium* and promoting the abundance of *Bacteroides*[20].

Utilisation of *in vitro* models of the infant gut microbiota eliminates some of the issues associated with *in vivo* studies. *In vitro* studies reduce issues with ethical restrictions and volunteer compliance, while enabling more frequent sampling and providing a simplified system to study the gut microbiota without host interference[21]. *In vitro* infant gut microbiota model systems have been employed to investigate the effect of probiotics[22], candidate probiotics[23], milk lipid hydrolysis products[24], iron[25], milk oligosaccharides[26–28], dietary polysaccharides and prebiotics[29–31]. Addition of short-chain fructo-oligosaccharides into an *in vitro* model of infant gut microbiota resulted in an increased abundance of the genus *Lactobacillus* while reducing the proportion of coliforms[29]. Shen *et al.* observed an increase in *Bifidobacterium* and *Bacteroides* upon addition of a prebiotic mixture of fructo-oligosaccharides and galacto-oligosaccharides into an *in vitro* model of infant gut microbiota[30].

Lack of dietary fibre in modern Western diets has been associated with changing the gut microbiota composition, functions, diversity and spatial arrangement[32–35]. Bridging this gap in dietary fibre intake is of increasing interest as a therapeutic modulation of the gut microbiota in order to improve metabolic and inflammatory health[36]. Whole grain products generally contain a high amount of dietary fibre[37]. Although, whole grain cereals are among frequently introduced first food to infants[15], the impact of cereals on infant gut microbiota is less well studied. In adults, consumption of whole grain maize based breakfast cereal promoted the growth of *Bifidobacterium*[38], whilst whole grain wheat cereal increased the abundance of *Bifidobacterium* and *Lactobacillus*/*Enterococcus* groups[39]. Consumption of whole grain barley and brown rice flakes increased the microbial diversity and reduced host markers associated with inflammation and postprandial glucose levels[40].

Given the increasing popularity of whole grain cereals as an early weaning food and the impact on gut microbiota and disease development[15], we chose to examine the effects of whole grain-based cereal products on the gut microbiota of infants. In this work, we investigated the effect of four commercially available cereal products, Weet-Bix™, Gluten free Weet-Bix™, Bellamy's organic baby rice cereal and Real good food-Organic baby oat cereal on infant gut microbiota and SCFAs using an *in vitro* infant gut microbiota model system.

Results and Discussion

Samples of four commercially available cereal products (wheat, sorghum, rice and oats based) were treated using a series of pH controlled enzyme additions and a dialysis step to simulate infant digestion. Digested cereal products were introduced into an anaerobic basal medium to examine the effects of the cereal products on the infant gut microbiota. The basal growth medium without any cereal addition was run in parallel as a control, this is referred to as the no added cereal control. All cereal and control cultures were inoculated independently with fecal homogenate obtained from a healthy infant. A total of six biological samples (one each from six different infants) were analysed. Cultures were sampled at 0, 24 and 48 hours and V4 region amplicons of the 16 S rRNA gene were sequenced. A total of 21,231,850 reads were generated. After quality filtering and rarefaction 35,095 reads per each of the 270 samples were used for further analyses (270 = 6 biological samples × 3 time points × 3 technical replicates for 5 experimental groups including 4 cereal treatments and no added cereal control).

Each biological sample had a unique initial gut microbial composition. The bacterial phyla *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* dominated the gut microbiota of all infants at 0 hours. However, the relative abundance of these phyla differed between individuals. Similar variations in the composition were observed at a family level (Supplementary Fig. S1). The relative abundance of the family *Veillonellaceae*, which is associated with milk polysaccharide digestion was significantly higher ($P < 0.05$) in breast-fed infants compared to the formula-fed (Table 1). In agreement with our observation, Fan *et al.* 2014 have found a higher abundance of *Veillonellaceae* in breast-fed infants compared to that in formula or mixed-fed infants[41].

The relative abundance of the family *Lachnospiraceae* was significantly higher ($P < 0.001$) in older infants (age > 6 months) compared to younger infants (age < 6 months). Samples obtained from older infants (age > 6 months) had a higher relative abundance of known plant polysaccharide digesting bacteria such as

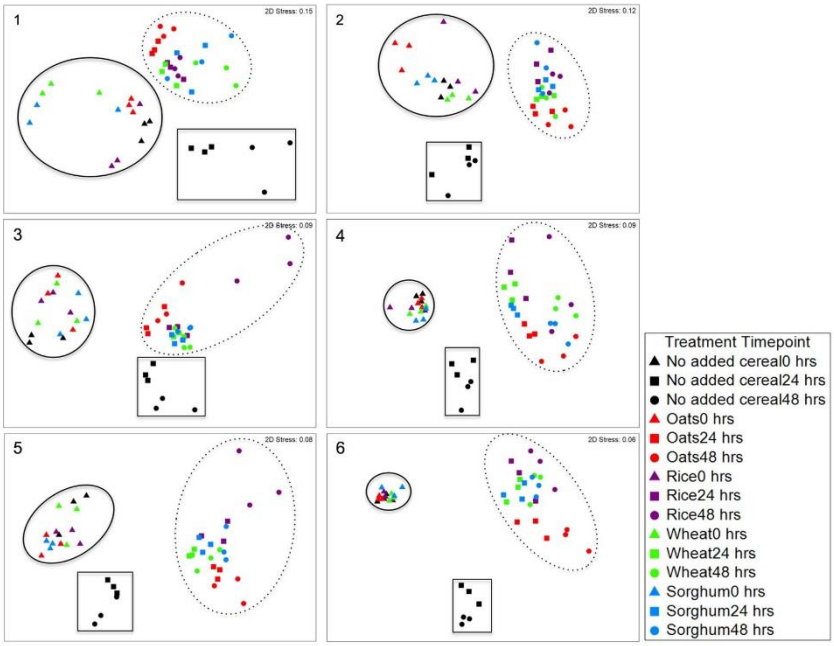

Figure 1. Ordination of the gut microbiota in each biological sample (1–6) at 0, 24 and 48 hours. Data is shown as Bray-Curtis similarity of Log (X + 1) transformed relative abundance based nMDS plots. Treatments and time points are colour coded as shown in the legend. All cereal additions shifted the community structure at 24 and 48 hours (dotted line circle) compared to the samples at 0 hours (solid line circle) and no added cereal control at 24 and 48 hours (solid line square).

Lachnospiraceae, *Ruminococcaceae* and *Bacteroidaceae*. Differences in the abundance of these bacterial families in the infant gut microbiota due to age are largely in agreement with previous studies[7,16,19,42,43].

Some bacterial families were highly variable between individuals. This is expected given that the composition of the infant gut microbiota varies depending on factors such as the mode of delivery (vaginal or caesarean section birth), usage of antibiotics, age, diet (breast milk or formula milk) and exposure to solid food[1,2,5]. The family *Coriobacteriaceae* was abundant in sample 2 (9.8%), 4 (4.1%) and 5 (14.4%) and not observed above 0.3% in other biological samples. The relative abundance of *Porphyromonadaceae* (12.2%) was high in sample 1, whilst sample 2 had a large proportion of *Enterococcaceae* (18.4%). In sample 4 *Streptococcaceae* was abundant (9%) and *Ruminococcaceae* was abundant in sample 6 (19.8%). The oldest biological sample (sample 6) showed the lowest relative abundance of *Enterobacteriaceae* (0.7%), whilst the lowest abundance of *Bacteroidaceae* (0.3%) was observed in biological sample (sample 3), obtained from an infant that had not been exposed to cereal grains.

All cereal additions altered the gut microbial composition. To determine the impact of different cereal additions on the gut microbiota at 0, 24 and 48 hours, non-metric multidimensional scaling (nMDS) plots were constructed based on the relative abundances of the Operational Taxonomic Units (OTUs) (Fig. 1). Samples at 0 hours in each biological sample clustered relatively close together irrespective of the treatments. All cereal additions resulted in different microbiota community structures at 24 and 48 hours compared to the samples at 0 hours and no added cereal control at 24 hours (global analysis of similarities (ANOSIM) $R > 0.7$, $P < 0.0001$) and 48 hours (global ANOSIM $R > 0.8$, $P < 0.0001$). The microbiota community structure of the no added cereal control also changed over time, however these remained distinct from the communities after cereal addition. The cereal additions showed similar shifts to each other in the nMDS plots (Fig. 1), and consistent with this there were no statistically significant differences in the microbial community structure between the cereal products.

The bacterial diversity in each sample was determined using a Shannon diversity index. Biological sample 3 had a significantly ($P < 0.0001$) lower Shannon index (4.6 ± 0.05) at 0 hours compared to all other biological samples (Shannon diversity index of samples 1, 2, 4, 5 and 6 ranged from 4.8 ± 0.1 to 5.0 ± 0.04). The low Shannon index value in sample 3 was primarily due to the dominance of a single OTU of the common infant gut bacterium, *Veillonella dispar* (OTU 585419, relative abundance at 0 hours: $43.6\% \pm 7.3\%$). The diversity indices between the treatments were similar at 48 hours in all biological samples, except for sample 3. The diversity of this sample increased significantly at 48 hours with the addition of rice ($P < 0.0001$).

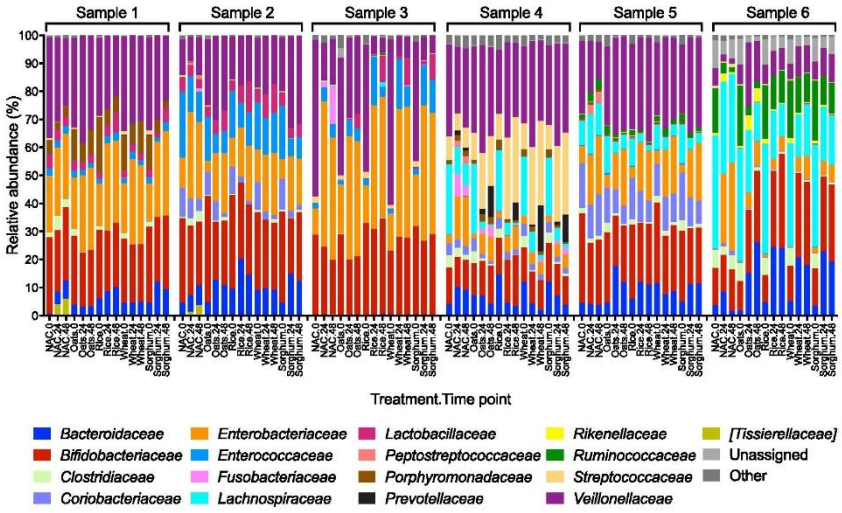

Figure 2. Family level taxonomic compositions of the microbial communities for each biological replicate. The relative abundances of the families were determined using QIIME and GraphPad Prism (V7). Each bar is labelled first by treatment, followed by time point (0, 24 and 48 hours). No added cereal control is abbreviated as NAC. Major bacterial families are shown in different colours as indicated in the legend. Bacterial identifications that were not assigned to a family are categorised as "Unassigned". Bacterial families that were not significantly differentially abundant comparing the treatment regimes in any of the six biological samples are categorised as "Other". Significance ($P < 0.05$) was determined using a Tukey's multiple comparisons test.

The relative bacterial abundance was examined at a family level and identifications across the samples were assigned into 33 bacterial families. Statistically significant differences in family abundance across the treatments in each biological sample were investigated using a two-way analysis of variance (ANOVA) test with Tukey's multiple comparisons test. This identified 17 families with significantly ($P < 0.05$) different abundances in at least one treatment and time point combination (Fig. 2, Supplementary Fig. S2 and Supplementary Table S2). The impact of cereal additions on the microbiota composition was highly variable between the biological samples. However, for each biological sample the relative abundance of at least one potential Carbohydrate-active enzymes (CAZymes) producing bacteria (families: *Bacteroidaceae*, *Bifidobacteriaceae*, *Lactobacillaceae*, *Prevotellaceae* and *Ruminococcaceae*) increased with addition each of the tested cereal products.

For three of the six biological samples the relative abundance of *Bacteroidaceae* increased upon addition of each of the four cereal products with the highest increase ($P < 0.01$) following addition of rice. In contrast, in biological sample 4 the relative abundance of this family significantly decreased ($P < 0.001$), while the abundance of *Prevotellaceae* significantly increased ($P < 0.001$) following all cereal additions. The families *Bacteroidaceae* and *Prevotellaceae* are members of the phylum *Bacteroidetes*, which are generally reported to degrade a wide range of dietary polysaccharides, due to their capacity to switch between energy sources depending on the availability[44–47].

In all biological samples, the abundance of *Veillonellaceae* was significantly higher ($P < 0.05$) with addition of oats compared to all other treatments. For four out of the six samples, the abundance of this family also increased after the addition of all other cereals. Family *Veillonellaceae* is associated with utilising partial breakdown products of bacterial polysaccharide digestion and producing propionate and acetate, likely due to its limited ability to digest complex carbohydrates[18,48–51]. Previous studies have also reported an increase in the abundance of this family following *in vitro* fermentation of specific complex polysaccharides by the infant gut microbiota[27,31].

The relative abundance of *Enterobacteriaceae* decreased following addition of each of the four cereal products, with the exception of biological sample 1, where this family significantly increased ($P < 0.0001$). According to previous studies, *Enterobacteriaceae* are usually more dominant in pre-weaned gut microbiota of younger infants and become less abundant due to weaning and age[3,19,52–54]. Therefore, the decrease in the abundance of *Enterobacteriaceae* with cereal supplementations may indicate the ability of the cereal products to aid the shift of the infant gut microbiota towards a mature status.

The relative abundance of *Bifidobacteriaceae* was significantly higher ($P < 0.0001$) with the addition of rice compared to other treatments. The family *Lactobacillaceae* was abundant in younger infants (age < 6 months) and the relative abundance significantly increased ($P < 0.05$) upon addition of rice. Previous observations of higher growth of *Bifidobacteriaceae* and *Lactobacillaceae* in the gut microbiota of adults and animal models upon addition of cereal grains[38–40], particularly, brown rice[55–58] are also in agreement with our results.

All four tested products have been obtained from cereal grains, which are naturally high in complex sugars such as starch, cellulose, arabinoxylans and glucofructans[59], while oats are particularly rich in β-glucans[59]. The

prevalence of *Bacteroidaceae*, *Bifidobacteriaceae*, *Lachnospiraceae* and *Lactobacillaceae* in all cereal additions is consistent with the ability of the members of these families to digest cellulose, starch and other polysaccharides[45,60]. The composition of the four tested cereal products varied in regards to dietary fibre, protein, iron, polyphenols and vitamins (Supplementary Table S1). Wheat and rice based cereal products are particularly rich in iron, which has been previously demonstrated to increase the abundance of enteropathogens in the family *Enterobactericeae* and modulate butyrate-producing bacteria[25,61,62]. While we did not observe significant changes in the abundance of the *Enterobactericeae* or butyrate-producing bacteria in samples with wheat supplementation, the abundance of potential butyrate-producing bacteria *Bacteroidaceae* and *Bifidobacteriaceae* was higher upon rice supplementation.

Biological sample 4, obtained from an infant who suffered from food allergies, showed a considerably higher initial relative abundance of *Streptococcaceae* than other biological samples. This sample displayed a notable expansion in the relative abundance of *Steptococcaceae* ($P < 0.0001$) upon all cereal additions, this family showed less than 1.0% relative abundance in other biological samples. As this is only a single individual, we cannot directly link the high abundance of the *Streptococcaceae* to the food allergies experienced by this individual. However, a high abundance of *Streptococcus* spp. in late infancy has been reported to be linked to allergic disease development[63].

Microbial composition was also studied at OTU level and significant differences were determined using a two-way ANOVA with Tukey's multiple comparisons test. Six OTUs that showed significantly different abundances ($P < 0.01$) between the treatments in at least three biological samples were identified (Supplementary Fig. S3 and Supplementary Table S3). All of these OTUs belonged to the families that are discussed above and showed similar trends in the relative abundances in each treatment.

Cereal products increased SCFA production. To investigate the effect of cereal addition on production of SCFAs, acetate, butyrate and propionate concentrations were measured from the samples collected at 0, 24 and 48 hours (Fig. 3, Supplementary Table S4). Addition of each of the four cereal products resulted in significantly higher ($P < 0.01$) concentrations of acetate across all biological samples at 24 and 48 hours compared to the no added cereal control.

Production of butyrate was higher following addition of wheat or sorghum for all biological samples except sample 3 at 48 hours. Butyrate was highly produced upon addition of rice and oats in at least four biological samples compared to the no added cereal control, however the increase in butyrate production upon supplementation with cereal products was statistically significant for only two biological replicates. Concentration of propionate was significantly higher ($P < 0.01$) in all cereal additions in biological samples 1, 2, 4 and 5 at 48 hours compared to the no added cereal control. The addition of oats significantly increased ($P < 0.05$) the concentration of propionate in biological samples 1, 3 and 6 compared to all other cereal additions.

The concentration of all three SCFAs positively correlated with the relative abundance of *Bacteroidaceae* (Spearman's $r = 0.21$, $P < 0.001$), whilst the concentration of acetate positively correlated with *Lactobacillaceae* (Spearman's $r = 0.22$, $P < 0.0001$) and concentration of propionate positively correlated with the relative abundance of *Veillonellaceae* (Spearman's $r = 0.20$, $P < 0.0001$). Each of these families are known to produce SCFAs[7,27,29,31,64]. Higher production of SCFAs with cereal additions is in agreement with a number of previous studies that have also demonstrated an increase in the production of SCFAs upon gut microbial fermentation of cereal grains[65–67]. Furthermore, elevated production of SCFAs is also a characteristic weaning induced change in the infant gut microbiota during maturation to an adult-like composition[7].

The pH of each of the culture vials with cereal additions at 48 hours showed significant reductions ($P < 0.001$) compared to the no added cereal control, which maintained the pH at the starting measurement of 7.0 ± 0.2 (Supplementary Fig. S4). Samples with rice demonstrated significantly lower ($P < 0.001$) pH levels compared to samples with oats, wheat and sorghum. pH has been previously been shown to impact gut microbiota composition, especially inhibiting the growth of pathogenic *Escherichia coli*[68]. The metabolic activities of the major SCFA producing bacterial groups such as *Bacteroidaceae*, *Bifidobacteriaceae* and *Lactobacillaceae* have previously been reported to reduce the pH in the large intestine[69,70]. The higher abundance of at least one of these SCFA producing bacterial families and lower abundance of the family *Enterobactericeae* upon addition of all tested cereal products may be linked with the reduction in the pH.

Predicted functional changes in response to cereal products. In order to investigate the effect of cereal additions on the functions of the gut microbiota, the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology functional profiles in each treatment at 0, 24 and 48 hours were inferred from the 16 S rRNA gene abundances using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt). This analysis predicted 12 functional pathways to be significantly differentially abundant in at least five biological samples following cereal additions (Fig. 4 and Supplementary Table S5). Based on the PICRUSt analyses, the functional category of fructose and mannose metabolism showed significantly decreased relative abundance ($P < 0.05$) in samples supplemented with oats. The Phosphotransferase system (PTS) functional category, responsible for membrane transport of simple carbohydrates, was significantly reduced ($P < 0.01$) in samples supplemented with oats, rice and wheat. There was a good positive correlation between the inferred relative abundance of the fructose and mannose metabolism functional group with the PTS functional group (Spearman's $r = 0.54$, $P < 0.0001$), this is consistent with the primary uptake mechanism for fructose and mannose being via PTS transporters[71]. The reduction in the inferred relative abundance of these two functional pathways upon cereal addition could be linked to the addition of more complex sugars such as starch, hemicellulose, cellulose and other polysaccharides from the cereal products. Previous studies have also demonstrated a decrease in the PTS and fructose and mannose metabolism in adults and animal models upon consumption of dietary fibre[72,73].

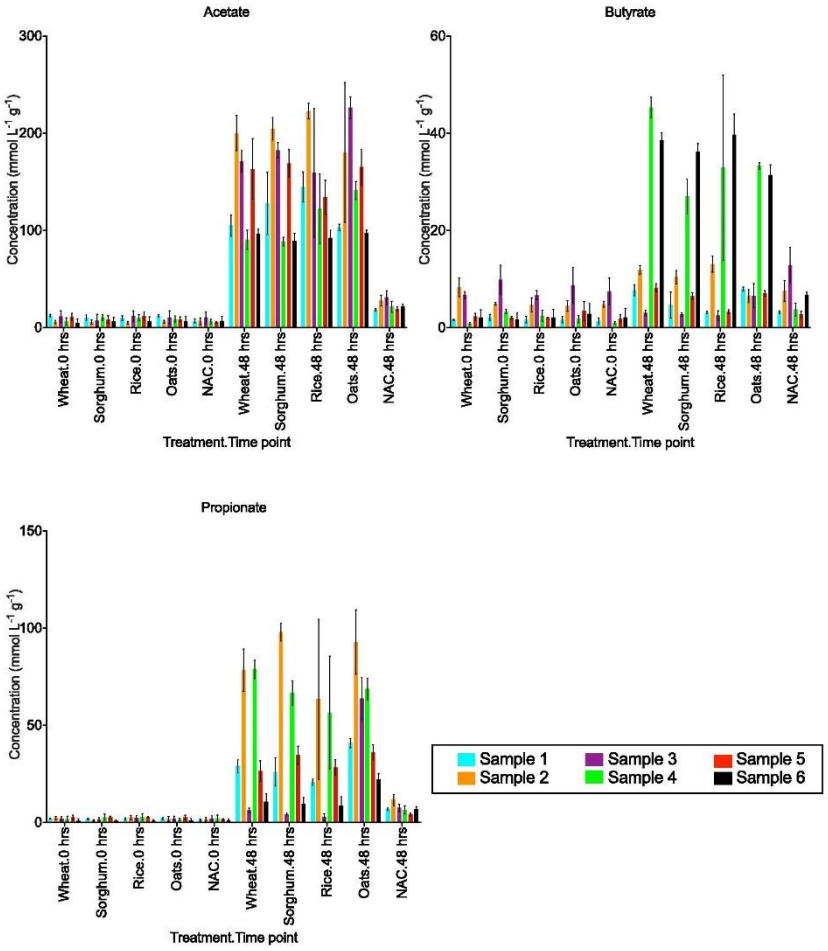

Figure 3. Concentration ($mmolL^{-1}g^{-1}$) of acetate, butyrate and propionate in each treatment at 0 and 48 hours. Concentration measurements at 24 and 48 hours for all three SCFAs were similar, therefore only 48 hours are shown. Mean ± SD concentration for all treatments with each biological sample (sample 1–6) denoted by colour-coded bars. No added cereal control is abbreviated as NAC. The concentrations and results of ANOVA with Tukey's multiple comparisons test for significance are provided in Supplementary Table S4.

The inferred relative abundance of functional pathways for glycan degradation increased ($P < 0.05$) following addition of the cereal products, which could be linked to the presence of plant protein N-linked glycans, due to the availability of glycoproteins in all cereals[74]. The relative abundance of this pathway correlated with the abundance of *Bacteroidaceae* (Spearman's $r = 0.51$, $P < 0.0001$). The correlation between glycan degradation and the family *Bacteroidaceae* is in line with the known ability of this family to digest a range of glycans[44,75]. Similar changes in these pathways have also been previously observed in animal models such as piglets, upon introduction to solid food[76].

The inferred abundance of functional groups for lipopolysaccharide biosynthesis significantly increased ($P < 0.05$) upon addition of oats, wheat and sorghum, with the highest increase observed with the addition of oats. The inferred relative abundance of this pathway correlated with the relative abundance of the Gram-negative *Veillonellaceae* (Spearman's $r = 0.47$, $P < 0.0001$), and negatively correlated with the Gram-positive *Lachnospiraceae* (Spearman's $r = -0.56$, $P < 0.0001$), *Rikenellaceae* (Spearman's $r = -0.51$, $P < 0.0001$) and *Ruminococcaceae* (Spearman's $r = -0.60$, $P < 0.0001$). This is in agreement with the occurrence of lipopolysaccharides in Gram-negative bacterial cell wall[77,78].

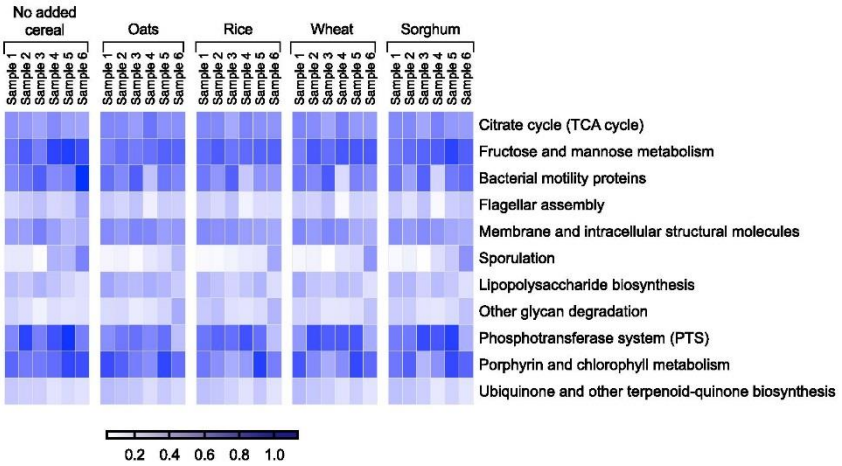

Figure 4. The predicted relative abundance of KEGG Orthology pathways for each sample with different cereal additions inferred using PICRUSt. The heat map shows the relative abundance of KEGG Orthology pathways (rows) with significant differences between treatments at 48 hours (columns) in at least five biological replicates. Significance was determined using an ANOVA with Tukey's multiple comparisons test. Biological samples (Sample 1–6) were analysed independently. Blue and white represent the highest and lowest relative abundance respectively. Intensity of the colour denotes the level of the relative abundance (as shown in the legend). The inferred relative abundance of the predicted functional pathways and results of tests for significance are provided in Supplementary Table S5.

Conclusions

We observed clear shifts in the infant gut microbiota upon addition of each of the cereal products into a large intestine simulating basal medium inoculated with a fecal sample. The relative abundance of the families *Bacteroidaceae*, *Veillonellaceae*, *Enterobacteriaceae*, *Bifidobacteriaceae*, *Lachnospiraceae* and *Lactobacillaceae* significantly changed following cereal supplementation. There were corresponding changes in the concentrations of short chain fatty acids. The concentration of acetate increased with each cereal, whilst the concentrations of butyrate and propionate significantly changed only in specific biological samples with specific cereal additions.

Supplementation with all four cereal products was observed to promote the growth of plant polysaccharide digesting bacteria, reduce the abundance of dominant families in the pre-weaned gut and increase the production of SCFAs. Therefore, these cereal products may have the potential to aid the establishment of a mature gut microbial community. Utilisation of an *in vitro* gut mimicking model system in the present study facilitated frequent sampling without host interference. However, extension of this work *in vivo* would be useful as it would eliminate the inherent limitations of batch culture gut microbiota model systems such as accumulation of bacterial metabolites due to absence of host interactions. Therefore, similar *in vivo* studies using biological samples from a narrower age range would provide further insight into the impact of cereal products on the gut microbiota and host health parameters associated with different weaning diets.

Methods

***In vitro* digestion of cereal products.** Cereal products used in this experiment are derived from whole grain wheat (Weet-bix™), whole grain sorghum (Gluten free Weet-Bix™), organic brown and white rice (Bellamy's organic baby rice cereal) and organic oats (Real good food-Organic baby oat cereal) (full nutritional profile and ingredient list provided in Supplementary Table S1). Weet-Bix™, Gluten free Weet-Bix™, Bellamy's organic baby rice cereal and Real good food-Organic baby oat cereal are referred as wheat, sorghum, rice and oats cereal products, respectively, here after.

All enzymes and reagents were purchased from Sigma Aldrich, Australia, unless otherwise stated. Wheat, sorghum, rice and oat based cereal products were purchased from a local Australian supermarket.

Wheat and sorghum based cereal products were ground with a mortar and pestle under sterile conditions prior to *in vitro* digestion. Each of the four cereal products and a sterile water (MilliQ, Millipore, Australia) sample as a no added cereal control was processed through simulated oral, gastric and small intestine digestion according to published protocols[79] with slight modifications. Lower concentrations of salivary alpha amylase (150 U/mL)[80], gastric pepsin (3125 U/mL)[81], small intestine bile salt (2.5×10^{-3} M), pancreatic trypsin (10 U/mL), chymotrypsin (2.5 U/mL), lipase (2,000 U/mL), colipase (4,000 U/mL), amylase (20 U/mL)[81,82] and a higher level of pH (3.0) in the gastric digestion step were maintained to accommodate the differences in infant digestive system[81]. Following the digestion, cereal products were dialysed at 5 °C in a 2000 MWCO dialysis membrane (Spectra/Por 6, Spectrum Labs) against a sterile NaCl (10 mM) dialysate for 12 hours, which was followed by an

additional 2 hour incubation with fresh dialysate[83]. Dialysed cereal products and the no added cereal control were frozen at −80 °C and freeze dried prior to use.

Preparation of the basal medium. A basal medium was used which was designed to simulate large intestine conditions. The composition of the basal medium per litre was: Peptone 0.5 g, yeast extract 0.5 g, $NaHCO_3$ 6 g, Hemin solution (0.05% (w/v) Hemin and 0.2% (w/v) NaOH) 1 mL, L-cysteine HCl 0.5 g, Bile salts 0.5 g, Tween 80 2 mL, Resazurin solution (0.1% (w/v)) 1 mL, Vitamin stock[84] 1 mL, K_2HPO_4 0.228 g, KH_2PO_4 0.228 g, $(NH_4)_2SO_4$ 0.228 g, NaCl 0.456 g, $MgSO_4$ 0.0456 g, $CaCl_2.2H_2O$ 0.0608 g and 1 mL trace mineral solution[85] with additional $NiSO_4.6H_2O$ (0.1 g/L), Na_2SeO_4 (0.19 g/L) and $Na_2WO_2.2H_2O$ (0.1 g/L). The pH of the medium was adjusted to 7.0 ± 0.2.

Preparation of the basal medium and subsequent culturing were performed under strict anaerobic conditions using a 25% carbon dioxide, 5% hydrogen and 70% nitrogen anaerobic chamber (Thermo Scientific model 1025 Forma). Anaerobic medium was aliquoted into airtight glass vials with rubber stoppers and aluminium lids prior to sterilisation.

Collection and preparation of fecal inocula. All experimental procedures and protocols were reviewed and approved by Macquarie University Human Research Ethics Committee (Reference number 5201400595) and all methods were performed in accordance with the relevant guidelines and regulations. One fecal sample each was collected from six healthy infants (4 female and 2 male) aged 5–11 months. None of the infants were given antibiotics in at least three months prior to sample submission. Infants were fed breast milk (n = 2), formula milk (n = 2) or both (n = 2). All infants were exposed to solid food prior to sample collection. Four infants were introduced to a wider range of food types compared with the other two infants (Table 1).

Fresh fecal samples were collected in a sterile container and immediately placed in an anaerobic jar (Anaero jar, Oxoid Limited, UK) with an Anaerogen sachet (Oxoid) and an anaerobic indicator (Oxoid). Samples were transported anaerobically and laboratory processing was commenced in less than two hours of collection. Fecal slurries were prepared from individual samples by homogenising in anaerobic sterile basal medium and filtering through a sterile nylon mesh cloth (985 µm) prior to using as an inoculum. Fecal slurry preparation was performed under strict anaerobic conditions as used for media preparation.

***In vitro* fermentation of the cereal products.** *In vitro* digested and freeze dried samples of wheat, sorghum, rice and oats based cereals were added into separate sterile anaerobic vials with the basal medium. A control sample was run in parallel with no added cereal. The final concentration of the cereal additions was maintained at 1% (w/v). Each of these vials were then inoculated with filtered fecal homogenate to obtain a final concentration of at least 0.6% (w/v) in a final volume of 50 mL (0.3 g feces per vial). Experiments were performed in triplicate for each of the fecal samples obtained from six healthy infants. All culture vials were anaerobically incubated at 37 °C with agitation (100 rpm). Aliquots (2 mL) from these cultures were harvested at 0, 24 and 48 hours of incubation and were stored at −80 °C prior to further analyses. The pH of the cultures at 48 hours were measured using pH indicator strips universal pH 0–14 and pH 4.5–10 (Dosatest, VWR, Australia).

Analysis of the gut microbiota. Harvested cultures were used to collect microbial cells by centrifugation at 20,238 × g for 15 minutes. Total community DNA was extracted from cell pellets using a FastDNA spin kit (MP Biomedicals) according to the manufacturer's instructions. The lysing matrix in the kit was replaced by Lysing matrix E (MP Biomedicals)[86]. The 16 S rRNA (V4 region) gene was amplified from extracted DNA using 515 (5′-GTGCCAGCMGCCGCGGTAA-3′) forward and 806 (5′-GGACTACHVGGGTWTCTAAT-3′) reverse primers with custom barcodes[87,88]. PCR amplification, amplicon quantification, purification and sequencing using an Illumina MiSeq V4 platform (2 × 250 bp paired-end sequencing) were conducted at the Ramaciotti Centre for Genomics, Australia.

Two independent Illumina Miseq sequencing runs were performed on all samples (n = 270) as technical replicates of sequencing. Quantitative Insights Into Microbial Ecology (QIIME) software (version 1.9.1)[89] was used to process the raw sequence data. Full length and high quality (-q 19 and with other default parameters) reads were used to determine OTUs pre-clustered at 97% similarity using an open-reference protocol against the Greengenes database (version 13_8)[90].

After confirming the reproducibility of the two Illumina MiSeq sequencing runs, raw data for each sample were combined and reanalysed using QIIME software according to the methods described above. This resulted in a total of 21,231,850 reads (mean 78,636 ± 16,684) prior to filtering out the OTUs with less than 0.005% reads. Reads per sample were rarefied at 35,095 reads prior to statistical analyses.

Functional prediction using PICRUSt. Functional genes in each treatment condition at 0, 24 and 48 hours were inferred from the 16 S rRNA gene sequences using PICRUSt, online galaxy version 1.1.0[91]. All *de-novo* OTUs were removed from the open-reference picked OTUs (filtered and rarefied) and those with Greengenes database (version 13_8) identifications were retained for analysis in PICRUSt. These new OTUs were normalised by the 16 S rRNA copy number and functional genes were inferred using KEGG Orthology genes[92]. The inferred KEGG Orthology genes were grouped into functional pathways at the third BRITE hierarchy level using PICRUSt. A total of 5,516,828,518 (mean 20,432,698 ± 4,553,675) KEGG Orthology genes were predicted. Each of the 270 samples was rarefied at 15,198,942 KEGG Orthology genes. Functional pathways inferred to have >10% higher/lower relative abundance in at least one cereal addition compared to the no added cereal control were identified. Biological samples were analysed individually and the inferred functional pathways that showed >10% change in at least five biological replicates were used for further statistical analysis.

Quantification of SCFAs. The supernatants (500 µl) of the samples collected at 0, 24 and 48 hours were spiked with an internal standard (4-methyl valeric acid). This was further diluted in a 70% (v/v) ethanol and 0.1% (v/v) trifluoroacetic acid (TFA) solution to obtain a final concentration of the internal standard in the mixture at 100 ppm. The solution was then vortexed and filtered through a 0.2 µm membrane (Millipore, Australia) prior to analysis using a gas chromatograph with a flame ionisation detector (GC-FID, Shimadzu GC-17A). Samples were separated on a 30 m × 0.25 × 0.5 µm i.d. HP-INNOWax fused silica column (Hewlett-Packard) as per the manufacturer's instructions. GC-FID analysis for each of the 270 samples was performed with further instrument specific technical triplicates (n = 810). SCFA concentrations were normalised for the weight of the fecal inoculum in each biological sample.

Statistical analyses. Statistical analyses of the gut microbiota sequence data were performed on filtered and rarefied OTUs using PRIMER-7 software package[93]. Non-metric multidimensional scaling (nMDS) plots were constructed based on Bray-Curtis similarity matrices of Log (x + 1) transformed abundance of the OTUs. One-way ANOSIM was performed with 9999 permutations using the Bray-Curtis similarity matrix for each biological sample. An ANOSIM R-value closer to 1 indicates a higher separation of the microbiota structure between samples, whilst R closer to 0 indicates a lower separation. The Shannon diversity index for each sample was determined based on the OTU abundance using the PRIMER-7 software package.

Bacterial families and OTUs with more than 1% relative abundance in at least three biological samples were used for further statistical analyses. Significant differences in the relative abundance of 16 S rRNA gene identifications (family and OTU level), relative abundance of inferred KEGG Orthology pathways, concentration of SCFAs, Shannon diversity indices and pH measurements between treatments were identified using GraphPad Prism (version 7) software (GraphPad Software, USA). Two-way ANOVA with Tukey's multiple comparisons tests were employed to compare each treatment. Biological samples were analysed individually.

The correlations between the relative abundance of bacterial families, SCFA concentrations, abundance of inferred KEGG Orthology pathways and pH measurements were determined using Spearman's correlation analyses (two-tailed test) on GraphPad Prism (version 7) software. Correlation analyses were performed between all bacterial families, SCFA concentrations, abundance of inferred KEGG Orthology pathways and pH measurements, however, results of tests where the Spearman's correlation (r) was $-0.2 > r > 0.2$ are presented.

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Author Contributions

H.K.A.H.G., S.G.T., I.T.P. and J.A. designed the study. H.K.A.H.G. prepared samples for the GC-FID and conducted all experiments including *in vitro* digestion, culturing, DNA extraction and bioinformatics analysis. R.W.W.C. performed the GC-FID quantification of SCFAs. All the statistical analyses were performed by H.K.A.H.G. H.K.A.H.G., S.G.T., I.T.P., N.P. and J.A. interpreted the results. H.K.A.H.G. drafted the manuscript with contributions of S.G.T. and I.T.P. All the authors read and approved the final manuscript.

Additional Information

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Competing Interests: J.A. is an employee of Sanitarium Health and Wellbeing, Australia, the producer of Weet-Bix™ and Gluten free Weet-Bix™. All other authors have no competing financial interests.

Accession codes: The 16S rRNA gene sequence data generated during this study are available on the GenBank Sequence Read Archive database under accession number SRP107068.

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3.3.2.2 Supplementary material

Title

Cereal products derived from wheat, sorghum, rice and oats alter the infant gut microbiota *in vitro*

Authors

Hasinika K.A.H. Gamage[1], Sasha G. Tetu[1*], Raymond W.W. Chong[1], John Ashton[2], Nicolle H. Packer[1], Ian T. Paulsen[1*]

Supplementary figures and tables

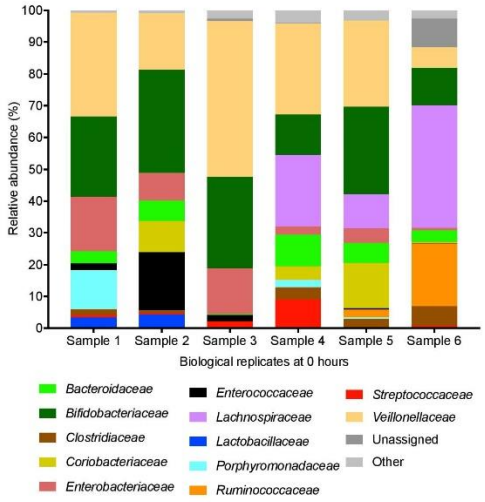


Figure S1 Family level taxonomic composition of the initial (at 0 hours) gut microbiota. The relative abundance at the family level was determined using QIIME and GraphPad Prism (V7). Bacterial identifications that were not assigned to a family are categorised as "Unassigned". Bacterial groups with less than 2% relative abundance in all biological samples (1-6) are categorised as "Other".

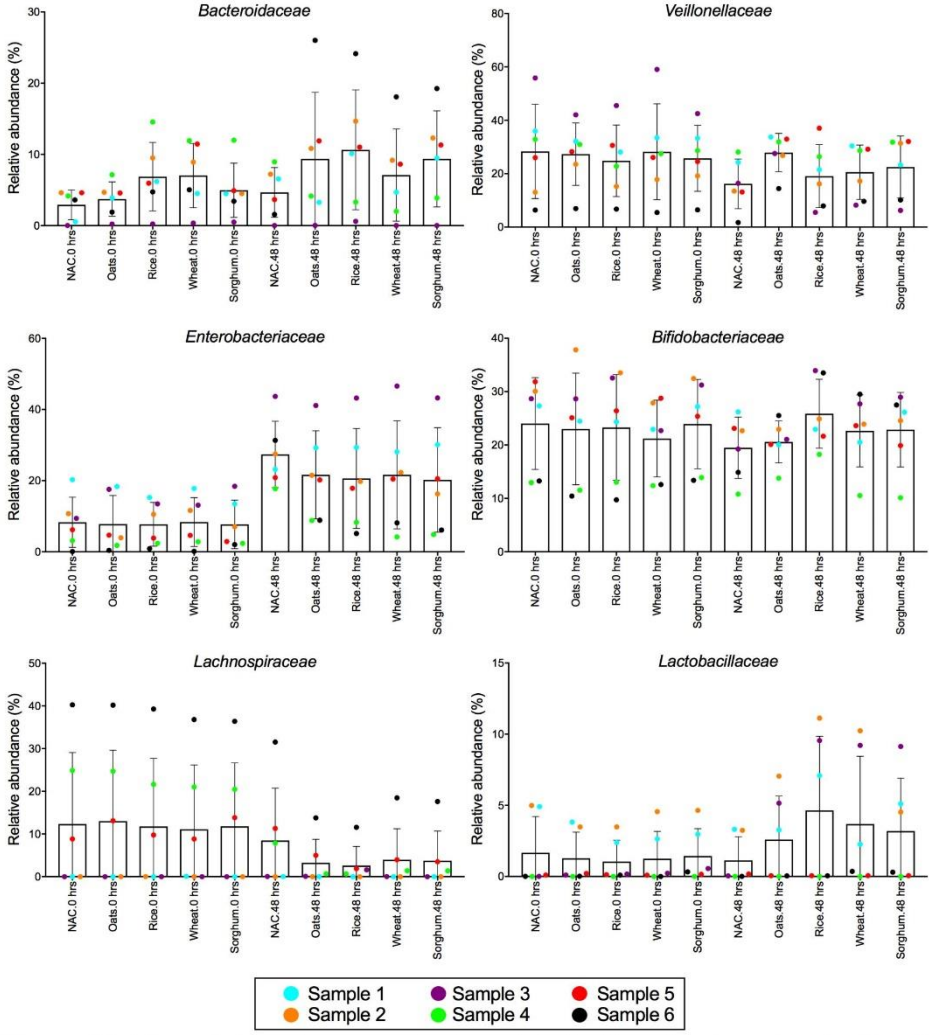


Figure S2 The relative abundance for bacterial families found to be significantly differentially abundant between treatments in at least three biological samples. Significance was determined using a Tukey's multiple comparisons test. Biological samples were analysed individually. The relative abundance of the families *Bacteroidetes*, *Veillonellaceae, Enterobacteriaceae, Bifidobactericeae, Lachnospiraceae* and *Lactobacillaceae* are shown. No added cereal control is abbreviated as NAC. In the bar graph, bars represent the mean relative abundance of all biological samples for each treatment and time point with ± SD. Mean relative abundance for each biological sample (sample 1-6) is denoted by colour-coded dots as shown in the legend. The relative abundance of these families and results of tests for significance are provided in Supplementary Table S2.

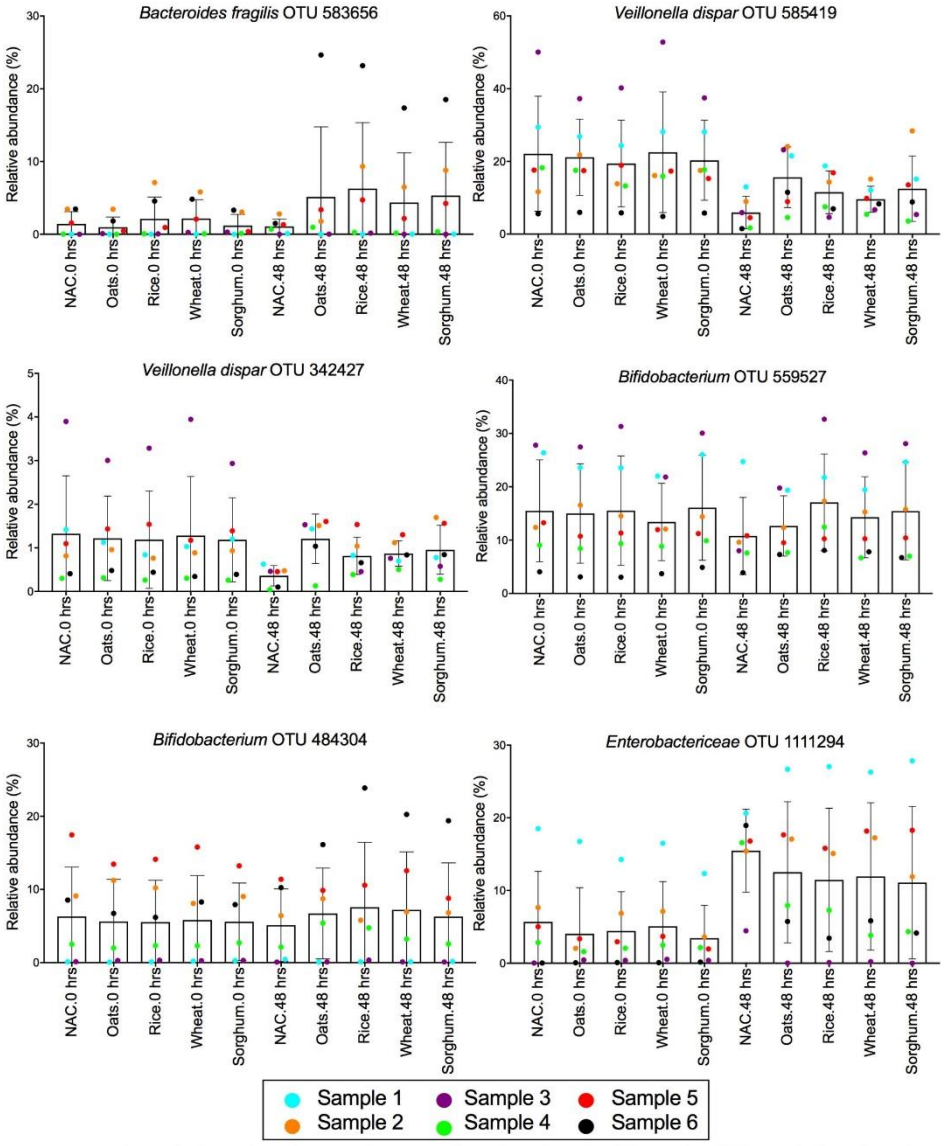


Figure S3 The relative abundance of OTUs found to be significantly differentially abundant between treatments in at least three biological samples. No added cereal control is abbreviated as NAC. Significance was determined using an ANOVA with Tukey's multiple comparisons test. Biological samples (sample 1-6) were analysed independently. Mean values with ± SD are mentioned. The relative abundance of the OTUs and results of tests for significance are provided in Supplementary Table S3.

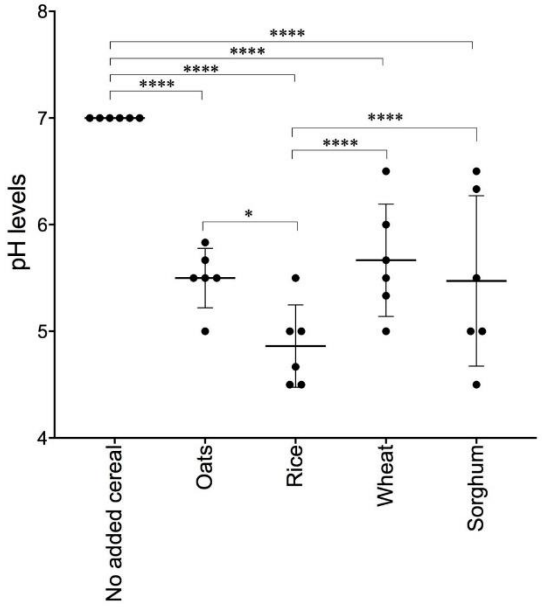


Figure S4 Measurements of pH for all cultures at 48 hours. Mean pH values for each of the three technical replicates in each of the six biological samples are indicated (dots). Bars represent the mean pH levels with ± SD for each treatment. Significance was determined using ANOVA with Tukey's multiple comparisons test (**** $P < 0.0001$).

Supplementary tables

Table S1 Nutritional information and ingredients of Weet-Bix, Gluten free Weet-Bix, Bellamy's organic baby rice cereal and Real good food-Organic baby oat cereal. NP-not provided.

Table S2 The relative abundance of bacterial families that were found to be significantly differentially abundant in at least three biological samples. Mean ± SD for each treatment and time point (0 and 48 hours) for biological samples (sample 1-6) are provided. Significance was determined using ANOVA with Tukey's multiple comparisons tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ and ns- not significant for each cereal addition compared to the no added cereal control at 48 hours.

Table S3 The relative abundance of bacterial OTUs found to be significantly differentially abundant and with more than 1% relative abundance in at least five biological samples. Mean ± SD for each treatment and time point (0 and 48 hours) for biological samples (sample 1-6) are provided. Significance was determined using ANOVA with Tukey's multiple comparisons tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ and ns- not significant for each cereal addition compared to the no added cereal control at 48 hours.

Table S4 Concentration of acetate, butyrate and propionate in each treatment at 0, 24 and 48 hours. Mean concentration per treatment for each biological sample (Sample 1-6) with ± SD is provided. Significance was determined using ANOVA with Tukey's multiple comparisons

64 tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ and ns- not significant for
65 each cereal addition compared to the no added cereal control at 48 hours.

66 **Table S5** The predicted relative abundance of KEGG Orthology pathways inferred using
67 PICRUSt for each biological sample (sample 1-6) with different cereal additions. Mean ± SD
68 for each treatment and time point (0 and 48 hours) are provided. Significance was determined
69 using ANOVA with Tukey's multiple comparisons tests. * $P < 0.05$, ** $P < 0.01$, *** $P <$
70 0.001, **** $P < 0.0001$ and ns- not significant for each cereal addition compared to the no
71 added cereal control at 48 hours.

72

3.3.3 SCFAs as key mediators between diet, the gut microbiota, and health

The results from both studies using the *in vitro* gut mimicking model demonstrate that dietary fibres with different chemical compositions are not equally fermented by the gut microbiota resulting variations in fermentation profiles. Though the use of the *in vitro* gut mimicking system provides a convenient method of simulating the environment of the lower intestine, the static nature of the system that does not involve the removal of degradation products via absorption across the gut lining prevents the model from being fully representative of the complex nature of the gastrointestinal tract.

Fibres with vastly different chemical compositions, such as NutriKane, Benefibre, and psyllium husk, exhibited distinct fermentation profiles while cereal products, which showed some similarities in composition, exhibited almost identical fermentation profiles despite containing different amounts of TDF. Both studies were consistent in demonstrating that different dietary fibre compositions can influence the production of SCFAs and are in agreement with other *in vitro* fermentation studies (Bach Knudsen, 2015; Noack et al., 2013; Timm et al., 2010). The physicochemical properties and composition of dietary fibres contributes to the production of SCFAs. Benefibre, a soluble wheat dextrin, was highly fermentable and exhibited the highest concentrations of all acetate, propionate and butyrate. Consistent with our results, arabinoxylan, a major component of dietary fibre in both psyllium husk, wheat, sorghum, and oats, has been shown to increase the production of acetate and butyrate (Hald et al., 2016). While acetic acid production is widely distributed across many bacterial families (Morrison and Preston, 2016), the production of propionic acid and butyric acid is more conserved to relatively few bacterial genera (Reichardt et al., 2014) and specific microbial compositions have been associated with increased abundance of propionic acid and butyric acid (Reichardt et al., 2018).

Epidemiological studies have shown an association between the increased consumption of dietary fibre and an improvement in gastrointestinal health marked by a reduced risk of irritable bowel

syndrome, colitis, and colorectal cancer (Kendall et al., 2010). Since humans lack the enzymes to digest most dietary fibres, these non-digestible carbohydrates enter the colon and undergo fermentation by the gut microbiota to produce SCFAs as the major bacterial metabolites (den Besten et al., 2013b; Nicholson et al., 2012). SCFAs in the gut possess both anti-inflammatory and anti-microbial activity and control various local physiological functions, dictating colonic mobility, colonic blood flow, and gastrointestinal pH, which can influence uptake and absorption of electrolytes and nutrients (Clausen and Mortensen, 1995; den Besten et al., 2013b; Scheppach et al., 1992). SCFAs have also been associated with a variety of wider functions including the maintenance of gut integrity, regulating immune function, and modulating host metabolism, appetite, and behaviour (Morrison and Preston, 2016; Ríos-Covián et al., 2016; Tan et al., 2014). By these physiological activities, accumulating evidence supports the role of SCFAs as key mediators between diet, the gut microbiota and health, with far reaching implications for the treatment and prevention of diet related diseases.

While the benefits of dietary fibre are well established in adults (Kendall et al., 2010; Nicholson et al., 2012), there is significantly less information regarding its effects in infants and young children. The majority of dietary recommendations for children have been extrapolated from adult studies (Edwards and Parrett, 2003; Kranz et al., 2012) and extreme caution has hindered the development of clear and practical guidelines for dietary fibre intake in infants children due to fear of malnutrition and mineral malabsorption (Dwyer, 1995; Ghisolfi, 2003). Dietary fibre intervention during weaning presents a window of opportunity to establish a healthy gut microbiota and to assist the programming of future metabolic and immune health (Goulet, 2015). While the effect of dietary fibre on infants and children is still relatively unexplored, studies have shown that factors such as diet, antibiotic usage, birth-mode, and breast-feeding status can have a profound effect on the gut microbiota (Yang et al., 2016). For example, infants who are exclusively breastfed possess reduced microbial diversity, while formula-fed infants tend to exhibit a more diverse microbiota (Azad et al., 2013; Backhed et al., 2015; Penders et al., 2006). The results of

the second study have shown that the infant gut microbiota responds to the addition of cereal products in a similar fashion to the adult gut microbiota as seen in the increased production of SCFAs. These results will assist with the development of new dietary fibre products targeted to children and aid in the establishment of new guidelines for dietary fibre intake in early development.

3.4 Chapter summary

Two separate studies were performed in determine the relationship between dietary fibre, the gut microbiota, and the production of SCFAs. The first study investigated the effect of three chemically different dietary fibres on the adult gut microbiota by measuring changes in microbial composition and associated SCFA production. The second study investigated the effect of four cereal products on the infant gut microbiota in the same way. Both studies support the use of the *in vitro* gut mimicking model, GC-FID analysis of SCFAs, and 16S rRNA sequencing as complimentary techniques in studying the effect of diet on the gut microbiota.

Both studies showed the gut microbiota of adults and infants responded to the addition of fermentable dietary fibres, causing an increase in the abundance of fibre digesters, such as members from the *Bacteroidaceae* and *Porphyromonodaceae* families. Soluble fibres such as wheat dextrin and psyllium, and cereal products made from wheat, sorghum, rice and oats were shown to be useful in stimulating the production of SCFAs. In contrast, NutriKane, which is classified as an insoluble fibre, was significantly less effective at stimulating SCFA production *in vitro*. These differences are most likely due to differences in the physiochemical properties of dietary fibres, such as solubility, that can interfere with microbial fermentation. High quantities of glucose and xylose, which are indicative of cellulose and hemicellulose, were characteristic of the NutriKane suggesting that these polysaccharides are significantly less fermentable than soluble fibre polysaccharides. Increasing the production of SCFAs is a key physiological activity of dietary fibres on the assumption that they reach the colon and are exposed to the gut microbiota. In conclusion, these results demonstrate that the composition of dietary fibres contributes significantly to the production of SCFAs *in vitro* and assist in understanding how dietary fibre can shape the gut microbiota composition and associated metabolites.

Chapter 4: Effect of diet and infection on intestinal health

4.1 Introduction

The intestinal tract facilitates the digestion and absorption of nutrients from the diet while maintaining an essential barrier against harmful substances and pathogens from the external environment. As such, intestinal health has a direct impact on overall health by contributing to nutrition, immune status and the state of the gut microbiota (Bischoff, 2011). Poor intestinal health includes a diverse range of conditions including nutritional malabsorption or intolerance, acute and chronic inflammation, infection and microbial dysbiosis. It is now known that the gut microbiota plays an important role in health and disease. Microbial dysbiosis, which is an imbalance of the microbiota, has been associated with many chronic diseases including inflammatory bowel disease, obesity, cancer, and autistic disorders (Zhang et al., 2015).

As discussed in the previous Chapter, through the production of SCFAs, dietary fibre contributes to the promotion of intestinal health. Specifically, the consumption of dietary fibre promotes the production of bacterial metabolites such as the short chain fatty acids (SCFAs) that contribute to the energy acquired from the diet and produce local effects including modulation of the gut microbiota and regulation of inflammatory processes, as well as having wider effects that influence host physiology (Tan et al., 2014). We hypothesised that the production of SCFAs could be used as a biomarker of intestinal health and reflect the dietary fibre content of the diet. Following on from our studies using *in vitro* gut mimicking systems (Chapter 3), in this Chapter we investigate the effect of dietary fibres on SCFA production in a mouse model system to determine their production *in vivo*.

The mucus layer is critical in mediating host-gut microbiota interactions by forming a semipermeable barrier that physically that separates and protects the epithelial cells of the host from food particles, chemicals, enzymes, and both commensal and pathogenic microbes

(Johansson et al., 2008; Ouwerkerk et al., 2013). Studies have shown that germ-free animals possess a mucus layer that is smaller in surface area in comparison to conventionally raised animals demonstrating an intimate relationship between the mucus layer and the gut microbiota (Gordon and Pesti, 1971; Johansson et al., 2015). The thickness of the mucus layer exhibits large fluctuations in thickness and composition along the gastrointestinal tract reaching a maximum in the colon of the lower intestine in proportion with the density of gut microbes (Atuma et al., 2001; Ermund et al., 2013; Johansson et al., 2008). Disruption of the mucus layer by loss of barrier integrity has been implicated in heightened susceptibility to infection (Desai et al., 2016; Lindén et al., 2008; Zarepour et al., 2013), the progression of inflammatory diseases including Crohns disease (Podolsky and Fournier, 1988) and ulcerative colitis (McGuckin et al., 2009; Van der Sluis et al., 2006), and even the development of colorectal cancer (Byrd and Bresalier, 2004; Velcich et al., 2002). Recent work has also established a role of the mucus layer in preventing microbial dysbiosis, a state of imbalance in microbial composition (Johansson et al., 2011b; McLoughlin et al., 2016; Ouwerkerk et al., 2013).

The mucus layer is a complex barrier composed primarily of water, mucins, bacteria, and antimicrobial products secreted by the epithelial cell lining (Johansson et al., 2011a). Mucins, which form the major component of mucus, are high molecular weight glycoproteins that are post-translationally modified with oligosaccharide chains or "glycans" covalently linked to the protein backbone. Glycans are composed of a variety of different monosaccharides including glucose (Glc), galactose (Gal), mannose (Man), fucose (Fuc), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), and N-acetylneuraminic or sialic acid (NeuAc) and other residues such as sulfate, phosphate and acetate that are sequentially attached by specific transferase enzymes (Brockhausen, 2010). These glycans are known as either *N*- or *O*-glycans due to their connection through the $-NH_2$ or -OH group of asparagine or serine/threonine amino acids respectively. The key differences between mucins and other glycoproteins are their large size

(200kDa – 200MDa) and tandem amino acid repeat regions rich in proline, serine and threonine residues (PTS regions) (**Figure 4-1**)(Kesimer and Sheehan, 2012).

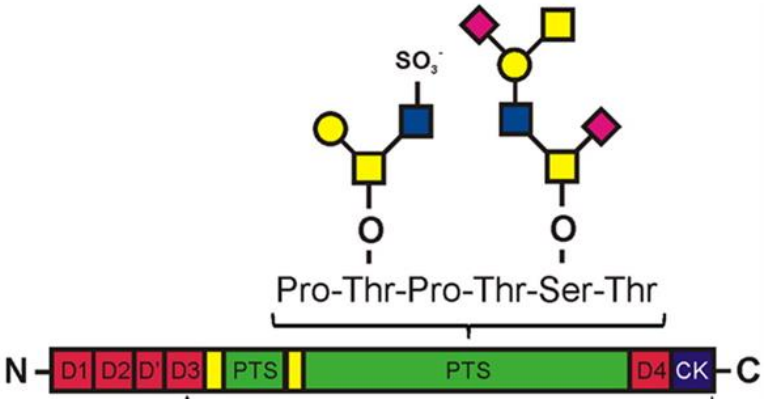


Figure 4-1. Protein structure of MUC2 and some typical *O*-glycans attached to serine (Ser) or threonine (Thr)[8]. The MUC2 structures is characterised by multiple Cysteine-rich domains (D1, D2, D', D3, D4) that are interspersed between Proline-Serine-Threonine-rich domains (PTS).

Serine and threonine residues provide potentially hundreds of *O*-glycosylation sites depending on the size of the PTS regions. Mucin glycosylation exhibits significant structural variation due to the sequential attachment of monosaccharides in various combinations and linkage types. The extensive glycosylation of mucins contributes to many of the physical properties that are characteristic of mucus such as its viscoelasticity, high water holding capacity, and gel forming ability (Cone, 2009), in addition to many other physiological functions (Bergstrom and Xia, 2013). In both the small and large intestine of mammals, MUC2 is the major secreted mucin (Allen et al., 1998).

While MUC5AC, MUC5B, and MUC6 are also secreted in smaller amounts, most of the chemical and physical properties of intestinal mucus are attributed to MUC2 (Kim and Ho, 2010). Membrane bound mucins such as MUC1, MUC3, MUC4, and MUC13 are also present and are displayed on the surface of epithelial cells comprising the "glycocalyx", a pericellular matrix that

[8] Adapted with permission from Elsevier, Arike, L., and G.C. Hansson. 2016. The Densely O-Glycosylated MUC2 Mucin Protects the Intestine and Provides Food for the Commensal Bacteria. *Journal of Molecular Biology*. 428:3221-3229. under licence number 4387021035622, 13-07-2018.

surrounds the cellular membrane of epithelial cells (Kim and Ho, 2010; Pelaseyed et al., 2014). The MUC2 protein contains approximately 5100 amino acids and has two central repetitive regions that are rich in *O*-glycosylation sites of which between 50 – 80% are occupied. When fully glycosylated MUC2 exhibits a size of approximately 2.5 MDa (Allen et al., 1998). In the colon, MUC2 is assembled and glycosylated in the goblet cells in the epithelium before being secreted into the lumen (Allen et al., 1998) forming two distinctly stratified layers: an inner layer that is tightly adherent to the epithelium and devoid of bacteria (Johansson et al., 2008), and an outer layer that is loose in structure and interacts with the gut microbiota (Johansson et al., 2011b). To maintain a consistent mucosal barrier, MUC2 is continuously secreted by goblet cells in a process whereby the inner layer is constantly being replaced as it moves towards the lumen and eventually replacing the outer layer (Hansson, 2012). This process has been proposed to protect the epithelium by flushing out invasive bacteria (Bergstrom et al., 2010).

Previous studies have established that MUC2 and its proper glycosylation are essential to mucus layer integrity and function. The absence of MUC2 through the deletion of the Muc2 encoding gene in genetic knockout mice has been shown to prevent the formation of the mucus layer, resulting in heightened susceptibility to pathogenic infection with enteric pathogens such as *Salmonella typhimurium* (Zarepour et al., 2013)and *Citrobacter rodentium* (Bergstrom et al., 2010), the spontaneous development of colitis, and to hasten the progression of colorectal cancer (Van der Sluis et al., 2006). Studies in mice lacking the transferases that attach Gal to the initial *O*-linked GalNAc to create Core 1 type *O*-glycans (**Figure 4-2**) resulted in dramatic thinning of the mucus layer that was associated with the development of severe colitis as a result of increased translocation of bacteria into the epithelium (Fu et al., 2011). Studies have also shown notable changes in the modification of MUC2 glycans such as smaller glycans, decreased sulfation and the increased presence of sialyl-Tn antigen in intestinal inflammatory disorders such as ulcerative colitis and Crohn's disease (Larsson et al., 2011; Morita et al., 1993; Ta et al., 1997). Furthermore, terminal modifications with fucose or sialic acid (NeuAc) provide different sites of interaction for

gut microbes and contribute toward the protective function of MUC2 (Hurd and Domino, 2004; Juge, 2012; Moonens and Remaut, 2017; Struwe et al., 2015). For example, the adherence of *Helicobacter pylori* is mediated by the BabA adhesin which binds to a glycan epitope known as the Lewis b blood group antigen (Le^b) found on the surface of epithelial cells in the stomach (Boren et al., 1993; Hansson, 2012; Moore et al., 2011). By this mechanism, MUC2 *O*-glycans act as bacterial binding targets encouraging the adhesion of pathogens to be transported away from the epithelium by continuous secretion (Bergstrom et al., 2010).

Though the necessity of MUC2 *O*-glycosylation is well established, comparatively little is known about the functional significance of the diversity and abundance of MUC2 *O*-glycans. Only a handful of studies have performed detailed glycomic analysis of MUC2 from either humans or mice (Arike et al., 2017; Holmén et al., 2013; Thomsson et al., 2012). These studies characterised the composition, structure and relative abundance of *O*-glycans attached to MUC2 from the colon of humans and mice. Significant differences between mouse and human MUC2 *O*-glycosylation were observed in the types of *O*-glycan Core structures Core 1 and 2 types found in mice and Core 3 and 4 types found in humans (**Figure 4-2**)(Capon et al., 2001; Robbe et al., 2006; Thomsson et al., 2012). Differences in the *O*-glycosylation of MUC2 were also seen in mice lacking the transferase enzymes necessary for Core 1 and Core 3 type *O*-glycans in comparison with wild type mice (Thomsson et al., 2012).

The epithelium of the gastrointestinal tract is highly exposed to rapid changes in the contents of the lumen due to the transient nature of the diet and the gut microbiota. Changes in diet composition have the potential to induce immediate changes to the mucus layer through direct effects such as nutritional availability and metabolic activity, as well as indirect effects through interactions with the gut microbiota. A small number of studies have employed techniques involving carbohydrate binding lectins and histochemical staining to study changes in glycosylation as result of high fat (Gupta et al., 1993) and dietary fibre intake (Hedemann et al.,

2005; Sharma et al., 1995; Tardy et al., 1995). By these methods, no change in Fuc, NeuAc, hexose (Gal or Glc) or hexosamines (GalNAc or GlcNAc) was observed when increasing dietary fat intake (Gupta et al., 1993).

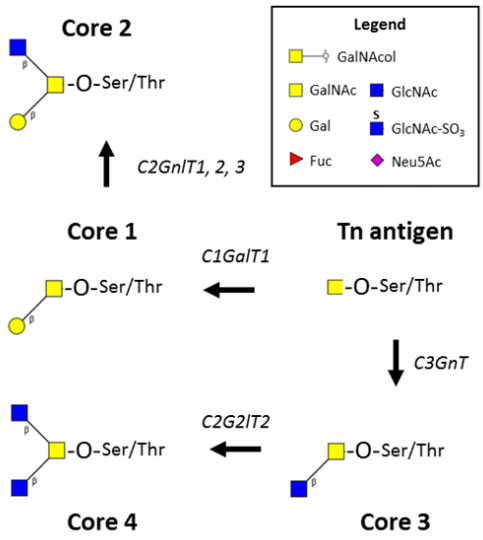


Figure 4-2. The four major *O*-glycan Core types are produced from the sequential attachment of monosaccharides to the initial GalNAc attached to serine or threonine residues in *O*-linkage. Attachment of Gal to the 3 carbon generates the Core 1 structure that is the foundation of the Core 2 structure. Attachment of GlcNAc to the 3 carbon instead generates the Core 3 structure that is the foundation of the Core 4 structure.

Introduction of pectin and cellulose to the diet increased the abundance of neutral sugar (Man, and Gal residues) content on glycoproteins from the small intestinal mucosa (Tardy et al., 1995). Furthermore, the introduction of dietary fibre has been shown to induce transient changes in the glycoprotein fucosylation pathway resulting in a decrease in fucose content in rats given cellulose and pectin after 4 days (Tardy et al., 1995). Particle size and diet morphology have also been shown to alter the charge state of mucin glycans with larger particle sizes associated with increased sulfation and NeuAc (Hedemann et al., 2005; Sharma et al., 1995). However, these studies exclusively examined the total glycocalyx of the intestinal epithelium and no studies to date have assessed specifically the changes in MUC2 glycosylation in response to physiological stimuli. We hypothesised that these overall changes in glycosylation in response to diet and microbiota would be reflected in the glycomic analysis of Muc2 from the gut epithelial surface. Here we present two

separate studies that investigated the effect of 1) dietary fat and fibre inclusion in the diet, and 2) infection with *Citrobacter rodentium*, on the glycosylation pattern of Muc2 in the colon of the mouse.

4.2 Materials and Methods

All chemicals and reagents were obtained from Sigma Aldrich (Australia) unless stated otherwise. Dowex AG-50W-X8 cation exchange resin was purchased from Biorad (Australia). Bond Elut OMIX C18 100 µL tips were purchased from Agilent Technologies (Australia). MilliQ water was obtained from a MilliQ system (Millipore).

4.2.1 Extraction of Muc2 from colonic mucus

Colons were surgically removed from euthanised mice and flushed with phosphate buffered saline (PBS) to remove faecal matter. Colons were opened longitudinally with surgical scissors and pinned down while the mucus was collected from the whole length of the colon using an in-house device that applied vacuum suction to a 1000µL tip. Mucus within the tips was there transferred to Eppendorf tubes using a table top microcentrifuge. For the extraction of Muc2, mucus samples were extracted five times with 200 µL of extraction buffer (6 M guanidinium hydrochloride, 10 mM Na_2PO_4, and 50 mM EDTA) while kept on ice following the protocol of (Carlstedt et al., 1993; Johansson et al., 2008). Samples were then centrifuged at 13 000 rcf at 4° C for 20 mins to obtain a partially purified Muc2-containing insoluble pellet. The supernatant was carefully removed by pipette and the residual pellet was washed twice with extraction buffer and centrifuged after each wash step. The remaining pellet containing Muc2 was washed with 1 mL of ethanol to remove residual extraction buffer and left to air dry.

4.2.2 Glycan release and purification

While *N*-glycans can be enzymatically released prior to β-elimination, the MUC2 pellet was considered incompatible with the PNGase F enzyme due to its insolubility. Instead, glycans were chemically released by alkaline reductive β-elimination. Approximately 1 mg of MUC2 (as

obtained from Section 4.2.1) was resuspended in 100 µL of 1 M sodium borohydride ($NaBH_4$) in 100 mM KOH and incubated at 50° C for 16 hours. The reaction was quenched with 5 µL of glacial acetic acid then desalted over a 50 mg bed volume of Dowex AG-50W-X8 (Biorad) strong cation exchange resin prepared on top of C18 Bond Elut OMIX 100 µL tips (Agilent Technologies). Released glycans were eluted with MilliQ water and dried in a SpeedVac rotary evaporator. The dried released glycans were resuspended three times in methanol acidified with 0.05% (v/v) acetic acid and dried to remove excess borate. As an additional purification step, desalted released glycans were resuspended in MilliQ water and loaded onto a 50 mg bed volume of porous graphitised carbon (PGC) prepared in Bond Elut OMIX C18 100 µL tips (Agilent Technologies). Tips were washed three times with 200 µL of MilliQ water to remove excess salts. Glycans bound to the PGC bed were then eluted with 500 µL of 50% (v/v) acetonitrile acidified with 0.05% (v/v) trifluoroacetic acid. Samples were dried and resuspended in 10 µL 10 mM ammonium bicarbonate pH 7.6 prior to PGC-LC-MS/MS analysis.

4.2.3 Glycan analysis by porous graphitised carbon-liquid chromatography electrospray ionisation tandem mass spectrometry (PGC-LC-ESI-MS/MS)

PGC-LC-ESI-MS/MS of released and purified glycans was performed using an established method according to Jensen *et al.* (2012). Glycans (obtained from Section 4.2.2) were separated using a porous graphitised carbon column (Hypercarb, 5 µm particle size, 0.18 mm I.D. x 100 mm). Separations were performed over a 45 min gradient of 0 – 90% (v/v) acetonitrile in 10 mM ammonium bicarbonate. The flow-rate was maintained at 2 µL/min using an online high-performance liquid chromatography system (Agilent 1100, Agilent Technologies, Inc., CA, USA) coupled to an electrospray ionisation ion trap mass spectrometer (ESI-MS, Agilent 6330, Agilent Technologies, Inc., CA, USA). The LC eluate was introduced directly into the ESI source. The capillary voltage was set to 3 kV. Dry gas was maintained at 300 °C. The MS spectra were obtained in the negative-ion mode with a scan range between *m/z* 300 and *m/z* 2200. While both *N*- and *O*-glycans are released simultaneously, *O*-glycans are the predominant form of glycosylation of

MUC2 and only masses belonging to potential *O*-glycans were considered for analysis. Though some odd masses were detected that may have been fragments of degraded *N*-glycans were detected by our PGC-LC-ESI-MS/MS analysis, we did not detect any masses corresponding to whole N-linked glycans containing the $Man_3GalNAc_2$ chitobiose core.

4.2.4 Assignment of *O*-glycan structures

The annotation of *O*-glycan structures was performed by manual interpretation of the tandem mass spectrometry (MS/MS) fragmentation spectra and comparison with previous studies on mouse MUC2 (Arike et al., 2017; Larsson et al., 2009; Thomsson et al., 2012). Manual interpretation of PGC-LC-ESI-MS/MS chromatograms and mass spectra was performed using ESI 1.3 Compass (Version 4.0, Bruker Daltonik, GmbH, Germany). For each glycan mass, an extracted ion chromatogram was generated allowing for multiple isomers to be identified by differences in retention time by porous graphitised carbon chromatography (Abrahams et al., 2018). An example of isomeric separation is shown in **Figure 4-3**

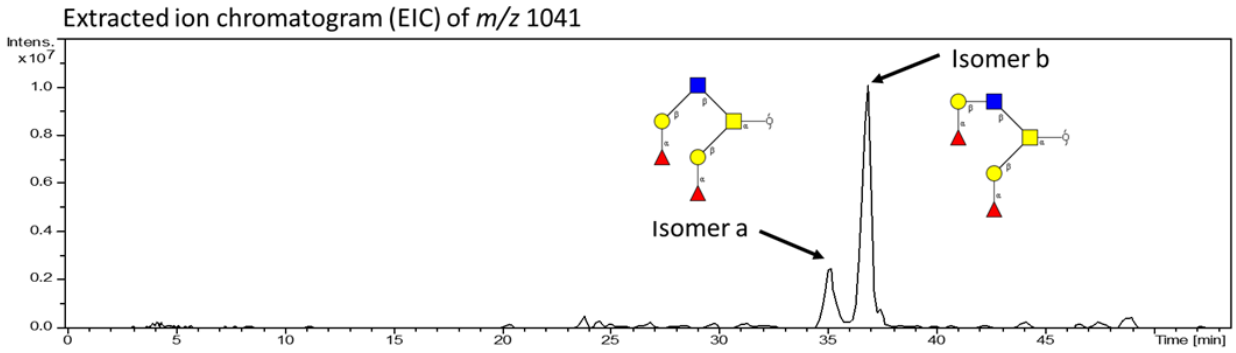


Figure 4-3. Extracted ion chromatogram (EIC) of glycan *m/z* 1041. Multiple peaks within the EIC of a single mass indicates the presence of structural isomers of the same mass and composition.

To assist in the annotation of glycan structures, we used GlycoMod, a web-based application (https://web.expasy.org/glycomod/), to calculate potential glycan compositions from the masses of compounds detected by the mass spectrometer and to exclude non-glycan masses. Potential glycan structures were proposed within the constraints of the glycan composition and using established pathways of glycan biosynthesis and MS^2 fragmentation analysis. Masses assigned as

glycan structures were subjected to MS^2 and compared to *in silico* fragmentation spectra generated using GlycoWorkbench 2 (Version 2.1), a software tool that assists in the annotation of the mass spectra of glycans by generating theoretical fragments masses from proposed glycan structures (Ceroni et al., 2008). The theoretical fragments were matched against the peak list of ions derived from the tandem mass spectra. For detailed structural annotation and verification, established diagnostic fragment ions were used to identify the loss of specific residues and cross-ring fragments in order to reconstruct the putative glycan structure (Everest-Dass et al., 2013; Karlsson et al., 2004; Robbe et al., 2006). Fragmentation ions are described according to the nomenclature proposed by Domon and Costello (1988) as shown in **Figure 4-4**. The reducing end of a glycan is the monosaccharide with a free anomeric carbon that is not involved in a glycosidic bond and attaches to the protein while monosaccharides on the non-reducing end are considered terminal residues. A, B, and C labels are used to describe fragments containing the terminal non-reducing end while X, Y, and Z labels represent fragments containing the reducing end.

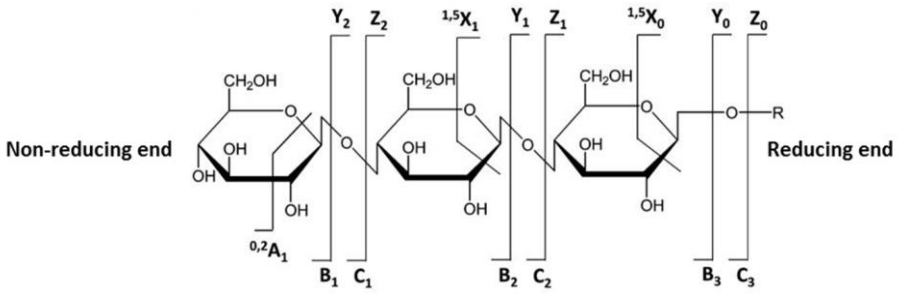


Figure 4-4. Nomenclature for ions generated from tandem MS fragmentation as proposed by Domon and Costello (1988)[9].

Subscripts indicate the position of the cleavage beginning from 1 from the non-reducing end (B and C ions) and 0 from the reducing end (Y and Z), while superscripts describe the position of the cross-ring cleavages occurring within carbohydrate rings. Fragment ions are described by their mass to charge ratio (*m/z*).

[9] Reproduced with permission from Springer Nature, Domon, B., and C.E. Costello. 1988. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. *Glycoconj J.* 5:397-409. under licence number 4292810480457, 19-02-2018.

The chemical release of *O*-glycans by reductive β-elimination results in the formation of vicinal diols followed by the reduction of the reducing end. The reducing end of released *O*-glycans is thus referred to as GalNAcol (**Figure 4-5**). Core 1 and 3 structures result from the attachment of either Gal or GlcNAc respectively to the hydroxyl group of the third carbon, referred to as the 3-arm. Branching occurs with the formation of Core 2 and 4 structures by the attachment of GlcNAc to Core 1 and 3 structures respectively onto the hydroxyl group of the sixth carbon to form the 6-arm. Core 2 structures can be identified by the presence of the prominent B type ions *m/z* 407, diagnostic of GlcNAcβ1-6GalNAcol following the loss of the 3-arm substituent or *m/z* 505, representative of the same fragmentation of the sulphated GlcNAcβ1-6GalNAcol (**Figure 4-6**, second panel). These ions are absent in the fragmentation of the Core 1 structure *m/z* 530 Fucα1-2Galβ1-3GallNAcol, which instead contains A and B type cleavage ions resulting from the loss of 3-arm substituents. While diagnostic ions can be useful in assigning many specific structural motifs, complete linkage information often cannot be obtained by mass spectrometry alone.

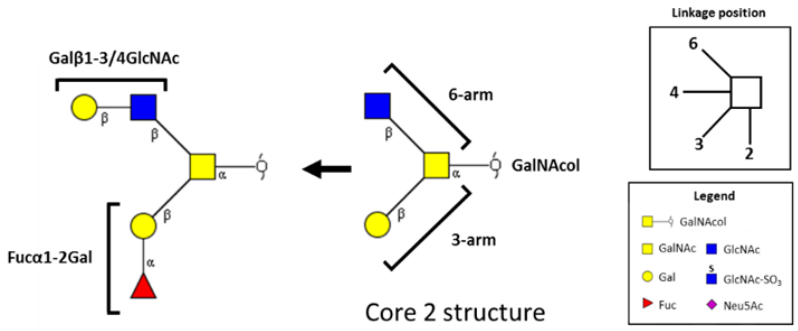


Figure 4-5. Summary of the SNFG graphical nomenclature for glycans (Varki et al., 2015). The orientation of lines dictates the linkage position of glycosidic bonds while the symbols indicate the type of monosaccharide or residue. Substitutions on the 6-carbon of GalNAcol build the 6-arm, while substitutions on the 3-carbon build the 3-arm.

For example, an ion corresponding to *m/z* M – 290 resulting from Y type cleavage is dominant in glycans containing a terminal sialic acid (NeuAc) resulting from the loss of a NeuAc that can often be observed as an *m/z* 290 B type ion. The position of NeuAc is more difficult to assign since NeuAc can be attached to the Gal of Core 1 and 2 type O-linked structures or the Gal on 3- or 6-arm extensions, however, linkage is almost always α2-3 since α2-6 linkages to Gal have not been

found in mammals (Brockhausen, 2010). Attachment of Fuc to Gal is confirmed by the presence of the A type cleavage ion *m/z* 205, which is diagnostic of terminal fucose resulting from the cross cleavage of Gal in a Fucα1-2Gal- chain (**Figure 4-6**, top panel). The *m/z* 205 ion was also accompanied by the C type *m/z* 325 or B type *m/z* 307 ion resulting from the loss of the entire Fucα1-2Gal chain with or without the glycosidic oxygen (18 difference) respectively (**Figure 4-6**, bottom panel). For larger structures, assignment of the Fucα1-2Galβ1-3/4HexNAc chain on the 6-arm of GalNAcol could be determined by the prominent *m/z* 570 A type fragment, or on the 3-arm by the *m/z* 715 Z type fragment depending on their relative intensities in the fragmentation spectra. Fucosylation of the 3-arm Gal also appeared to prevent any further extension and was a good indicator of 6-arm extension. The extension of Core 2 structures with GlcNAc or GalNAc can be difficult to assign by fragmentation alone due to the formation of identical theoretical fragments. In these cases, we referred to previous studies that have either characterised the *O*-glycan structures on MUC 2 or identified the specific glycosyltransferases in the colon of the mouse to assist in probable linkage assignment (Arike et al., 2017; Larsson et al., 2009; Thomsson et al., 2002; Thomsson et al., 2012). For example, only one fucose transferring enzyme (α1-2fucosyltransferase, *Fut2*) has been identified in the mouse colon. The specific activity of *Fut2* thus allowed assignment of fucose (deoxyhexose) residues to galactose in α1-2 linkage and is consistent with our fragmentation spectra (Arike et al., 2017). Sulfation is catalysed by a single enzyme, Sulfotransferase 4 (*Chst4*) (Arike et al., 2017), and attaches sulfate to the 6-carbon of the 6-arm GlcNAc of Core 2 type *O*-glycans.

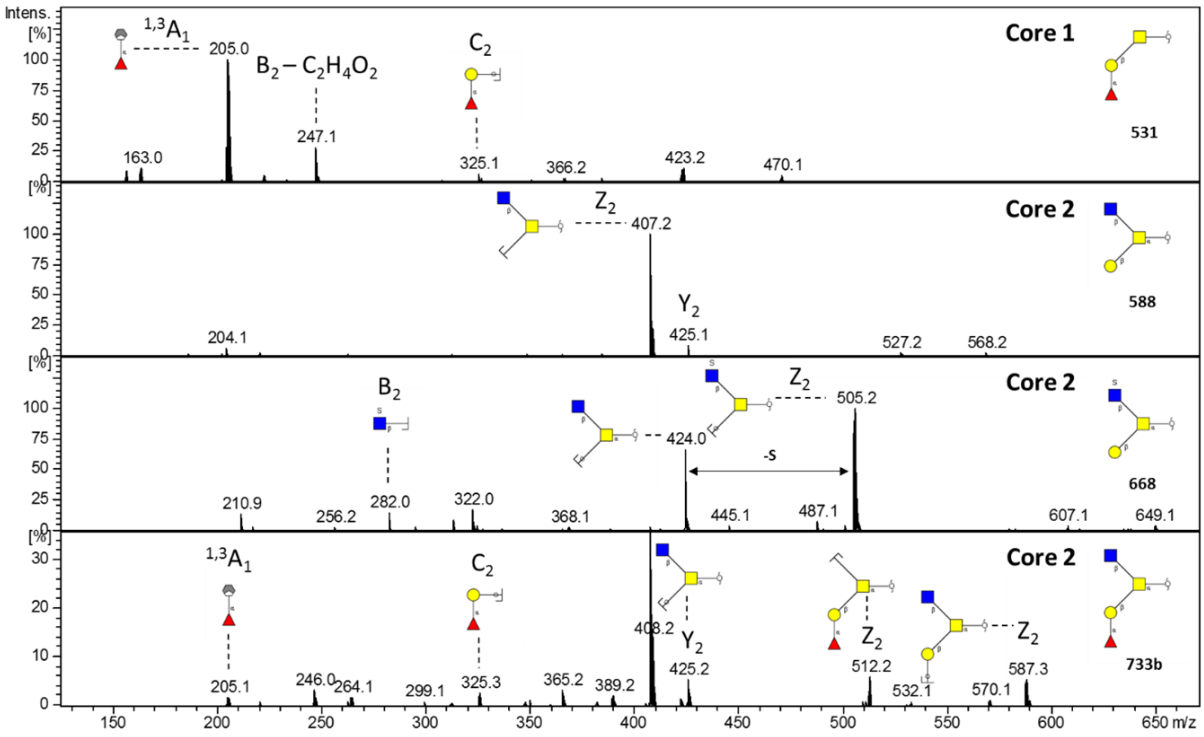


Figure 4-6. Tandem MS fragmentation spectra of *m/z* 530, 587, and 667 showing some diagnostic ions used in the assignment of Core 1 and Core 2 structures. The *m/z* 407 (GlcNAcβ1-6GalNAcol) fragment resulting from the loss of the 3-arm substituent was a good indicator of Core 2 types structures

4.2.5 Relative quantification of glycan abundance

Once structures were assigned, an extracted ion chromatogram (EIC) was generated for each individual glycan to isolate specific masses from the mass spectral data allowing identification of potential isomers and providing clean chromatograms of compounds of interest with which to calculate relative abundance. EICs and peak areas were calculated by integrating the area under the curve. The relative abundance of glycan structures was obtained using Skyline (Version 3.7.0.11317, MacCoss Lab, UW) for assisted peak peaking, generation of extracted ion chromatograms (EICs) and integration of EIC peak area following the protocol of Ashwood et al. (2018). Individual peak areas were then divided by the total peak area of all glycans detected to obtain a relative % abundance of each *O*- glycan.

4.2.6 Statistical analysis

All data are shown as mean ± the standard error of the mean (SEM) unless stated otherwise. Statistical analysis was performed using GraphPad Prism 7.0.3 (GraphPad Software Inc.) Statistical significance of differences was assessed using two-way analysis of variance (ANOVA) and multiple comparisons with Tukeys testing.

4.3 Results and discussion

4.3.1 Study 1 design: Effect of high fat diet and dietary fibres on SCFA production and Muc2 glycosylation

As part of a major collaborative work within "Nutraceuticals from Sugarcane" project in the Australian Research Council Industrial Transformation Training Centre for Molecular Technology in the Food Industry, a multiple diet *in vivo* mouse feeding study was performed to investigate the effect of high fat and dietary fibre inclusion on the physiology of the mouse. Design and running of the animal experiments was a collaborative effort involving the candidate, two other PhD students, and a Post doctorate researcher. A variety of samples including whole blood, liver, faeces, small intestine, caecum and lower intestine, were collected and distributed among the collaborators to measure changes in physiology (SCFAs), liver proteome, gut microbiome, and mucus layer glycome of the mouse, research areas that have been implicated in the maintenance of intestinal health. The C57BL/6 mouse is the most well-known and widely used inbred mouse strains originating from the C57BL parent strain. The C57BL/6 sub strain is commonly used for *in vivo* studies on physiology, immunology, and the development of new genetically deficient mice (Perlman, 2016) and is susceptible to diet induced obesity and type 2 diabetes (Champy et al., 2008).

Mice were fed one of four different diets: a standard rodent diet (NC, 4.8% fat), a high fat diet (HF, 60% fat), a high fat diet modified with 4.7% (w/w) NutriKane (HF-NK, 60% fat), or a high fat diet modified with 4.7% (w/w) Benefibre (HF-BF, 60% fat). Standard (NC and HF) and

custom-made mouse diets (HF-NK and HF-BF) were purchased from Specialty Feeds, (Glen Forrest, Western Australia). HF-NK and HF-BF diets were nutritionally identical to the HF diet (Diet SF02-006) except that 4.7% cellulose that was replaced with an equal percentage of either NutriKane, an insoluble dietary fibre derived from sugarcane, or Benefibre, a soluble dietary fibre derived from wheat. Basic nutritional information for each diet is provided in **Table 4-1**. The chemical composition of the NK and BF fibre supplements is described in Chapter 2 of this thesis. High fat content was achieved using canola oil (10 g/Kg), cocoa butter (40 g/Kg), and clarified butter fat (10 g/Kg). Digestible carbohydrate was supplied by sucrose (10.6 g/Kg). A full breakdown of the HF diet is provided in the Supplementary (Section 4.5).

Table 4-1. Nutritional information of the NC*, HF, HF-NK and HF-BF diets used in this study.

	Diets			
Content (% dry weight)	NC*	HF	HF-NK	HF-BF
Protein	20.0	19.4	19.4	19.4
Total Fat	4.80	60.0	60.0	60.0
Digestible carbohydrate	59.4	16.0	16.0	16.0
Dietary fibre*	16.4	4.70		
Fibre additive	-	-	4.70	4.70

*Ingredients include: Wheat, barley, Lupins, Soya meal.

During the study, mice were housed in a temperature-controlled room on a 12/12 hr dark-light cycle with 2 mice per cage and free access to food and water. Cages were changed weekly to provide clean bedding and fresh feed. Ten-week-old male C57BL/6 mice were separated into two groups and given either the NC (n = 10) or HF diet (n = 42) to observe the effect of high fat on mouse physiology (**Figure 4-7**). After 16 weeks, the HF group was separated into 3 groups that were given either HF (n = 14), HF-NK (n = 14) or HF-BF (n = 14) diet until the end of the 33-week experiment to determine the effect of dietary fibre inclusion on the high fat fed mice. Body weight and feed consumption per cage were measured weekly. At specific points during the experiment blood samples were collected via the submandibular vein and faecal samples were

collected. Changes in diet, group sizes and sample schedule are summarised in Error! Reference source not found.. After 33-weeks, mice were sacrificed, and samples were collected.

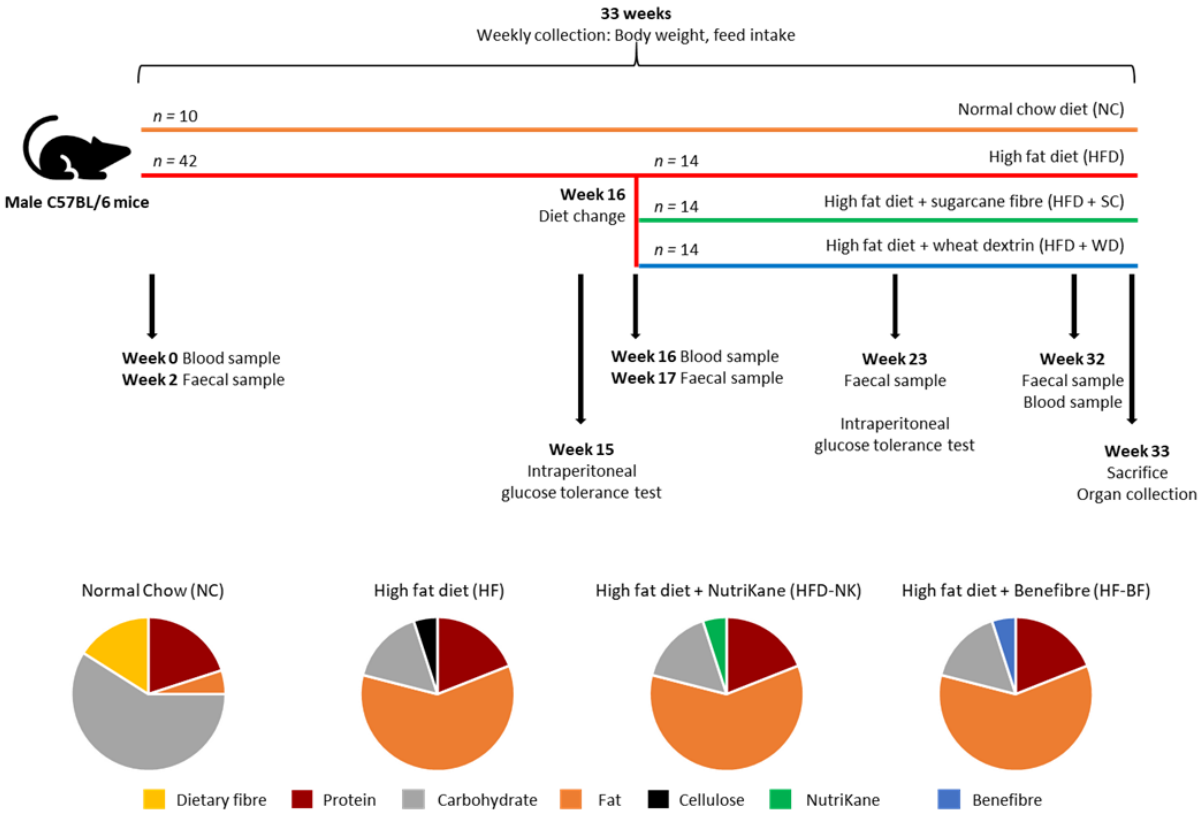


Figure 4-7. Graphical representation of the mouse feeding schedule illustrating changes in diet and group size and the timing of sample collection and tolerance tests. Pie charts represent the nutritional content of the four different diets emphasising the differences in fat content between NC and high fat diets (HF, HF-NK, HF-BF). The 16% dietary fibre of the NC diet is included in the carbohydrate content.

Whole blood was collected via the retro-orbital sinus following deep anaesthesia with isoflurane. Mice were then euthanised by spinal dislocation and the liver and intestinal tract were carefully removed. The intestinal tract was separated into ileum, caecum, and colon and the intestinal contents was removed by flushing with phosphate buffered saline. The ileum, caecum, and colon were then placed into separate tubes and snap frozen on dry ice. All procedures were performed in accordance with animal ethics approval (Number: 5201500129 - Dr Kautto - (Collaborative) Effect of Nutrikane on immune system and gut microbiome in lean, obese and diabetic mice). Whole blood and liver were given to Daniel Bucio Noble to measure changes in blood biomarkers such as cytokines, and the liver proteomes respectively. Faeces and gut contents were given to

Hasinika Hewawasam Gamage to measure the diet induced changes in the composition of the gut microbiome. Faecal samples were used by me to measure the concentration of SCFAs in faeces using the method optimised in Chapter 3 of this thesis. Finally, the colon was used by the candidate to determine changes in mucus layer glycosylation.

4.3.1.1 Effect of high fat and dietary fibre total weight gain and glucose tolerance

After 33 weeks of feeding, the average body weight of the HF-NK but not HF or HF-BF groups was significantly higher than the NC group (**Table 4-2**). In general, we observed that weight gain was more pronounced in one mouse per cage suggesting that co-housing may have introduced dominant/submissive behaviour. However, both mice were given free access to food and feed intake per mouse was calculated by dividing total feed intake per cage by the number of mice present.

Feed intake per mouse was significantly higher in the NC group than the HF groups suggesting differences in palatability with a greater preference for the NC diet than the HF diets. This was in contrast to previous studies that have reported a hedonic preference in animals for fat that increases with fat concentration (Bake et al., 2014; Imaizumi et al., 2000). When converted to energy intake, only the HF-NK and HF-BF, but not HF groups, were significantly higher than the NC group.

Fasting blood glucose levels (FBGL) were measured at week 4, 15, 23, and 33. Initial FBGLs were between 5.9 to 6.8 mmol L^{-1} with no significant difference between groups. There was no significant difference between groups by the end of the experiment though FBGLs had increased to 8.0 to 8.7 mmol L^{-1} (144 to 157 mg/dl) (**Table 4-2**) suggesting that glucose intolerance was acquired by the NC group. Using the threshold of 200 mg/dl as diagnostic of diabetes (Surwit et al., 1988), we can conclude that none of these diets clearly induced type 2 diabetes.

Table 4-2. Body weight, food and energy intake, fasting blood glucose levels (FBGL) in mice fed; high fat diet (HF), high fat diet supplemented with NutriKane (HF-NK), high fat diet supplemented with Benefibre (HF-BF), and normal chow (NC). FBGL in mg/dl were calculated by multiplying mmol L^{-1} by 18. Values are mean and standard deviation. Values in **bold** were significantly different to NC when compared by two-way ANOVA and Tukeys testing.

	Groups							
	HF		HF-NK		HF-BF		NC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Initial body weight (g)	26.6	1.2	27.1	1.6	27.7	1.3	26.1	1.3
Final body weight (g)	36.7	6.1	**40.2**	8.3	37.2	7.8	34.5	3.5
Total weight gained (g)	10.1	6.1	**13.1**	8.2	9.5	7.4	8.4	2.9
Feed intake (g/week/mouse)	**15.6**	0.7	**16.3**	0.6	**16.8**	0.9	25.0	1.2
Energy intake (Kcal/week)	**89.4**	3.9	**93.6**	3.3	**96.5**	4.9	83.7	4.2
Initial FBGL (mmol L^{-1})	5.9	1.8	6.1	0.9	6.8	2.0	6.3	1.0
Final FBGL (mmol L^{-1})	8.0	1.6	8.6	1.9	8.5	1.9	8.7	2.2
Initial FBGL (mg/dl)	106	32	110	17	122	37	114	18
Final FBGL (mg/dl)	144	30	154	34	154	35	157	40

4.3.1.2 High fat and dietary fibre modification alter the production of SCFAs

To investigate the effect of high fat and dietary fibre on the metabolic output of the gut microbiota, we determined the concentration of SCFAs in the faecal contents of mice fed a normal diet (NC), high fat diet (HF), or high fat diet modified with NutriKane (HF-NK) or Benefiber (HF-BF). Faecal samples were collected at weeks 2, 16, 23, and 32. SCFAs were extracted from faecal pellets with acidified ethanol (0.01% v/v TFA) and concentrations were determined by GC-FID (refer to method in Chapter 3). Differences in SCFA production were apparent by the second week. By week 16, the NC diet produced 2.5, 1.9, and 6.6 times higher concentrations of Ace, Pro, and But respectively in comparison to the HF diet (**Figure 4-8**).

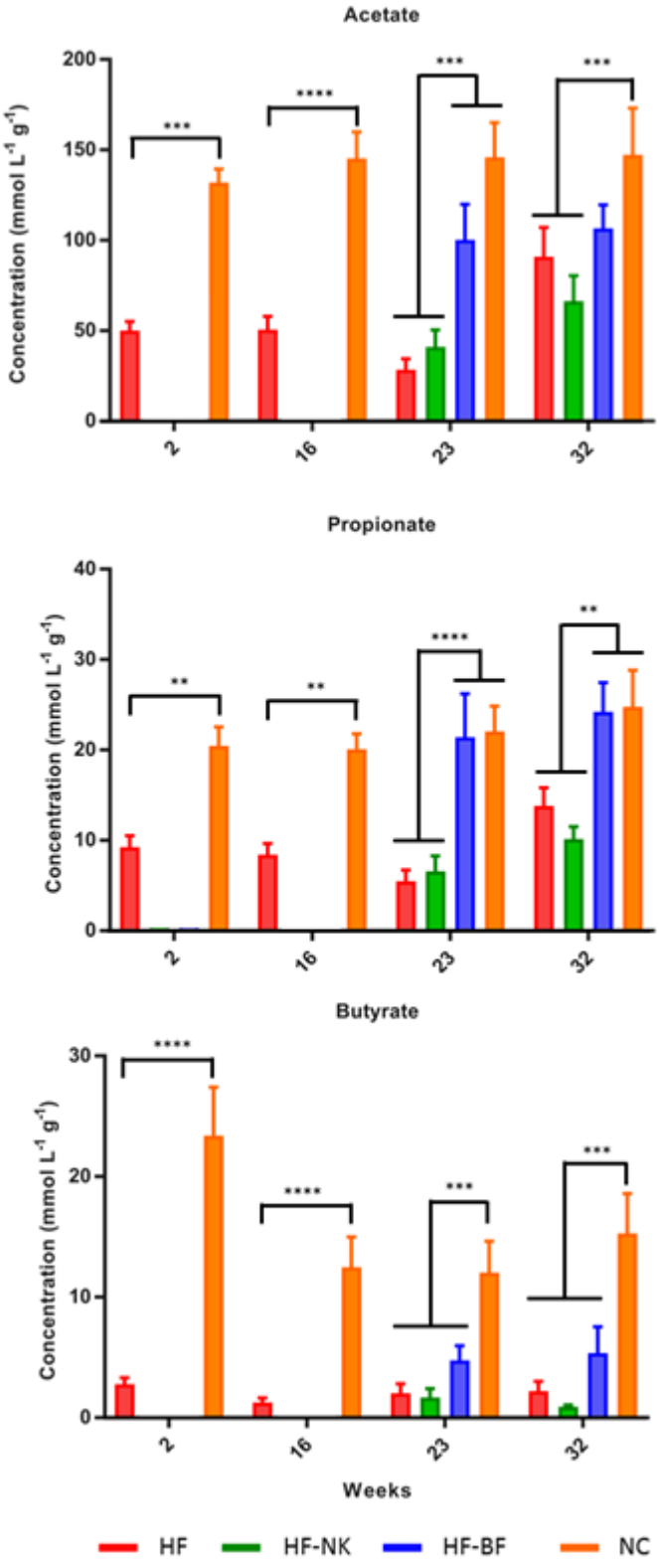


Figure 4-8. Concentrations of acetate, propionate, and butyrate in faecal samples collected at weeks 2, 16, 23, and 32 as determined by GC-FID. At weeks 2 and 16, mice in HF, HF-NK and HF-BF groups are shown grouped together since separation occurred after week 16. At weeks 23 and 32, mice were fed their respective HF, or HF modified diets and are grouped accordingly. Bars represent the group mean ± the standard error of the mean (SEM). NC n = 9, HF n = 14, HF-NK n = 14, HF-BF n = 14.

The lower SCFA concentrations with the HF diet suggest that the 60% fat diet is less fermented than the NC diet and this is consistent with observations that during periods of low fermentable fibre intake, microbial fermentation is reduced, resulting in low SCFA yields (Murphy et al., 2010; Murugesan et al., 2018; Ríos-Covián et al., 2016.). Upon the introduction of Benefiber to the HF diet (HF-BF), Ace, Pro and But concentrations significantly increased by 111%, 146% and 194% times respectively by week 23 to the point where Ace and Pro concentrations were similar to that of the NC diet. This is consistent with our findings from the *in vitro* gut-mimicking system (Section 3.3.1) that demonstrated the high fermentability of Benefiber.

In contrast, the introduction of NutriKane to the HF diet (HF-NK) did not significantly increase SCFA concentrations compared to the HF diet at week 23. Again, these results are consistent with our *in vitro* findings in Section 3.3.1 that demonstrated the low fermentability of NutriKane due to its insoluble nature. At week 32, we saw no change in SCFA concentrations from week 23 in the NC and HF-BF groups. However, at week 32, Ace and Pro concentrations significantly increased by 151% and 98% respectively in the HF group, and 49% and 42% in the HF-NK group. The butyrate concentration in all three HF diets remained low throughout the entire experiment. The increase in Ace and Pro concentrations at week 32 may represent a long-term effect of high fat diet on the fermentative capacity of the gut microbiota, suggesting a mechanism by which the production of important metabolites like SCFAs could be maintained.

Previous work by Desai et al. (2016) has shown that in the absence of fermentable dietary fibre, specific members of the gut microbiota can shift their metabolic specificity to instead degrade host derived polysaccharides as a primary carbon source and may explain why SCFA production increased slowly over time. Alternatively, we may be observing the acquisition of a HF diet-induced microbiota capable of increased SCFA production and enhanced energy harvest that is responsible for dysbiosis associated obesity. Previous studies have shown that a high fat diet induces changes in gut microbiome structure characterised by an increase in the Firmicutes to Bacteroides ratio (Hildebrandt et al., 2009; Lu et al., 2016; Murphy et al., 2015; Zhang et al.,

2012). This HF diet-induced dysbiosis of the gut microbiome has been linked to the development of obesity and related metabolic disorders (Cani et al., 2008; Duca et al., 2014; Shen et al., 2013). The most convincing evidence for this association comes from the work of Duca et al. (2014) that showed that transfer of obese-prone microbiota to germ free rats replicated the characteristics of the obese-prone phenotype such as weight gain, adiposity and inflammation.

Our results in the mouse correlate well with our *in vitro* study (Section 3.3.1) in demonstrating that fermentable dietary fibres such as Benefiber can increase the production of SCFAs in high fat and low fermentable fibre conditions in an *in vivo* setting. The production of SCFAs by the gut microbiota contributes to intestinal health by modulating the intestinal environment, controlling the composition of the gut microbiota, and regulating host metabolism (Ríos-Covián et al., 2016). SCFA production also has been shown to have effects on overall health, with previous work showing that SCFAs, particularly propionate and butyrate, protect against HF diet-induced obesity (den Besten et al., 2015; Lin et al., 2000; Lu et al., 2016). HF diet-induced obesity is believed to be caused by an increased capacity of the HF diet-induced microbiome for energy harvest and storage through the increased fatty acid metabolism and altered energy intake characterised by greater abundance in SCFAs (Schwiertz et al., 2010; Wolf and Lorenz, 2012). However, there are conflicting reports on the changes in SCFA production regarding obesity with some studies reporting higher concentrations in obese subjects compared to lean subjects (Schwiertz et al., 2010; Turnbaugh et al., 2006) and other studies like ours that report reduced SCFA concentrations in response to HF diet compared to normal control diets (Murphy et al., 2010). These discrepancies may be the result of different shifts in microbial cross-feeding patterns, diet compositions, and genetic background. Overall, our results highlight the need for further studies to conclusively define the role of SCFAs in intestinal and general health.

4.3.1.3 Muc2 contains predominantly Core 1 and 2 type O-glycans

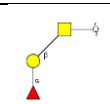
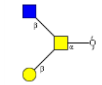
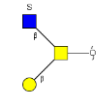
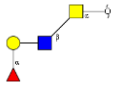
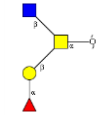
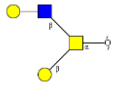
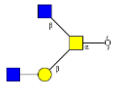
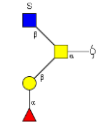
Mucus layer glycosylation was determined by PGC-LCMS analysis of glycans released by reductive β-elimination from Muc2 partially purified by precipitation with guanidinium

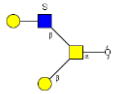
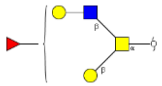
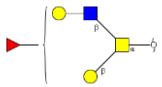
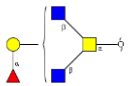
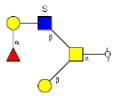
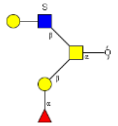
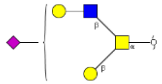
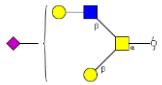
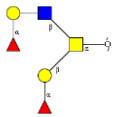
hydrochloride (Larsson et al., 2009). Mucus acquired by vacuum suction sampling from surgically removed mouse colon which was considered the preferable method to obtain mucus since physical scraping is more likely to harvest the epithelium and unsecreted stored Muc2. Partial purification of Muc2 using guanidinium hydrochloride precipitation has been shown to isolate a range of other proteins that are closely associated with Muc2 (Kesimer and Sheehan, 2012). Without separation by electrophoresis (Larsson et al., 2009), these associated proteins could still be present in our samples and complement to Muc2 *O*-glycans present in our analysis.

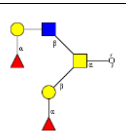
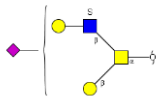
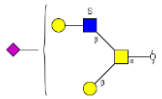
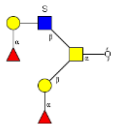
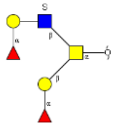
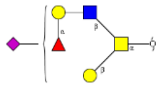
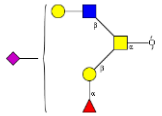
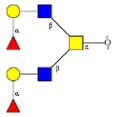
Glycan isomers were structures were assigned using MS/MS fragmentation as previously described in Section 4.2.4. Individual glycans are referred to by their neutral mass [M] calculated by multiplying their mass to charge ratio (*m/z*) by the charge status and correcting for the loss of protons. A total of 29 *O*-glycan compositions were identified with 38 unique structures annotated and accounted for by multiple isomeric structures. Identified glycan compositions, their proposed structures, and relative abundance are summarised in **Table 4-2**.

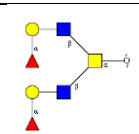
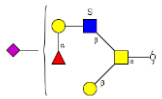
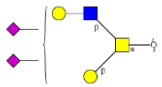
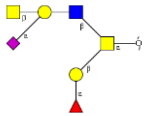
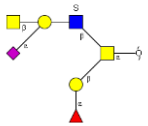
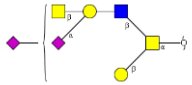
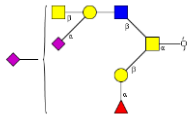
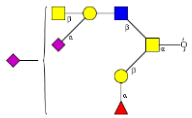
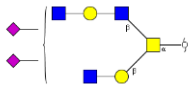
The glycans 531 Galβ1-3GalNAcol, 734b Fucα1-2Galβ1-3(GlcNAcβ1-6)GalNAcol and 814 Fucα1-2Galβ1-3(SO_3-6GlNAcβ1-6)GalNAcol were highly abundant (7 – 31%) in all samples which was consistent with previous studies (Arike et al., 2017; Holmén et al., 2013). The remaining *O*-glycans were generally found in low abundance (<5%) or were completely absent depending on the sample. While a high degree of individual variation between samples was observed for specific glycans, the glycosylation pattern of Muc2 was consistent within each diet (**Figure 4-9**). Muc2 carried a mixture of neutral and acidic *O*-glycans that were predominantly Core 1 and Core 2 type structures. The highly abundant (7 – 22%) 531 Fucα1-2Galβ1-3GalNAcol glycan was the main Core 1 structure present in all samples. Only one Core 3 and three Core 4 structures were identified. Altogether, these were extremely low in abundance (<2%) or absent in some samples. Our results are highly consistent with previous studies showing the prevalence of Core 1 and Core 2 type glycans (Arike et al., 2017; Holmén et al., 2013; Thomsson et al., 2012).

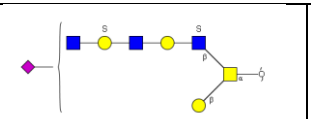
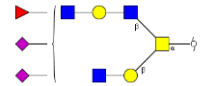
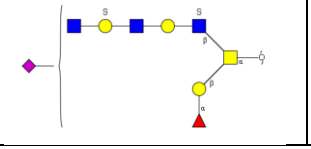
Table 4-3. Summary of *O*-glycans released from Muc2 obtained from the colon of mice fed different diets. NC: standard diet, HF: high fat diet, HF-NK: high fat diet modified with NutriKane, HF-BF: high fat diet modified with Benefibre. Values provided are in % relative abundance .

Mass (m/z)			Composition	Core	Proposed structure*	NC	HF	HF-NK	HF-BF
$[M-H]^-$	$[M-H]^{2-}$	[M]				Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
530		531	(Hex)1 (HexNAc)1 (Deoxyhexose)1	1		12.6 ± 4.4	12.0 ± 6.6	19.0 ± 7.4	15.8 ± 9.1
587		588	(Hex)1 (HexNAc)2	2		5.4 ± 3.2	5.3 ± 3.9	14.3 ± 8.1	11.6 ± 8.3
667		668	(Hex)1 (HexNAc)2 (Sulph)1	2		4.5 ± 2.6	2.5 ± 2.4	8.1 ± 4.3	9.7 ± 7.4
733a		734a	(Hex)1 (HexNAc)2 (Deoxyhexose)1	3		2.0 ± 2.6	1.9 ± 3.2	4.2 ± 4.3	2.6 ± 2.6
733b		734b	(Hex)1 (HexNAc)2 (Deoxyhexose)1	2		6.4 ± 4.6	13.9 ± 5.4	15.5 ± 7.1	21.0 ± 10.2
749		750	(Hex)2 (HexNAc)2	2		1.2 ± 1.2	0.1 ± 0.2	0.5 ± 0.8	0.0 ± 0.0
790		791	(Hex)1 (HexNAc)3	2		0.5 ± 0.7	0.2 ± 0.4	0.3 ± 0.7	0.5 ± 0.7
813		814	(Hex)1 (HexNAc)2 (Deoxyhexose)1 (Sulph)1	2		11.7 ± 7.9	13.9 ± 7.6	19.8 ± 11.3	20.6 ± 8.0

829	830	(Hex)2 (HexNAc)2 (Sulph)1	2		1.4 ± 2.0	0.2 ± 0.4	0.2 ± 0.5	0.2 ± 0.5
895a	896a	(Hex)2 (HexNAc)2 (Deoxyhexose)1	2		1.9 ± 1.1	2.8 ± 1.7	0.8 ± 1.9	1.1 ± 1.9
895b	896b	(Hex)2 (HexNAc)2 (Deoxyhexose)1	2		2.9 ± 2.1	1.3 ± 1.5	0.4 ± 1.1	1.2 ± 1.9
936	937	(Hex)1 (HexNAc)3 (Deoxyhexose)1	4		1.3 ± 1.9	1.4 ± 1.2	0.0 ± 0.0	0.5 ± 0.8
975a	976a	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (Sulph)1	2		4.2 ± 3.2	3.1 ± 2.8	0.4 ± 0.4	0.6 ± 0.8
975b	976b	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (Sulph)1	2		0.9 ± 1.2	1.2 ± 1.5	0.8 ± 1.2	0.0 ± 0.0
1040a	1041a	(Hex)2 (HexNAc)2 (NeuAc)1	2		0.9 ± 1.4	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
1040b	1041b	(Hex)2 (HexNAc)2 (NeuAc)1	2		4.6 ± 2.1	4.5 ± 3.8	0.0 ± 0.1	0.1 ± 0.2
1041	1042	(Hex)2 (HexNAc)2 (Deoxyhexose)2	2		9.8 ± 4.1	10.3 ± 5.3	0.1 ± 0.3	0.2 ± 0.4

1041		1042	(Hex)2 (HexNAc)2 (Deoxyhexose)2	2		0.7 ± 0.8	1.3 ± 1.5	0.0 ± 0.0	0.1 ± 0.1
1120a		1121a	(Hex)2 (HexNAc)2 (NeuAc)1 (Sulph)1	2		1.4 ± 1.5	0.4 ± 1.0	0.0 ± 0.0	0.1 ± 0.3
1120b		1121b	(Hex)2 (HexNAc)2 (NeuAc)1 (Sulph)1	2		7.0 ± 3.6	9.0 ± 4.7	1.1 ± 0.8	1.4 ± 1.0
1121		1122a	(Hex)2 (HexNAc)2 (Deoxyhexose)2 (Sulph)1	2		0.3 ± 0.4	0.1 ± 0.2	0.0 ± 0.1	0.1 ± 0.2
	560	1122b	(Hex)2 (HexNAc)2 (Deoxyhexose)2 (Sulph)1	2		0.3 ± 0.3	0.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
1187a		1188	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1	2		0.9 ± 0.6	0.9 ± 0.7	0.0 ± 0.1	0.2 ± 0.3
1187b		1188	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1	2		1.0 ± 0.6	1.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0
1245a		1246a	(Hex)2 (HexNAc)3 (Deoxyhexose)2	4		0.8 ± 0.9	1.0 ± 1.1	0.2 ± 0.6	0.0 ± 0.1

1245b		1246b	(Hex)2 (HexNAc)3 (Deoxyhexose)2	4		2.9 ± 2.7	1.2 ± 0.8	0.0 ± 0.0	0.1 ± 0.2
1266		1267	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 (Sulph)1	2		0.9 ± 0.5	0.8 ± 0.6	0.0 ± 0.0	0.1 ± 0.2
1332		1333	(Hex)2 (HexNAc)2 (NeuAc)2	2		0.9 ± 1.0	0.7 ± 0.7	1.5 ± 0.9	1.7 ± 1.8
1390		1391	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1	2		0.5 ± 0.5	1.0 ± 0.7	1.0 ± 0.8	1.3 ± 1.6
	734	1470	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 (Sulph)1	2		7.0 ± 3.0	2.3 ± 0.8	7.3 ± 5.9	4.0 ± 3.0
	766	1535	(Hex)2 (HexNAc)3 (NeuAc)2	2		0.4 ± 0.6	0.1 ± 0.3	2.8 ± 1.7	1.5 ± 1.5
	840a	1682a	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2	2		1.5 ± 1.8	1.8 ± 1.5	0.0 ± 0.0	2.2 ± 1.6
	840b	1682b	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2	2		0.5 ± 0.7	0.4 ± 0.4	0.3 ± 0.4	0.2 ± 0.2
	868	1739	(Hex)2 (HexNAc)4 (NeuAc)2	2		0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.2

884	1770	(Hex)3 (HexNAc)4 (NeuAc)1 (Sulph)2	2		1.5 ± 1.8	0.9 ± 0.8	0.4 ± 0.8	0.4 ± 0.3
941	1885	(Hex)2 (HexNAc)4 (Deoxyhexose)1 (NeuAc)2	2		0.6 ± 1.2	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
957	1916	(Hex)3 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 (Sulph)2	2		0.2 ± 0.4	0.4 ± 0.6	0.0 ± 0.0	0.4 ± 0.7

*Proposed structures were based on comparison with the structures reported in previous studies (Arike et al., 2017; Thomsson et al., 2012).

4.3.1.4 Dietary fibre but not high fat content induces changes in Muc2 glycosylation

To determine the effect of diet on Muc2 glycosylation, we compared the glycosylation profile of Muc2 obtained from the colon of mice fed one of four diets: a standard rodent diet (NC), a high fat diet (HF), a high fat diet modified with NutriKane (HF-NK), and a high fat diet modified with Benefibre (HF-BF). To our knowledge, this is the first study to report the detailed characterisation of diet induced changes in Muc2 glycosylation in the mouse colon. Previous studies have shown that dietary fibre intake increases the thickness of the mucus layer (Brownlee et al., 2003; Hedemann et al., 2009; Jamroz et al., 2006; Knudsen, 2014). Additionally, diet and nutrition has proposed to influence glycosylation. While an increase in fat from 10% to 26% of the diet did not alter the mucin composition of the intestine (Sharma et al., 1995), introduction of pectin and cellulose has been reported to increase the abundance of neutral monosaccharides and decrease the abundance of fucose in the glycan component of small intestinal glycoproteins (Tardy et al., 1995). However, these studies focussed on the epithelial surface of the gastrointestinal tract and their observations may not apply to secreted mucins such as Muc2.

Two-way analysis of variance (ANOVA) using Tukeys tests to perform comparisons of each glycan found between each group identified nine *O*-glycans that were significantly different between the four different diets (**Figure 4-9**). The glycosylation patterns of the HF and NC groups were surprisingly similar with only one glycan, the 734b Fuc-Gal-3(GlcNAc-6)GalNAcol isomer, significantly higher in abundance in all three high fat diets (HF, HF-NK, HF-BF) compared to NC suggesting that this glycan could be associated with changes in diet composition. The 734b isomer could be associated with high fat content or changes in dietary fibre content, however, which of these is true is difficult to determine without knowing the precise composition of each diet.

Similarities were also seen between the HF-NK and HF-BF groups that both expressed lower glycan diversity and a higher abundance of glycans smaller than the 814 Fuc-Gal-3(SO_3-GlcNAc-6)GalNAcol glycan. Across all four diets, the 531 Gal-3GalNAcol, 588 Gal-3(GlcNAc-

6)GalNAcol, 668 Gal-3(SO_3-GlcNAc-6)GalNAcol, and 814 Fuc-Gal-3(SO_3-GlcNAc-6)GalNAcol glycans were significantly higher in HF-NK and HF-BF compared to HF and NC while the 1042a Fuc-Gal-3(Fuc-Gal1-3/4GlcNAc-6)GalNAcol, 1042b Fuc-Gal-3(Fuc-Gal1-3/4GlcNAc-6)GalNAcol, and 1121a Fuc-Gal-3(Fuc-Gal1-3/4(SO_3)GlcNAc-6)GalNAcol were higher in HF and NC compared to HF-NK and HF-BF.

To further assess the glycosylation patterns, we used two-dimensional principal component analysis (PCA), a multivariate chemometric technique that reduces the dimensionality of a dataset to give a set of "principal components" to best explain and portray variation (Hervé and J., 2010; Wold et al., 1987). The goals of PCA are to 1) extract the most important information from the data table; 2) compress the size of the data set by keeping only this important information; 3) simplify the description of the data set; and 4) analyse the structure of the observations and the variables (Hervé and J., 2010).

The two dimensional (2D)-PCA plot showed clustering and tight overlap of the HF-NK and HF-BF groups indicating similarities between the glycosylation patterns of the two groups in support of our previous observations (**Figure 4-10**). The NC and HF groups also grouped together indicating again their similarities, though this clustering showed less overlap. The NC and HF groups were clearly separated from the HF-NK and HF-BF groups supporting our earlier observation that HF was similar to NC, and HF-NK was similar to HF-BF (**Figure 4-9**). The similarities in glycosylation of HF and NC was surprising considering the large difference in diet composition. According to our results, increasing dietary fat content from 4.8% to 60% and decreasing digestible carbohydrate content from 60% to 16% caused no significant changes in Muc2 glycosylation.

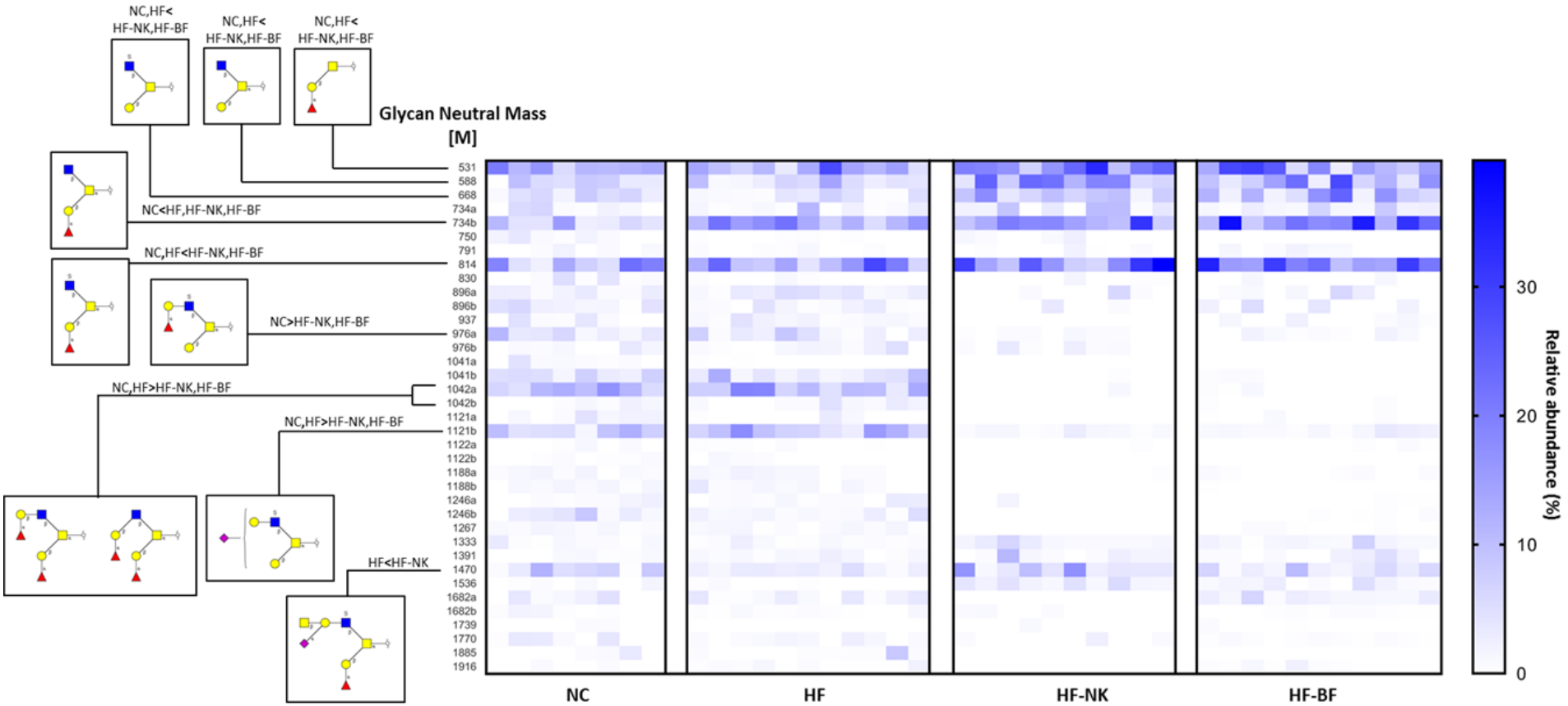


Figure 4-9. Un-clustered heat map illustrating the relative abundance of Muc2 *O*-glycans from the colon of mice fed NC, HF, HF-NK, and HF-BF diets. Each column represents an individual mouse. Glycans are listed according to their neutral mass [M] with isomers labelled alphabetically according to their retention time order (a elutes before b). Relative abundance is represented by shading with darker shades representing higher abundance. The structures of *O*-glycans that were found to be significantly different between any of the groups by two-way ANOVA and multiple comparison by Tukeys testing are shown on the left. For each glycan that was significant, < or > symbols are used to describe the difference between diets, a comma (,) is used to describes diets that are not significantly different from each other

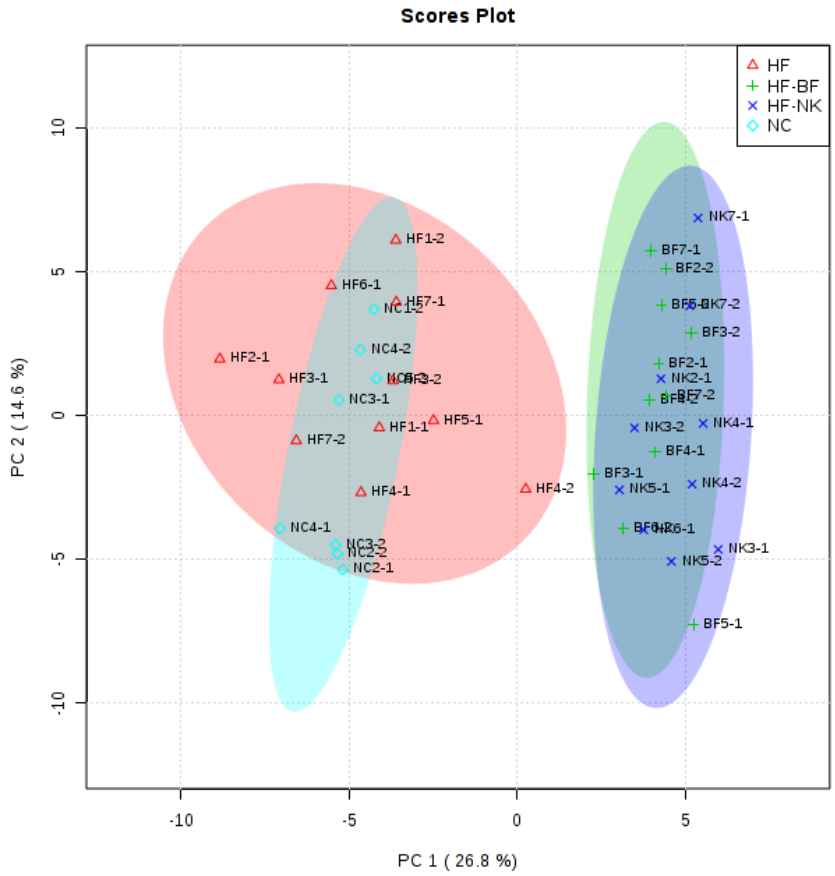


Figure 4-10. Two-dimensional principal component analysis (2D-PCA) plot of the *O*-glycosylation pattern of Muc2 from mouse colon. Significant overlap between NC and HF diets, and HF-NK and HF-BF diets respectively was indicative of how alike the glycosylation patterns were between diets. *n*NC = 8, *n*HF = 11, *n*HF-NK = 10, *n*HF-BF = 11.

Conversely, the similarities between the Muc2 glycosylation of high fat diets supplemented with different fibres (HF-NK and HF-BF) suggests that replacement of 5% cellulose with NutriKane or Benefibre in a HF diet has a greater effect than altering fat content. The considerable variation between the HF and the dietary fibre modified groups (HF-NK and HF-BF) was unexpected since these three diets shared the same high amounts of fat (60%). This was initially postulated to have a larger impact on Muc2 colon glycosylation. The similarity between Muc2 glycosylation in the HF-NK and HF-BF fed mice was also surprising because although Nutrikane and Benefibre are both dietary fibres, the former is classified as an insoluble fibre while the latter is completely soluble. The chemical differences between soluble and insoluble dietary fibre classifications are quite significant (as shown in Chapter 2) and have been shown to impart unique physical properties (Lunn and Buttriss, 2007), induce characteristic physiological responses (Brownlee, 2011), and

alter the composition of the gut microbiota in an *in vitro* gut mimicking system (Chapter 3). For example, soluble fibres are readily fermented by the gut microbiota while insoluble fibres are less well fermented and instead physically stimulate the gut lining and contribute to faecal bulking (Brownlee, 2011; Guillon and Champ, 2000). How two completely different types of dietary fibre with vastly different physical properties can induce similar changes in Muc2 glycosylation is not currently understood. The lack of clear distinctions between the four diets may have been influenced by sampling of the entire length of the colon. Previous studies have established that Muc5ac and Muc2 *O*-glycosylation of C57BL/6 mice exhibits regiospecific changes along the gastrointestinal tract (Holmén et al., 2013). Furthermore, the proximal and distal mouse colons have been shown to differ considerably and it is likely that pooling together the two sections of the colon may have masked diet dependent glycosylation patterns.

4.3.1.5 16S rRNA sequencing of the faecal microbiota

To determine if the different diets were inducing changes in the composition of the gut microbiota that could be related to the differences in MUC2 glycosylation, we (Hasinika H. Gamage) obtained the results from 16S rRNA sequencing of faecal samples from individual mice collected at 0, 17, 23, and 32 weeks of the mixed-diet feeding study (**Figure 4-18**, supplementary material). DNA sequencing clearly showed significant differences in microbial community structure between the HF, HF-NK and HF-BF and the standard diet (NC) from as early as 17 weeks. Additionally, the HF, HF-NK and HF-BF groups appeared more alike each other than to the NC. These similarities in microbial community structure were not reflected in Muc2 glycosylation patterns that instead showed similarities between HF and NC, and between HF-NK and HF-BF. These results would suggest that the changes in microbial community structure were not consistent with overall changes in Muc2 glycosylation. However, we must acknowledge that 16S rRNA sequencing was performed on the faecal microbiota and is not truly representative of the mucus adhered microbiota that are more associated with modulation of glycosylation pathways and the enzymatic degradation of host glycans due to their proximity to the host in the mucus layer.

4.3.1.6 Dietary intervention alters *O*-glycosylation terminal epitopes

Muc2 *O*-glycans from mouse colon were found to carry a variety of terminal residues including fucose (Fuc), sialic acid (NeuAc), and sulfate, with some glycans containing a combination of two or all three types of residues. Fucose as part of the Blood group H antigen (Fucα1-2Gal) was highly abundant with approximately 72% of all glycans detected containing at least one fucose in this epitope (**Figure 4-11**). Consistent with previous studies (Arike et al., 2017; Thomsson et al., 2012), the other fucose containing epitopes such as the Lewis and sialyl Lewis antigens that are common in humans (Capon et al., 2001) were not found in our analysis most likely due to mice not possessing the necessary fucosyltransferases.

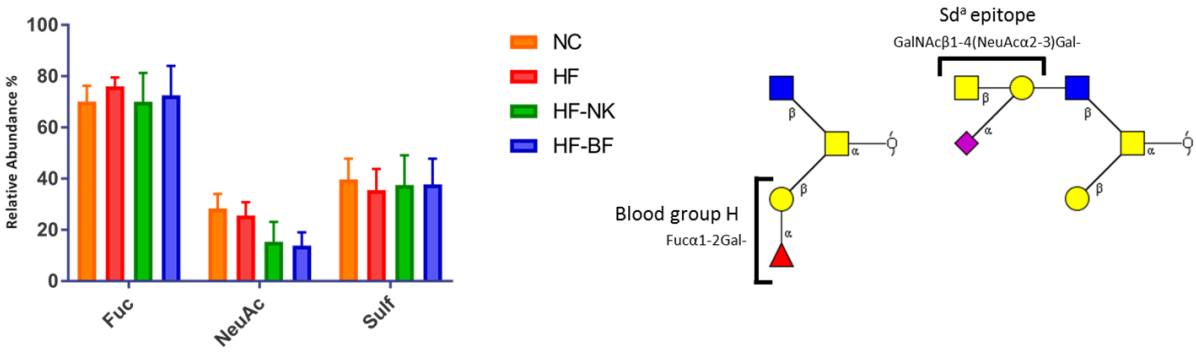


Figure 4-11. Bar graph illustrating the relative abundance of terminal residues across the different groups. The structure of the Blood group H antigen and Sd[a] epitope are also shown on the right. Fuc: Fucose, NeuAc: N-Acetylneuraminic acid (Sialic acid), Sulf: Sulfate. Values are the group mean ± the SD.

Terminal sialic acid (NeuAc) was much less abundant than fucose with approximately 19% of glycans containing at least one NeuAc residue. When present, NeuAc was found attached to Gal, however, due to the limitations of MS fragmentation, the linkage of NeuAc was not determined. NeuAc was also be found as part of the Sd[a] antigen (**Figure 4-11**), a non-ABO blood group epitope that is more common in the human gastrointestinal tract (Capon et al., 2001). There appeared to be a slight decrease in abundance of *O*-glycans containing NeuAc upon the replacement of 4.7% (w/w) cellulose with an equal percentage of NutriKane or Benefiber. Sulfate was more abundant than NeuAc but less abundant than fucose with approximately 38% of glycans containing at least one sulfate residue. Sulfate was found attached to the 6-arm GlcNAc when only one sulfate was

present. In the case of two sulfate residues, Gal on a 6-arm extension was the usual site of attachment. Since Muc2 glycans can contain combinations of these terminal residues we then sorted the identified *O*-glycans into categories according to types of terminally attached residues and plotted the relative abundance across the NC, HF, HF-NK, and HF-BF groups As before, Fuc was the most abundant type terminal residue followed by the combination of Fuc and Sulf (**Figure 4-12**). In general, the HF and NC groups were almost identical to each other in terms of terminally attached residues and HF-NK and HF-BF shared an almost identical glycosylation pattern in the types of terminally attached residues. We found a significant increase in abundance of glycans containing only Gal or GlcNAc as terminal residues in the HF-NK and HF-BF groups compared to the HF and NC groups (**Figure 4-12**) indicating that the abundance of terminally attached fucose, NeuAc or Sulf was reduced with both dietary fibre inclusion.

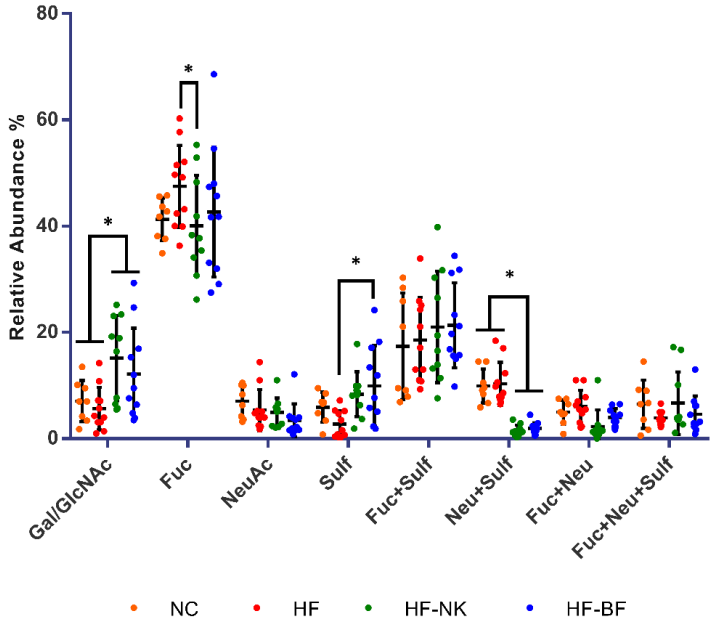


Figure 4-12. MUC2 *O*-glycans grouped by the type of terminal residues. Gal/GlcNAc represent *O*-glycans that terminate in either Gal or GlcNAc and do not contains Fuc, NeuAc or Sulf. Each point represents a single mouse. Bars represent the mean ± the SD.

Glycans containing only Fuc as terminal residues appeared to increase in abundance in HF compared to NC, however, this was not statistically significant. Compared to HF, the HF-NK group exhibited a significant decrease in the abundance of fucosylated glycans. Since the

fucosylated glycans in our samples could contain one or two Fuc residues, we further separated them into groups by the number of Fuc residues. The HF-NK and HF-BF groups exhibited a higher abundance of monofucosylated (**Figure 4-13**, Fuc) glycans compared to NC and a lower abundance of difucosylated (**Figure 4-13**, Fuc-Fuc) glycans compared to both HF and NC.

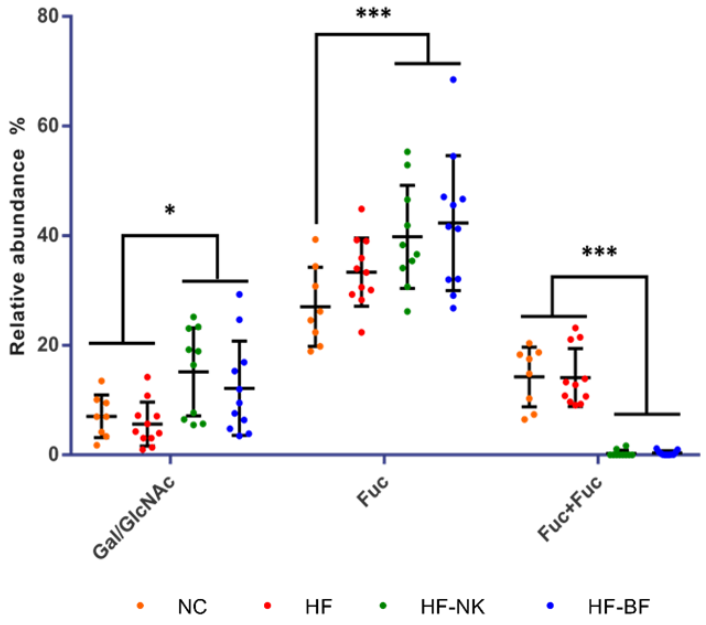


Figure 4-13. Comparison of *O*-glycans containing terminal Gal/GlcNAc, one fucose (Fuc) or two fucose (Fuc+Fuc) residues. Each point represents a single mouse. Bars represent the mean ± the SD.

Fucosylated epitopes such as the blood group antigens are highly immunogenic preventing successful red blood cell transfusions between incompatible individuals (Hooper and Gordon, 2001). Fucosylated glycans are also known as key mediators in host-microbe interactions by acting as potential binding sites for bacterial adhesins (Pickard and Chervonsky, 2015; Pickard et al., 2014). Fucose is also known to influence the composition of the gut microbiota by regulating commensal colonisation (Pickard and Chervonsky, 2015) and studies have shown that mice lacking the *Fut2* enzyme exhibit significantly different microbiota compositions than the wild type (Kashyap et al., 2013). Furthermore, *Fut2* deficient mice are more susceptible to infection with *Salmonella typhimurium* (Goto et al., 2014), *Citrobacter rodentium* (Pham et al., 2014), and

Candida albicans (Hurd and Domino, 2004), implying that terminal α1-2 fucosylation has an inhibitory role against pathogen adhesion. Our results showed no effect of high fat on Muc2 fucosylation, however, upon the addition of both NK and BF dietary fibres, monofucosylated glycans increased while difucosylated species decreased in abundance. The purified forms of dietary fibre in the HF, HF-NK, and HF-BF diets, which were cellulose, NutriKane, and Benefiber, respectively, may indicate that the inclusion of purified forms of dietary fibre prompts a signal that alters fucosylation patterns with the purpose of applying selective pressure to the gut microbiota.

No differences were seen in glycans containing only NeuAc or glycans with Fuc+NeuAc or all three terminal residues. However, there was a significant decrease in the abundance of glycans containing NeuAc and Sulf in both HF-NK and HF-BF compared to the HF and NC diets (**Figure 4-12**, NeuAc+Sulf). This may also explain the slight decrease in total NeuAc observed earlier. Of the three glycans that comprised this group, the 1121b [NeuAc]Galβ1-3(Galβ1-3/4(SO_3-6)GalNAcol *O*-glycan was significantly higher in abundance in HF and NC compared to HF-NK and HF-BF. NeuAc is well known as a component of binding epitopes for a number of bacterial and viral pathogens (Varki, 2008) and the presence of NeuAc as part of the Sd^a epitope has been implicated in the adhesion of microbes (Rausch et al., 2011). Additionally, knockout of *B4GalNT2*, which is required for Sd^a synthesis, altered the composition of the gut microbiota suggesting involvement in microbial selection (Staubach et al., 2012). The Sd^a epitope is also involved in a variety of other physiological processes including inhibition of gastrointestinal cancer cell metastasis (Kawamura et al., 2005) and mediating embryo attachment in porcine primordial germ cells, although how this relates to the gastrointestinal tract is unclear (Klisch et al., 2011).

The identification of the glycohydrolase 33 (GH33) family of sialidases that are present in many gut microbes suggest involvement in bacterial selection and cross feeding mechanisms (Corfield et al., 1992; Tailford et al., 2015). For example, the prominent commensal *Bacteroides thetaiotaomicron* lacks the necessary pathway for NeuAc metabolism despite possessing the GH

sialidase to cleave it (Marcobal et al., 2013). The removal of terminal NeuAc is thought to be an initial step in mucin degradation since the presence of NeuAc may prevent the action of other GHs (Tailford et al., 2015). The microbial consumption of NeuAc has been shown to contribute to intestinal inflammation by enabling the proliferation of pathogenic *Escherichia coli* and *Enterobacteriaceae*, which consume NeuAc despite not possessing the machinery required to liberate it from glycans (Huang et al., 2015). Studies have also demonstrated the radical scavenging activity of NeuAc containing mucins in protecting the mucosal surfaces of the gastrointestinal and respiratory tracts (Ogasawara et al., 2007). Considering the variety of microbial interactions with NeuAc, it is possible that the observed changes in NeuAc substitution may be the result of bacterial degradation of the mucous layer.

An increase in sulfation was observed in HF-NK and HF-BF compared to HF and NC though some of these comparisons were not statistically significant (**Figure 4-12**). Our results indicate that the inclusion of NK or BF dietary fibres in a high fat diet appears to increase the sulfation of Muc2 *O*-glycans in mice. Sulfation of *O*-glycans plays a role in cell adhesion and bacterial binding, and in the regulation of many biosynthetic pathways (Brockhausen et al., 1997). Sulfate is thought to be important for the barrier function of gastrointestinal mucins and studies have shown that the presence of sulfate hydrolysing enzymes increases the susceptibility of mucins to degradation by bacterial GHs (Corfield et al., 1992; Rho et al., 2005; Tsai et al., 1995). Mucin sulfation has also been implicated in ulcerative colitis with control patients exhibiting lower amounts of sulfate than patients with severe inflammation (Corfield et al., 1996). Furthermore, regiospecific differences in Muc2 *O*-glycosylation between the distal and proximal mouse colon are characterised by many mono- and disulfated structures in the distal colon and fewer sulphated glycans in the proximal colon (Holmén et al., 2013).

Accumulating evidence suggests that mucin glycosylation is influenced by the gut microbiota in one of two mechanisms: 1) modulation of host pathways involved in glycosylation, and 2) bacteria

trimming of host glycans by enzymatic degradation. A recent study in pigs demonstrated that supplementation with a mixture of probiotic bacteria induced significant changes in the *N*- and *O*-glycan composition of mucins secreted from the Brunner's glands in the small intestine indicating that changes in the composition of the gut microbiota have an effect on the glycosylation of intestinal mucins (Accogli et al., 2018). This study utilised a combination of lectins (carbohydrate binding proteins with epitope specificity) to measure changes in glycan composition but this method does not provide information on the rest of the carbohydrate chain. Studies in mice have shown that those raised under germ free conditions exhibit considerably different glycosylation patterns in the intestinal tract characterised by reduced terminal fucosylation and less extended structures compared to conventionally raised mice (Bry et al., 1996; Freitas et al., 2002). Furthermore, subsequent colonisation of germ-free mice with specific or conventional gut microbes has been shown to restore the glycosylation pattern to that of conventionally raised mice by activation of enzymes such as *Fut2* (Bry et al., 1996), and transferases attaching Gal and NeuAc (Arike et al., 2017; Johansson et al., 2015). These studies provide compelling evidence for a function of the gut microbiota in modulating the biosynthetic pathways involved in mucin glycosylation, though precisely how these microbes control this process at a distance is still unknown.

Alternatively, the enzymatic depolymerisation of host glycans could provide an alternative explanation for the diet-induced differences in Muc2 glycosylation that we observed. Commensal bacteria, such as *B. thetaiotaomicron* and *Akkermansia muciniphila*, occupy an environmental niche as mucus adherent microbes and are known to possess an array of bacterial GHs that specifically degrade mucin polysaccharides as an additional energy source (Arike and Hansson, 2016; Flint et al., 2012; Marcobal et al., 2013; Tailford et al., 2015). Bacterial degradation of Muc2 *O*-glycans could potentially explain the increased abundance of shorter glycans in the HF diets modified with both dietary fibres though this is inconsistent with the findings of Desai et al. (2016) who demonstrated that the absence of plant polysaccharides stimulates certain gut microbes to

harvest the host mucus layer as an alternative carbon source. In order to obtain Muc2 for glycomic analysis, we collected mucus from the colon of mice by vacuum suction. Mucus samples were then treated with guanidinium hydrochloride to obtain an insoluble Muc2 pellet partially purified from other components. It has previously been shown that guanidinium hydrochloride extraction selectively precipitates the firm inner layer but not the loose outer layer of mucus (Johansson et al., 2008) meaning that our glycomic analysis was performed on Muc2 from the inner layer of mucus. Considering that the outer mucus layer is inhabited by commensal gut microbes while the inner mucus layer is largely devoid of bacteria (Johansson et al., 2008), there would have been minimal contact between Muc2 and the microbiota thus reducing the chance of enzymatic degradation. However, this does not rule out the secretion of carbohydrate degrading enzymes into the inner mucus layer.

In summary, our results indicate that the presence of dietary fibre and different forms of purified dietary fibres, such as NK and BF, can induce significant changes in the *O*-glycosylation pattern of Muc2, specifically by increasing the abundance of shorter glycans and reducing the abundance of difucosylated and sialylated structures. This is the first study to demonstrate that diet can alter Muc2 glycosylation although the mechanism by which this is possible is still unclear. Glycans comprise up to 80% of MUC2 by weight and have been implicated in the maintenance of mucus layer function and integrity, selection of the gut microbiota, and the prevention of inflammatory diseases such as severe ulcerative colitis. These findings suggest that dietary factors can influence the composition of this critical component of the mucus layer and could be useful in developing diet driven approaches to manage issues such as intestinal dysbiosis, susceptibility to infection, and the progression of inflammatory conditions like ulcerative colitis that rely on mucus layer function.

4.3.2 Effect of fibre supplementation on high fat diet-induced changes in the mouse (Paper 4)

The work described in Study 1 above is my contribution to the following manuscript currently being prepared for submission to Proceedings of the National Academy of Sciences of the United States of America (PNAS). Hasinika K.A.H. Gamage, Daniel Bucio-Noble, Liisa Kautto, Anandwardhan Hardikar were equally involved in experimental design, animal handling and sample collection. Hasinika K.A.H. Gamage performed the DNA extraction and bioinformatic analysis. Daniel Bucio-Noble performed the proteomic analysis determination of plasma biomarkers. Malcolm S. Ball, Sasha G. Tetu, Nicolle H. Packer, Mark Molloy, Ian T. Paulsen were involved in the experimental design and interpretation of data.

Interactions between host and gut microbiota following dietary interventions

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Abstract

Increasing the consumption of dietary fibre has been proposed to alleviate the progression of non-communicable diseases such as obesity, type 2 diabetes and cardiovascular disease, yet the effect of dietary fibre on host physiology remains unclear. In this study, we performed a multiple diet feeding study in C57BL/6J mice to compare high fat and high fat modified with dietary fibre diets

on host physiology and gut homeostasis by combining proteomic, metagenomic, metabolomic and glycomic techniques with correlation network analysis. We observed significant changes in physiology, liver proteome, gut microbiota and SCFA production in response to high fat diet. Dietary fibre modification did not reverse these changes but was associated with specific changes in the gut microbiota, liver proteome, SCFA production and colonic mucin glycosylation. Furthermore, correlation network analysis identified gut bacterial-glycan associations.

Introduction

Consumption of high fat diets has been associated with an increased incidence of obesity (1), type 2 diabetes (2), and metabolic syndrome (3). Conversely, increasing the intake of dietary fibre is associated with a decreased incidence of colorectal cancer (4) and cardiovascular disease (5), and the prevention of type 2 diabetes (6). Despite the widespread availability of dietary fibre products in the market, limited work has been conducted on their efficacy at the molecular level. While observational studies have been useful in demonstrating long-term effects, the molecular mechanisms by which dietary changes can influence health status remain unclear.

Several animal models, such as the C57BL/J6 mouse, have been developed to investigate these mechanisms. This mouse strain develops symptoms of increased weight gain and glucose intolerance when exposed to a high fat diet making it a useful model of high fat diet induced obesity and type 2 diabetes (7, 8). Previous studies in these mice have targeted the proteome of the liver due to its central role in the maintenance of whole-body glucose and lipid metabolism (9, 10), and the consumption of a high fat diet has been shown to significantly alter the liver proteome, specifically, pathways associated with fatty acid and carbohydrate metabolism, inflammation and antioxidant defence (10, 11).

Previous studies have demonstrated that consumption of high fat diets induces characteristic changes in the gut microbiota such as an increase in the abundance of the families

Erysipelotrichaceae and Rikenellaceae and a decreased abundance of Lachnospiraceae, Lactobacillaceae, Ruminococcaceae and Prevotellaceae (12-15). Intake of high fat diets also generally lowers the gut microbial species diversity and gene richness (12, 16). This type of microbial dysbiosis has been associated with obesity and other metabolic disorders (17). The production of bacterial metabolites, such as short chain fatty acids (SCFAs), plays a major role in host energy capture through the breakdown of dietary fibre and regulation of lipogenesis, gluconeogenesis and inflammation (18). High fat diet intake is typically associated with lower concentrations of SCFA (19).

The colonic mucus layer is an important mediator of host-gut microbial interactions. While a link between the colonic mucin glycosylation profile and composition of the gut microbiota in mice and humans has been defined (20). almost nothing is known about the relationship between mucin glycosylation and specific gut bacteria. Mucin glycans are involved in immune defence by serving a binding decoy targets for gut bacteria (21). The removal of specific epitopes such as Fucose also influences the composition of the gut microbiota (22, 23), thus the presence or absence of specific residues may apply selective pressure that alters the composition of the gut microbiota.

In this study, we investigated the modification of high fat diets with NutriKane and Benefiber, two dietary fibre products derived from sugarcane fibre and wheat dextrin, respectively. The aim of this study was to determine the effects of these fibre modifications on host physiology, liver proteome, gut microbiota, SCFA production and glycosylation of colonic mucin using a combination of proteomics, genomics, metabolomics, glycomics. Furthermore, we performed correlation network analysis and found previously unidentified associations between the gut microbiota and mucin glycans.

Materials and methods

Animal trial and sample collection

All experimental procedures were approved by the Animal Ethics committees in the University of Sydney, Australia and Macquarie University, Australia. A total of 50, 11-week old male C57BL/6J mice were fed either a normal chow (n=9) or a high fat diet (n= 41) for 17 weeks, following this the high fat diet group was randomised into three dietary groups; high fat diet (HF), high fat diet modified with NutriKane (HF-NK) or high fat diet modified with Benefiber (HF-BF). Nutritional information and ingredients of experimental diets are provided in Table S1. Intraperitoneal glucose tolerance tests (IPGTT) were performed at week 17 and 23. Samples and measurements were obtained as mentioned in Fig. S1, and the animals were euthanised in week 32. Further information on the experimental design and diets are provided in SI Materials and Methods.

Quantification of circulating markers of inflammation in plasma

Cytokine and diabetes markers quantitation was determined according to the manufacturer's protocol (Bio-rad, Australia).

Mass spectrometry-based proteomics analysis

Perfused liver (10 mg) was subjected to LC-MS/MS analysis employing SWATH-based proteomics as the quantitation strategy. Library and SWATH-MS data were acquired in a 6600 Triple TOF mass spectrometer coupled with an Ekspert 415 LC system (Sciex, Australia). Database searches included trypsin as digestion enzyme and carbamidomethylation as fixed cysteine modification, reversed database search was enabled to allow false discovery rate (FDR) calculation, and protein global FDR was established at 1%. Information extracted from SWATH MS peak areas was performed using Peak View Version 2.1 with SWATH MicroApp 2.0 (Sciex) using the library search output file. Perseus Version 1.5.5 was employed for statistical analysis.

16S rRNA gene amplicons sequencing and bioinformatics analysis

Total community DNA was isolated from faecal samples collected at week 0, 17, 23 and 32, and the V4 region of 16S rRNA gene was amplified and sequenced using an Illumina MiSeq platform.

Demultiplexed raw sequence data was processed using Quantitative Insights Into Microbial Ecology (QIIME) software (Version 1.9.1) and Operational Taxonomic Units (OTUs) were determined at 97% similarity using an open-reference protocol against the Greengenes (Version 13_8) database. Statistical analysis of the gut microbiota sequencing data was conducted using PRIMER-7 software package and Distinct phylotypes (families and OTUs) between dietary groups were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method (online Galaxy Version 1.0).

Quantification of SCFAs

Acetate, propionate and butyrate concentrations in faecal samples collected at week 17, 23 and 32 were determined by GC-FID. All measurements were normalised for the weight of faecal samples used for SCFA quantification.

Mucin extraction and O-glycan characterisation

Mucus was obtained by vacuum suction from the entire length of opened colons. MUC2 was obtained from colonic mucus by precipitation with GuHCl. O-glycans were released by reductive β-elimination and subjected to PGC-LC-ESI-MS/MS analysis according to a previously established protocol (24). O-glycans were separated on an Agilent 1100 LC system coupled to an Agilent 6330 ESI-MS (Agilent Technologies, Inc., USA). Glycan compositions were calculated using Glycomod (https://web.expasy.org/glycomod/) and glycan structure was assigned by manual interpretation of the tandem MS fragmentation spectra. Glycan peaks were quantified by relative abundance using Skyline (Version 3.7.0.11317, MacCoss Lab, UW) for assisted peak peaking, generation of extracted ion chromatograms (EICs) and integration of EIC peak area. Glycan structures were drawn using Glyco Workbench 2 (Version 2.1).

Correlation network analysis

We conducted pairwise correlations analyses between significantly differentially abundant gut bacterial OTUs and glycans. Pearson correlation coefficients were determined using Hmisc R package (25), and correlation networks were constructed using Cytoscape software (Version 3.6.1).

Detailed Material and Methods are provided in SI Materials and Methods.

Results and Discussion

Physiological response to high fat diets

At the end of the 32-week feeding period there were no significant differences in the body weight of the four groups (Fig. S2A) though the mass of the epididymal white adipose tissues was somewhat higher in the HF, HF-NK and HF-BF groups compared to the NC group (Fig. S3). The HF, HF-NK and HF-BF groups exhibited significantly lower ($P < 0.0001$) feed consumption in comparison to the NC group (Fig. S2B), while calculated energy intake was the same across the four groups (Fig. S2C). IPGTT data at week 17 showed that glucose concentrations were higher in the HF diet indicating the development of glucose intolerance (Fig. S2D and F) and transition to HF-NK or HF-BF provided no improvement at week 23 (Fig. 1A and B).

We then sought to determine whether specific plasma biomarkers were altered by the consumption of HF, HF-NK or HF-BF diets. The cytokines GM-CSF and IL-1β, which have been associated with oxidative stress (26), were significantly decreased in the HF-NK and HF-BF groups compared to the NC group (Fig. 1C and D). The concentration of diabetes markers GLP-1, insulin and PAI-1 was significantly higher in HF, HF-NK and HF-BF groups compared to the NC group with no difference between HF, HF-NK and HF-BF (Fig. 1E, F and G). Increased levels of GLP-1, insulin, and PAI-1 have been previously associated with induced obesity in high fat diet fed animals (27, 28). Several other tested plasma biomarkers showed no significant difference between the four groups (Fig. S4).

Diet induced changes in liver protein expression after HF and fibre modification

Quantitative information of the liver proteome was extracted for 2,388 proteins from a spectral library using SWATH-MS. Hierarchal clustering showed significant differences in the liver protein expression between NC and HF fed groups, with no significant difference between HF, HF-NK and HF-BF groups (Fig. 2A). A total of 155 proteins were found to be significantly different between HF and NC groups (Tables S2A). These included proteins linked to fatty acid and carbohydrate metabolism, inflammatory promotion and antioxidant regulation (10, 11, 29, 30).

In comparison to the HF group, the expression of 9 proteins was significantly higher in the HF-NK group, including selenocysteine synthase (SecS), which is associated to selenocysteine insertion (31), and expression of 13 proteins was significantly lower, including STAT3 and a 15kDa Selenoprotein (SEP15) (Fig. 2B, Table S2B). Interestingly, ethanol extracts of NK positively modulate the expression of Selenoprotein H (SELH) in vitro (32) suggesting a potential role of NK in the regulation of selenium-associated proteins. STAT3 is established to regulate inflammatory mechanisms and tumour progression in the liver of mice fed a high fat diet (33, 34). Comparing HF-BF to HF, the expression of 16 proteins, including UDP-glucoronosyltransferase 1-9 (UGT1A9), was significantly higher, while the expression of 25 proteins, including Pyruvate dehydrogenase kinase isoform 2 (PDK2), was significantly lower (Fig. 2B, Table S2C). UGT1A9 and PDK2 are associated with xenobiotic metabolism (35) and glucose metabolism in response to fat accumulation, respectively (36, 37).

Addition of fibre products altered the gut microbiota community and composition

Sequencing of the 16S rRNA gene amplicons was used to determine changes in the gut microbiota in response to dietary interventions. The overall gut microbiota structure in the NC group was significantly different ($P < 0.0001$, PERMANOVA) compared to the HF group (Fig. 3A). Transition to HF-NK or HF-BF did not significantly alter the microbiota structure. The alpha-

diversity, as determined by Shannon diversity and Simpson's evenness indices, were significantly higher in the NC group compared to HF, HF-NK and HF-BF groups, with no significant difference between HF, HF-NK and HF-BF (Fig. 3B).

LEfSe analysis was used to identify the bacterial families (Fig. S5) and OTUs (Fig. 4) that responded to HF, HF-NK, or HF-BF. Comparing HF to NC, the relative abundance of 25 OTUs in the families Erysipelotrichaceae, Bacteroidaceae, Alcaligenaceae, Clostridiaceae, and Verrucomicrobiaceae, was significantly higher. Concurrently, the relative abundance of 55 OTUs in the S24-7, Lachnospiraceae, Prevotellaceae, and Clostridiales was significantly lower in the HF group compared to the NC group. These HF-induced changes in the gut microbiota are consistent with several studies (12-15).

Comparing HF-NK to HF, the relative abundance of 7 OTUs in the families Lactobacillaceae, Clostridiales, Lachnospiraceae and Ruminococcaceae was significantly higher. While the relative abundance of 5 OTUs in the families Bacteroidaceae, Clostridiaceae, Lachnospiraceae and Erysipelotrichaceae was significantly lower. In response to HF-BF the relative abundance of 11 OTUs in the families Porphyromonadaceae, Bacteroidaceae, Rikenellaceae, Lactobacillaceae, was significantly higher compared to the HF group. Whereas, the relative abundance of 3 OTUs in Clostridiaceae and Erysipelotrichaceae was significantly lower. Both HF-NK and HF-BF diets were associated with a decrease in the relative abundance in Clostridiaceae, Erysipelotrichaceae and Bacteroidaceae, all of which are typically abundant in mice fed a high fat diet (38-40). The OTUs that were more abundant in fibre modified diets (HF-NK, HF-BF) belong to families that have been associated with fibre digestion (41-46). Furthermore, these OTUs were product dependent, potentially due to distinct variations in the chemical nature of NutriKane and Benefiber (46).

Dietary modifications alter SCFA production

Faecal concentrations of the SCFAs acetate, butyrate and propionate were determined by GC-FID. In comparison to NC, the HF diet was associated with significantly lower concentrations of all three SCFAs (Fig. 5). Comparison of HF with HF-NK found no significant differences in SCFA concentrations while comparison with HF-BF was associated with a significant increase in propionate concentration at weeks 23 and 32. Supplementation with dietary fibre typically increases the production of short chain fatty acids, which are the major fermentation products from gut microbial degradation of dietary fibre, and are significantly reduced upon consumption of a high fat diet (47, 48) (19, 49).

Addition of dietary fibre alters the glycosylation of the colonic mucus layer

Reductive β-elimination and LC-MS was used to release and characterise the O-glycan structures attached to MUC2, the main component of the colonic mucus layer. A total of 37 unique O-glycan structures were detected as presented in Table S5. Glycans are referred to by their molecular weight with isomers distinguished alphabetically. The majority of glycans detected were Core 2 structures that contain GlcNAc β1-6 linked to the GalNAc of Core 1 structures (Gal β1-3 GalNAc). Only one Core 1 structure (531) was detected. When comparing NC and HF, there were no significant differences in glycosylation except for glycans 734b and 976a (Fig 6). In contrast to NC and HF, the HF-NK and HF-BF diets were associated with an increase in the relative abundance of smaller glycans (≤ 814 M). These results suggest that replacement of 4.7% (w/w) cellulose with NutriKane or Benefiber has a greater influence than high fat content on glycosylation of MUC2.

The state of the mucus layer is critical to intestinal health and a loss of integrity has been shown to precede the onset of certain diseases including colitis and even colorectal cancer (50-52). Changes in the mucin glycosylation as result of pectin and cellulose intake have been previously

reported (53-55). However, these studies exclusively examined the total glycocalyx of the intestinal epithelium and no studies to date have assessed specifically the changes in MUC2 glycosylation in response to physiological stimuli.

The gut microbiota demonstrated significant correlations with colonic mucin glycans

Pairwise correlation analysis between OTUs and O-linked glycans identified significant diet dependent associations. These were used to construct a network consisting of 372 correlations and 109 nodes (diets, OTUs, glycans) (Fig. S7). We then generated subnetworks by extracting OTU nodes that were directly connected to specific glycans (Fig. 7). These subnetworks suggest that OTUs within the families S24-7 and Prevotellaceae are positively correlated with glycans containing Gal β1-3/4 linked to the GlcNAc of Core 2 O-glycans and negatively correlated with glycans without this extension (Fig 7A). Furthermore, OTUs within the families Porphyromonodaceae and Rikenellaceae were positively correlated with glycans lacking this extension on the 6-arm. The presence of terminal epitopes (Fuc or NeuAc) was associated with the abundance of OTUs in the families Clostridiaceae and Lactobacillaceae (Fig 7B). Specifically, OTUs in Clostridiaceae were positively correlated with glycans 1042a and 1121b and negatively correlated with 588 and 668. The OTUs in Lactobacillaceae were negatively correlated with glycans 1041b, 1042a, 1121b and positively correlated with glycans 531, 588 and 668. Core type may also be significant since OTUs in the family Alcaliganaceae were positively correlated with glycans 588, 668, 814, which are all Core 2 structures, and negative correlated with glycan 531, the only Core 1 structure (Fig. 7C).

As the main component of the colonic mucus layer, mucins and their attached glycans serve as binding targets for bacterial adhesins (21), provide an alternative carbon source for commensal bacteria (20, 56), and influence microbial diversity through the application of selective pressures (22, 23). Our results suggest a relationship between the abundance of OTUs and specific glycans. These correlations could potentially be explained by three mechanisms: 1) bacterial degradation

of glycans, 2) bacterial induced modulation of host glycosylation, and 3) microbial selection by host glycosylation patterns. Targeted downstream experiments will be useful in understanding the biological mechanisms of these correlations.

Conclusion

In this work we applied several omics techniques and correlation network analysis in an unprecedented effort to explore the molecular basis of physiological changes in response to dietary intervention. We observed significant changes in the gut microbiota, SCFA production, liver proteome, glucose tolerance and specific biomarkers of diabetes, in response to a high fat diet with fewer changes in response to dietary fibre modification. In contrast, dietary fibre modification was associated with significant changes in mucin glycosylation and this was further correlated with specific gut bacteria via correlation network analysis. The application of multiple omics techniques in conjunction with correlation network analysis highlighted the extreme complexity physiological responses to diet, however, several bacterial-glycan relationships were identified. The intimate affiliation between gut bacteria and mucin glycans is critical yet incredibly unexplored. Our study suggests that gut microbiota studies and glycomic analysis should become common practice in the field of gut microbiota research.

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Author Contributions

HKAHG, RWWC, DBN, LK, MSB, MM, NHP and ITP designed research. HKAHG, RWWC, DBN, LK and AH performed research. HKAHG, RWWC, DBN analysed data; HKAHG analysed gut microbiota and correlation network analysis data, RWWC analysed metabolomic and glycomics data and DBN analysed proteomics and plasma biomarker data. HKAHG, RWWC and DBN wrote the paper.

Figures

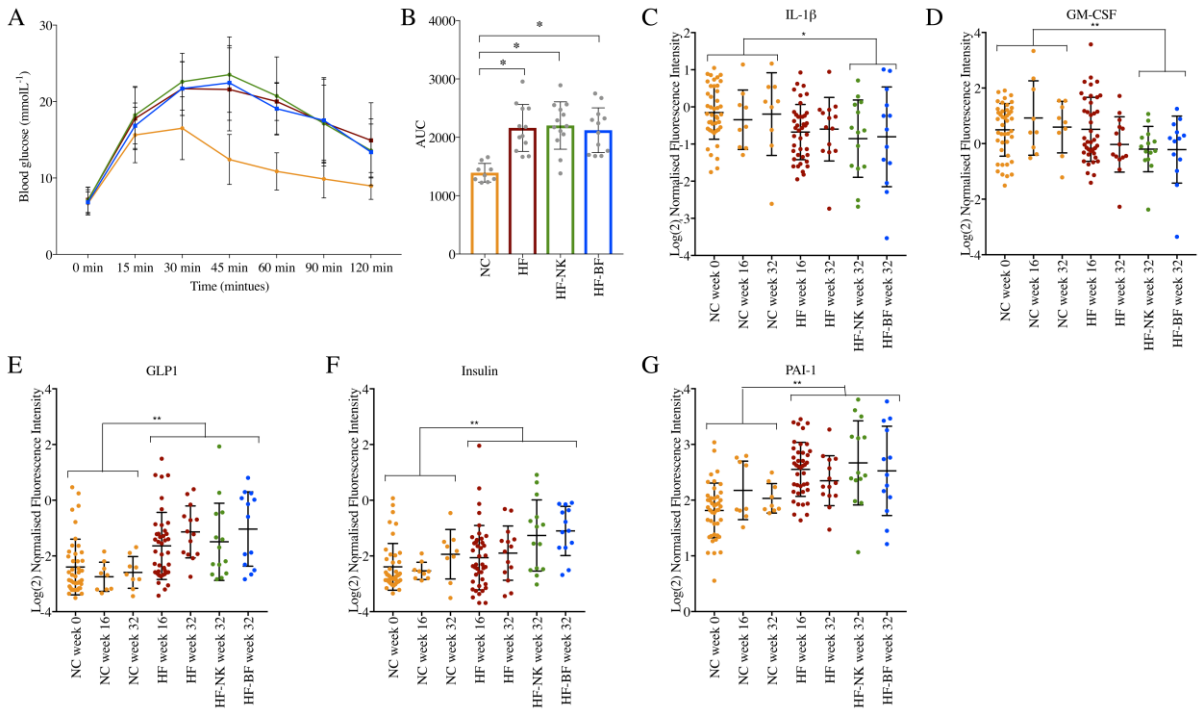

Figure 1 The glucose tolerance and plasma biomarker expression of mice in each dietary group. (A) Blood glucose levels and (B) area under curve (AUC) of IPGTT conducted at week 23. Plasma samples were obtained at baseline (week 0, NC n=50), week 16 (NC n=9 and HF n=41), and week 32 (NC n=9, HF n=14, HF-NK n=14 and HF-BF=13). Values expressed as log(2) normalised fluorescence intensity. (C) GLP-1, (D) Insulin, (E). PAI-1, (F) GM-CSF and (G) IL-1β. Mean values with ± SD are shown.* $P < 0.05$, ** $P < 0.01$.

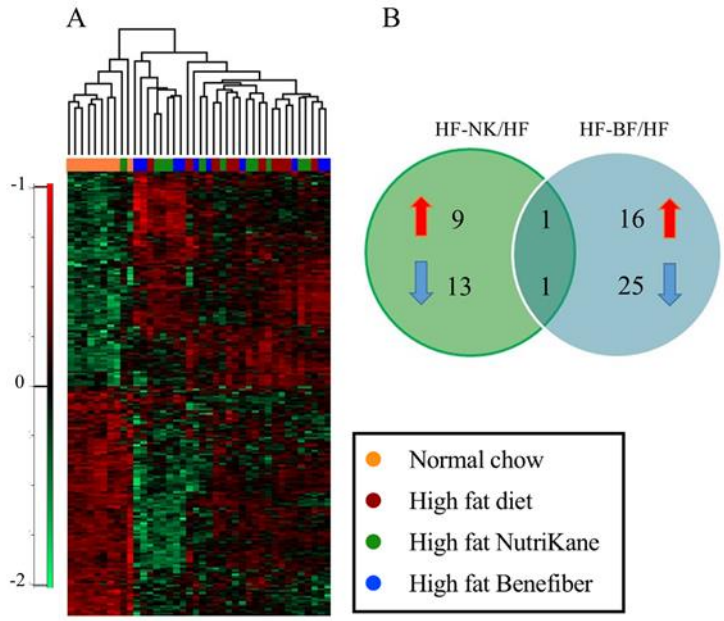

Figure 2 MS-based hepatic protein expression of mice in each dietary group. (A) Hierarchical clustering, (NC n=9, HF n=10, HF-NK n=11 and HF-BF=10) based on ANOVA multiple sample test, Permutation based FDR 0.05. Up- and down-regulation of proteins are denoted by red and green, respectively. (B) The number of differentially up- and down-regulated proteins in response to NK and BF modification compared to the HF group. Students t-test, $P < 0.05$, fold change > +/- 1.5.

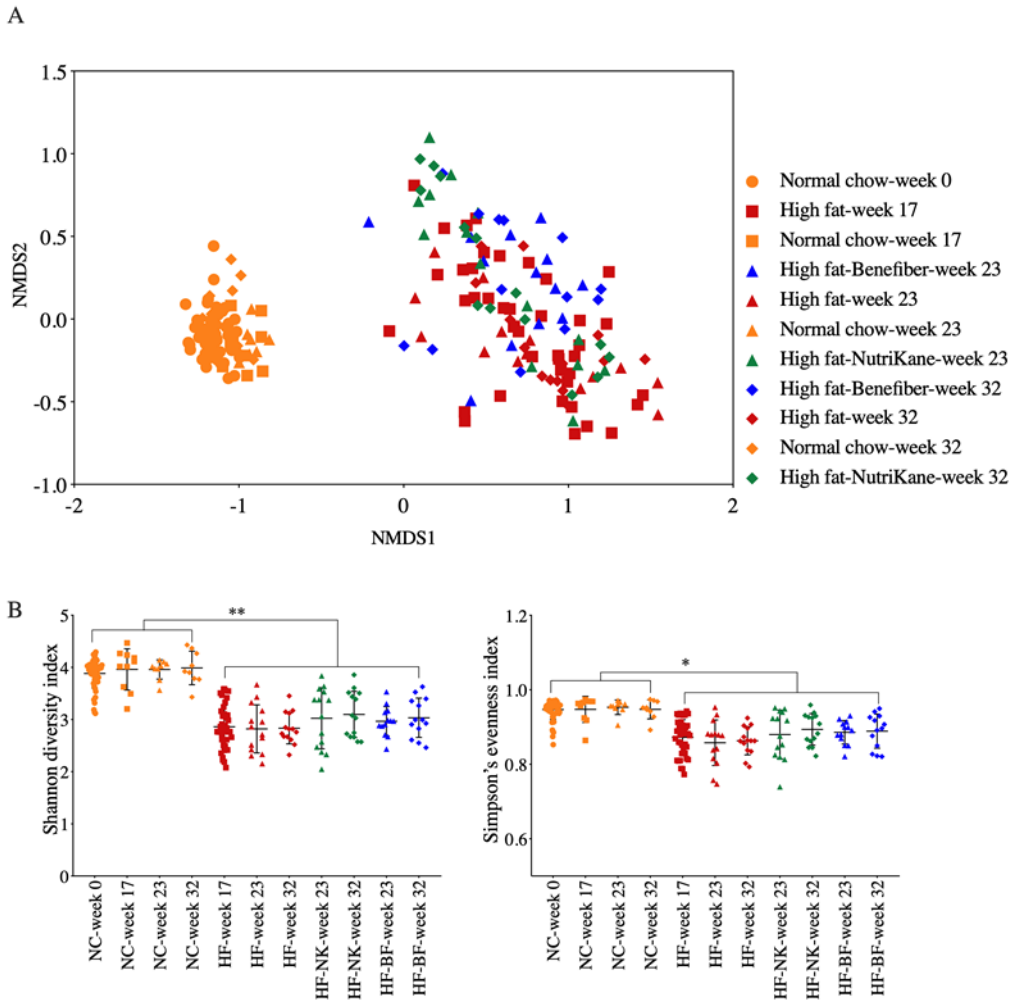

Figure 3 Ordination and alpha diversity of the gut microbiota in mice fed each of the four diets at weeks 0, 17, 23 and 32. (A) Ordination of the gut microbiota shown as a Bray-Curtis similarity based nMDS plot. (B) Gut microbial diversity and evenness shown as Shannon diversity and Simpson's evenness indices, respectively. Mean values with ± SD are shown. ** $P < 0.01$.

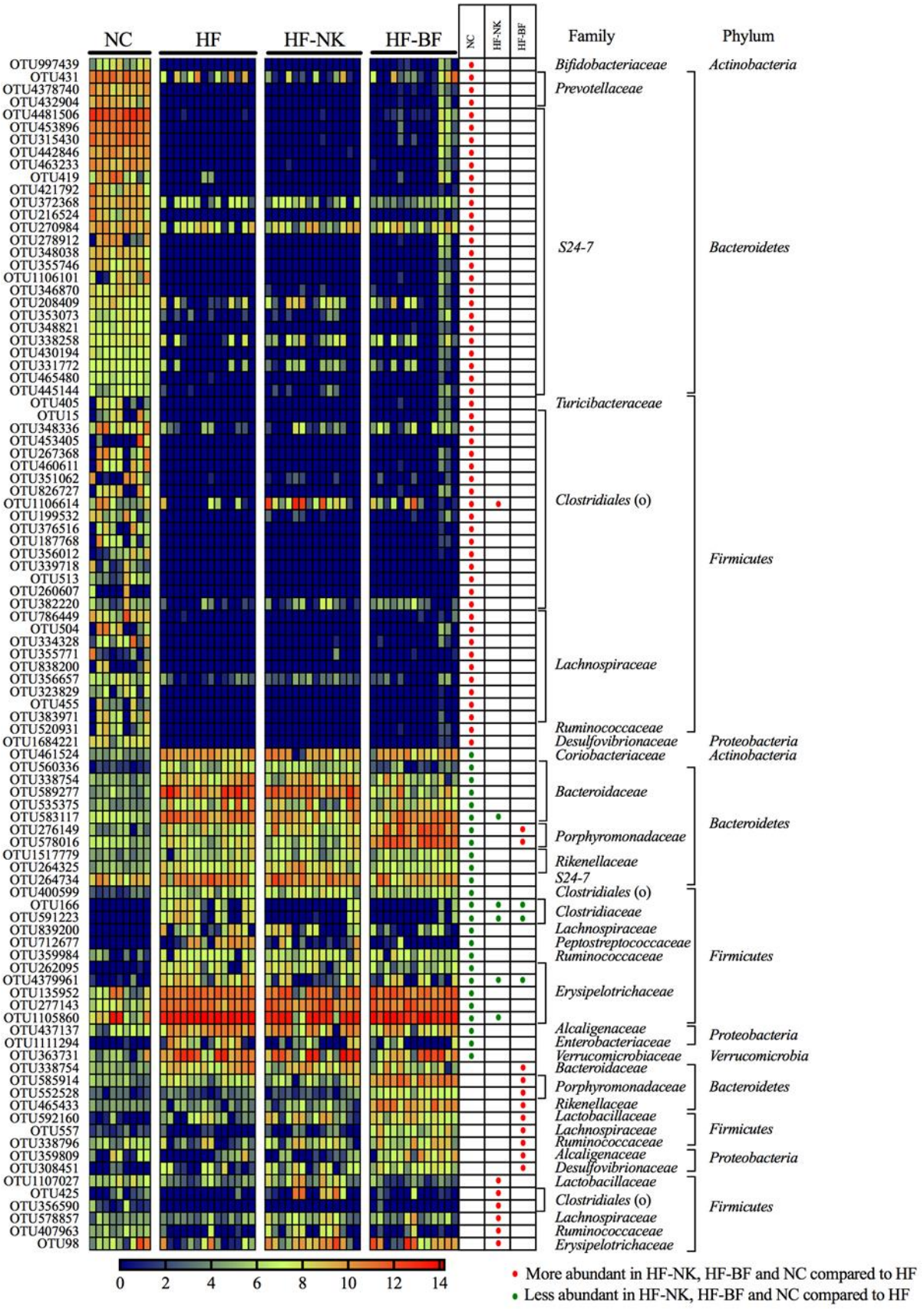

Figure 4 The relative abundance (Log10 transformation) of the OTUs that were significantly different between dietary groups, shown per mouse. Data was obtained through LEfSe analyses

between HF vs NC, HF vs HF-NK and HF vs HF-BF groups. Rows in the heat map correspond to the abundance of the OTUs and columns correspond to individual mice in each dietary group. Red and blue denote the highest and lowest relative abundance as per the legend. The changing direction of the relative abundance of the OTUs in comparison to the HF fed group is shown in the table, red and green dots denote more and less abundant compared to the HF group, respectively. The taxonomy of the OTUs (family and phylum) are shown on the right. The relative abundance of the these OTUs per mouse is provided in Table S2.

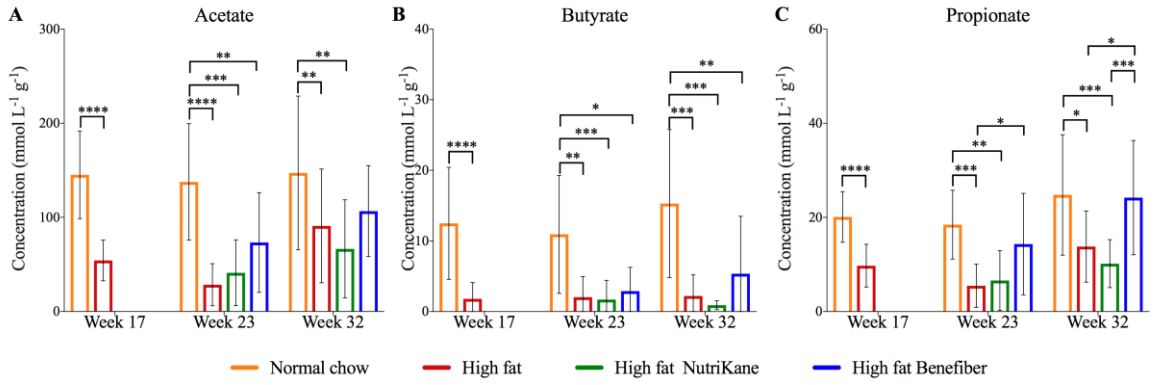

Figure 5 Concentration (mmolL-1g-1) of acetate, butyrate and propionate per mouse in each dietary group at week 17, 23 and 32. Mean values with ± SD are shown. **** P < 0.0001, *** P < 0.001, ** P < 0.01 and * P < 0.05. The concentrations per mouse are provided in Table S4.

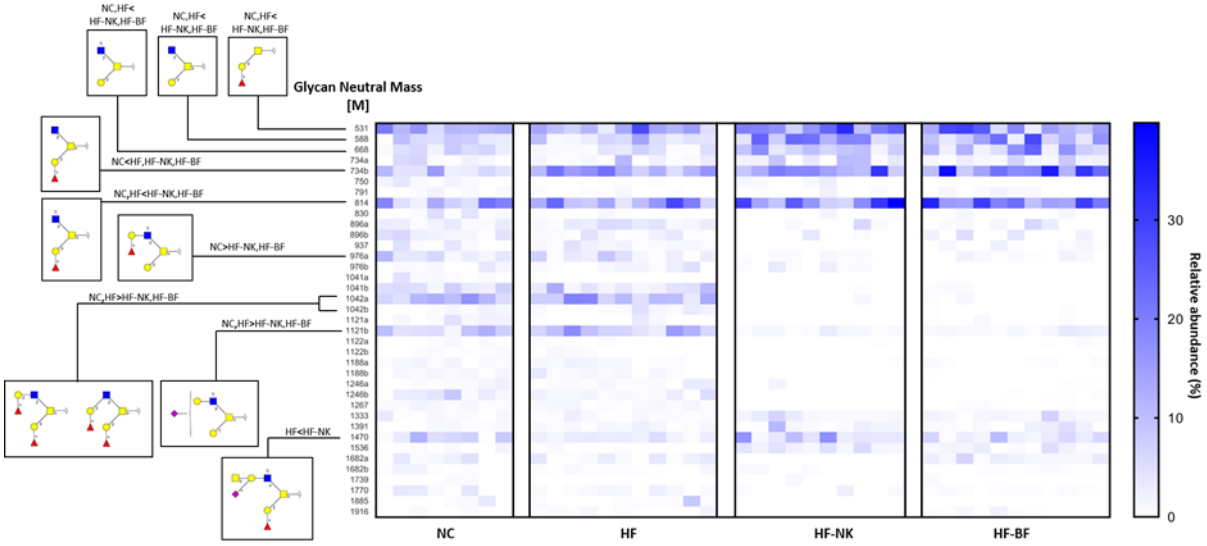

Figure 6 Unpaired heatmap illustrating the relative abundance of O-linked glycans released from the colonic mucin of mice given a normal chow (NC), high fat (HF), high fat modified with NutriKane (HF-NK), or high fat modified with Benefiber (HF-BF) diet. Glycans are listed in rows by their neutral molecular mass with structural isomers distinguished alphabetically. Each column represents a single mouse. Glycans with relative abundance significantly different between diets are presented on the left.

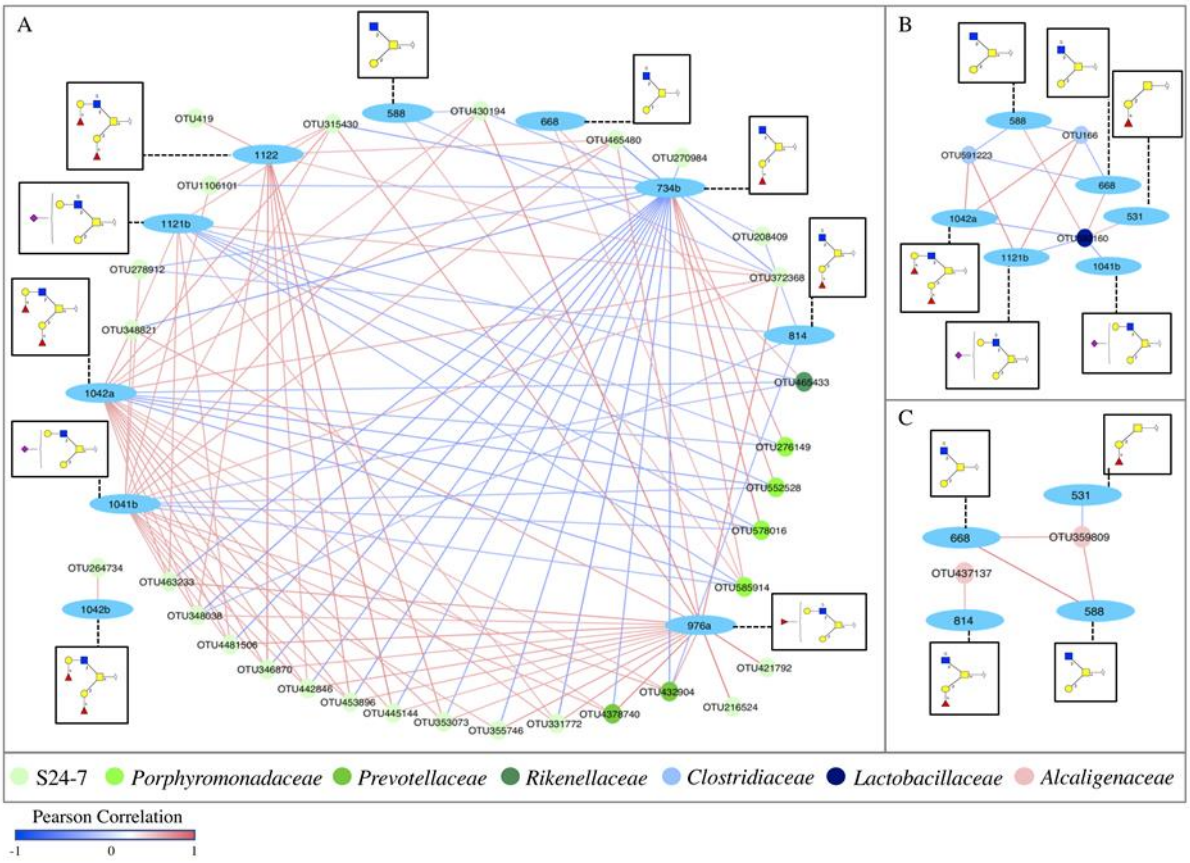

Figure 7 The correlation networks showing associations between the relative abundance of specific gut bacterial OTUs and colonic mucin glycans. The OTUs are shown in colour-coded dots based on the bacterial family. Glycans are shown in light blue ovals with the neutral molecular mass, structural isomers distinguished alphabetically. A positive and negative correlation is presented with a red and blue line, respectively. The intensity of the colour denotes the strength of the correlation. (A) The OTUs in the families S24-7, Prevotellaceae, Porphyromonodaceae and Rikenellaceae showed correlations with glycans containing Gal β1-3/4 linked to the GlcNAc of Core 2 O-glycans. (B) The presence of terminal epitopes (Fuc or NeuAc) was associated with the abundance of OTUs in the families Clostridiaceae and Lactobacillaceae. (C) The associations between the core type and OTUs in the family Alcaliganaceae.

Supplementary Figures and Table legends

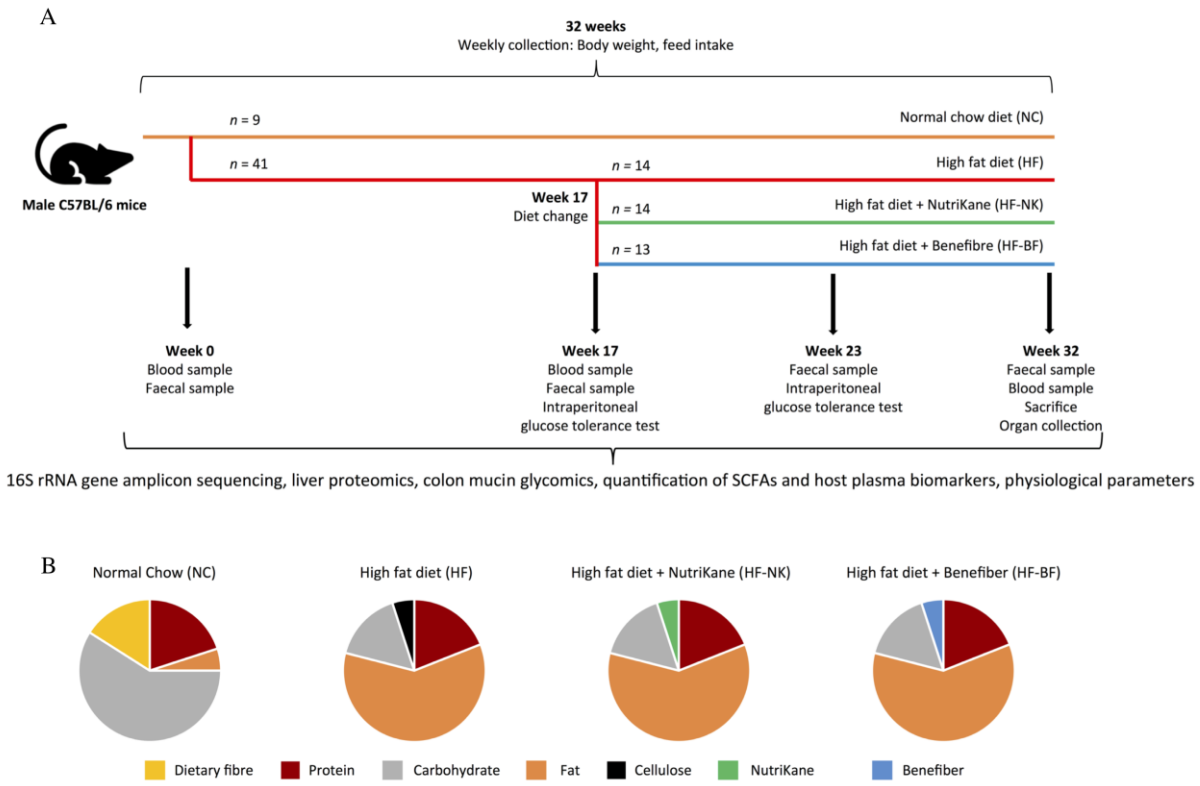

Figure S1 The experimental design. **(A)** The timeline of the experiment, 50 C57BL/6 mice were randomised into one of two dietary groups, normal chow (NC) or high fat diet (HF) at week 0. After 17 weeks, a subset of the HF group was further randomised into, high fat modified with NutriKane (HF-NK) or high fat modified with Benefiber (HF-BF) diets. The time and type of samples/measurement collected are indicated. **(B)** The nutrient composition of each diet. Nutritional information and ingredients of experimental diets are provided in Table S1.

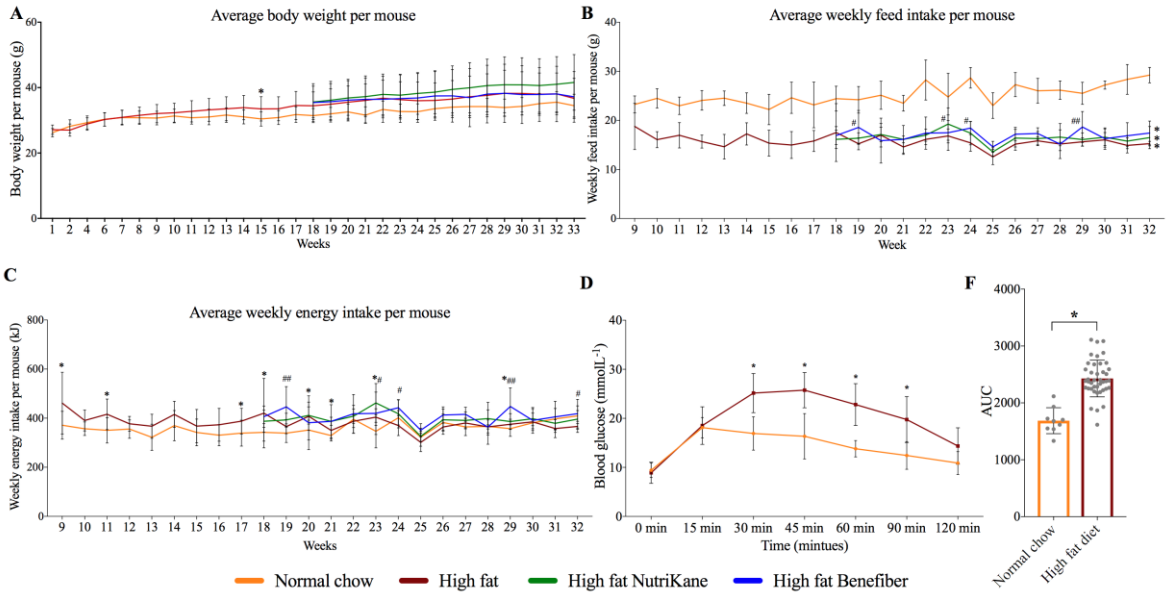

Figure S2 Weekly **(A)** feed intake, **(B)** energy intake and **(C)** body weight per mouse. Mean values per dietary group with ± SD are shown. **(D)** Blood glucose levels and **(E)** area under curve (AUC) of IPGTT conducted at week 17. Significance was determined based on Bonferroni's multiple comparisons tests, (**P* < 0.05) compared to normal chow.

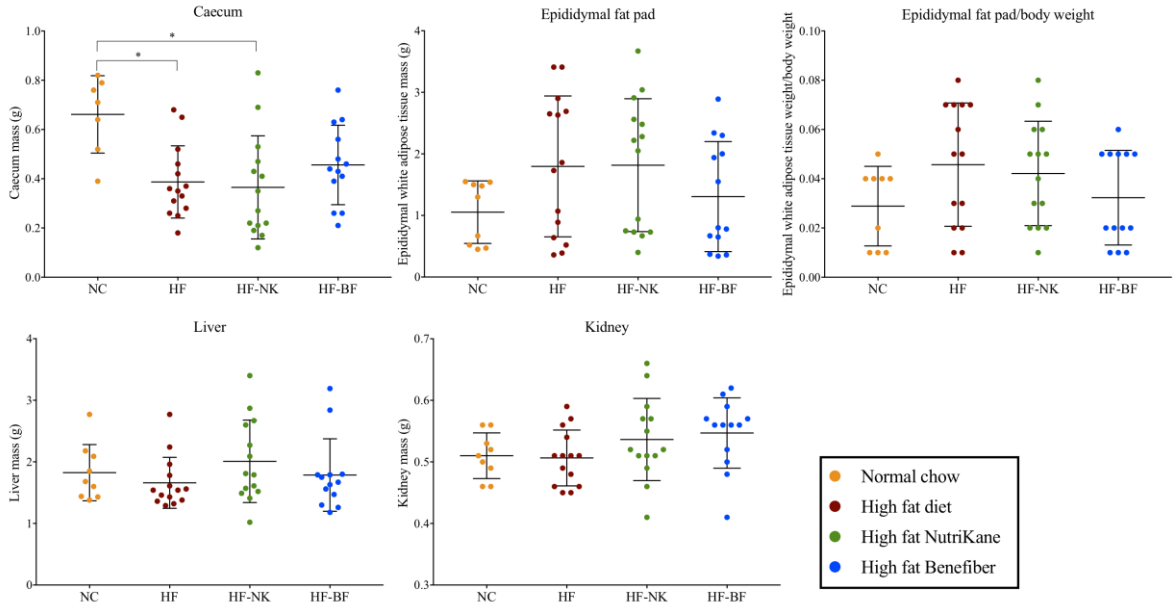

Figure S3 Mass of the organs excised from mice at week 32. The mass of the caecum, epididymal white adipose tissue, liver, kidneys and ratio between epididymal white adipose tissue mass and body weight per mouse is shown (mean ± SD). Significance (* *P* < 0.05) was determined using a Mann-Whitney.

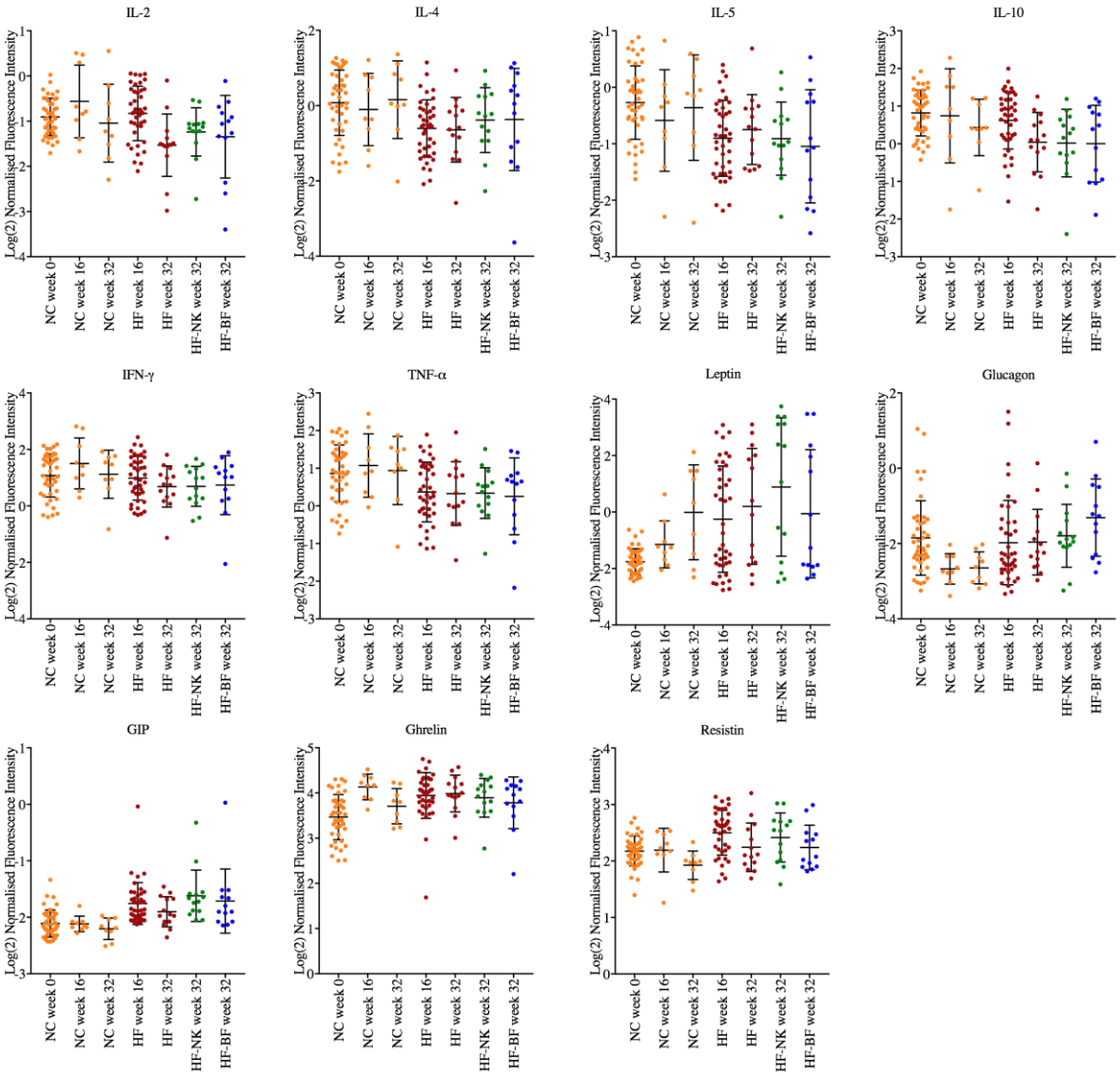

Figure S4 Plasma marker expression in response to dietary changes. Plasma samples were obtained at baseline (week 0, NC n=50), week 16 (NC n=9 and HF n=41), and week 32 (NC n=9, HF n=14, HF-NK n=14 and HF-BF=13). Values expressed as log(2) normalised fluorescence intensity.

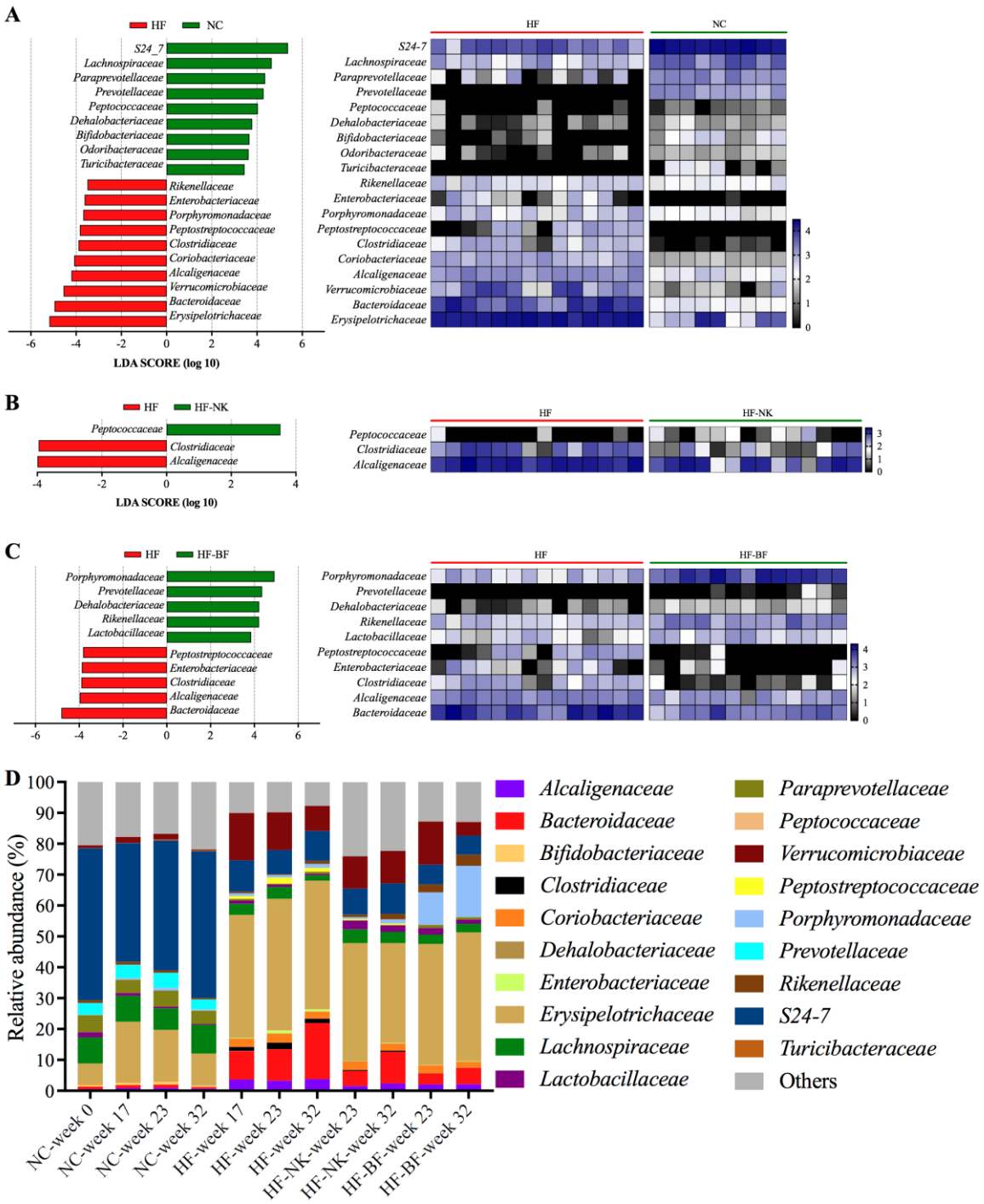

Figure S5 Key bacterial families of the gut microbiota responding to dietary changes at week 32. Data was obtained using LEfSe analyses between **(A)** HF vs NC, **(B)** HF vs HF-NK and **(C)** HF vs HF-BF groups. The left histogram shows the LDA scores computed for each bacterial family and the right heat map shows the relative abundance (Log_{10} transformation) of the families in each mouse. In the heat map, rows correspond to bacterial families and columns correspond to individual mice in each dietary group. Blue and black denote the highest and lowest relative abundance as shown in the legend. **(D)** The relative abundance (%) of these key bacterial families at week 0, 17, 23 and 32.

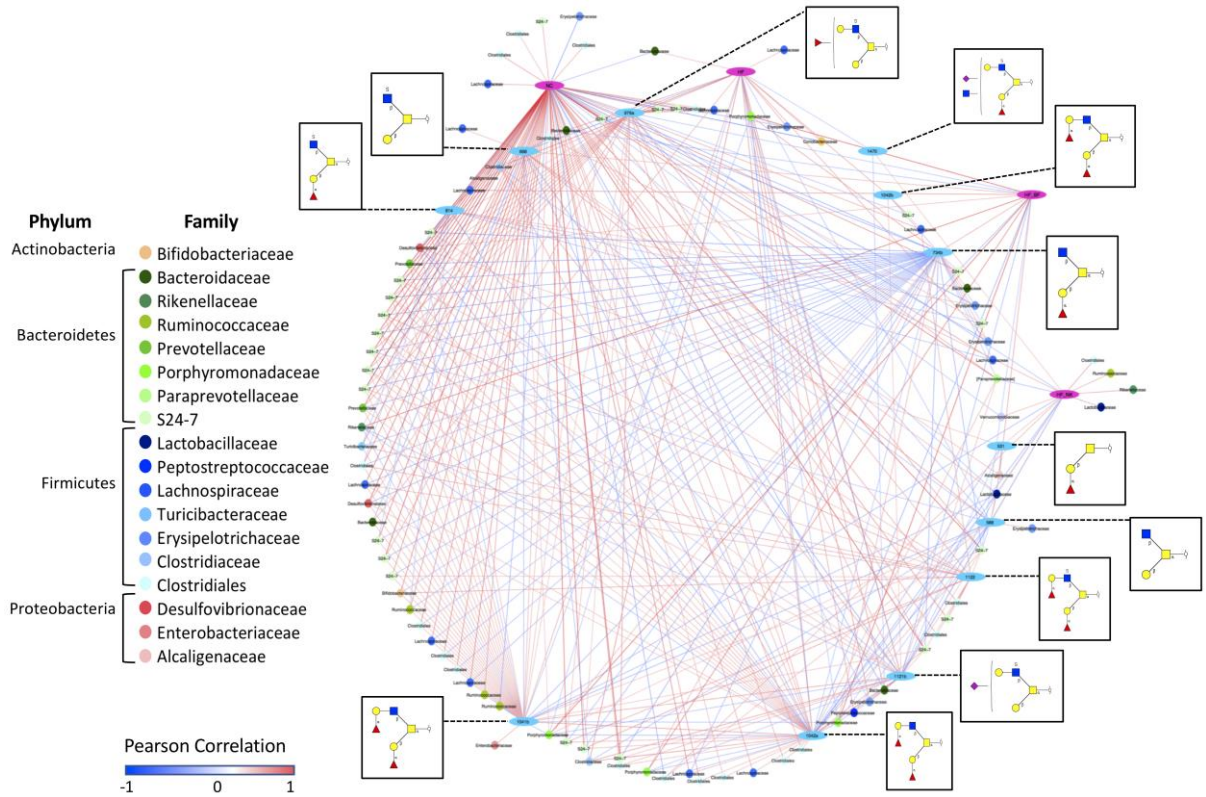

Figure S7 The correlation network showing associations between specific gut bacterial OTUs, colonic mucin glycans and dietary groups. The OTUs are shown in colour-coded dots based on the bacterial family and phylum. Glycans are shown in light blue ovals with the neutral molecular mass, structural isomers distinguished alphabetically. The four dietary groups; normal chow (NC), high fat (HF), high fat diet modified with NutriKane (HF-NK) and high fat diet modified with Benefiber (HF-BF) are shown in magenta ovals. A positive and negative correlation is presented with a red and blue line, respectively. The intensity of the colour denotes the strength of the correlation.

Supplementary tables

Table S1 Nutritional information and ingredients of experimental diets. **(A)** Composition of normal chow and high fat diet (high fat diet supplemented with NutriKane or Benefiber were produced replacing cellulose of the high fat diet with 5% (w/w) each product). **(B)** Ingredients and nutritional information of NutriKane and Benefiber.

Table S2 Significantly regulated proteins expressed as **(A)** NC/HF, **(B)** HF-NK/HF, **(C)** HF-BF/HF and ratios. Two-sample t-test ($P < 0.05$), fold change>1.5.

Table S3 The relative abundance of bacterial OTUs that were found to be significantly differentially abundant between dietary groups at week 32. Data was obtained based on LEfSe analyses between NC vs HF, HF vs HF-NK and HF vs HF-BF groups. LEfSe analyses were performed with following parameters; Kruskal-Wallis test among classes ($P< 0.05$), Wilcoxon test between classes ($P < 0.01$) and the threshold on the logarithmetic LDA score for discriminative features > 3.0.

Table S4 Concentration of acetate, butyrate and propionate in each treatment at week 17, 23 and 32. Measurements per mouse in each dietary group are provided.

Table S5 Glycan abundance

Table S6 The Pearson correlation coefficient (*R*) of pair-wise correlation comparisons between the relative abundance of gut bacteria and colonic glycans. Only significantly correlated comparisons are shown ($P < 0.05$).

Supplementary Materials and Methods

Animal handling

All experimental protocols and procedures were reviewed and approved by the animal ethics committee, University of Sydney, Australia (reference no: 2014/611) and the animal ethics committee, Macquarie University, Australia (reference no: 5201500129).

Fibre products used in this study are derived from dried whole sugarcane stem and pectin (NutriKane) and wheat dextrin (Benefiber). NutriKane was produced and provided by Gratuk technologies Pty Ltd, Australia and Benefiber (GlaxoSmithKline, Australia) was purchased from a local Australian supermarket.

A total of 50 11-week old male C57BL/6J mice (Animal Resource Centre (ARC), Canning Vale, WA, Australia) were cohoused (two per cage) under monitored temperature (20-26 °C), humidity (40-60%), light and dark cycle (12 hour-12 hour) and with ad libitum access to water and feed during the experiment.

Following two weeks acclimatisation on a normal chow (containing 14.0 total kJg-1, 12% of total energy from fat) mice were randomised into two groups based on the body weight at week 0. One group (n=9) was fed a normal chow as a healthy control during the overall experiment. The second group (n=41) was fed a high fat diet (containing 24.0 total kJg-1, 81% of total energy from fat for a period of 17 weeks. Following this, the high fat diet fed group was further randomised into three groups based on the body weight and area under curve of the intraperitoneal glucose tolerance test at week 17. These groups were fed a high fat diet (n=14), high fat diet modified with NutriKane (n=14) or high fat diet modified with Benefiber for further 15 weeks, details of the experimental design are provided in Fig. S1.

All experimental diets were produced by Speciality feeds, WA, Australia. Custom-made high fat diets with fibre additions contained 4.7% (w/w) of either NutriKane or Benefiber as a replacement of 4.7% (w/w) cellulose in the control high fat diet (compositions of the four experimental diets and nutritional details of NutriKane and Benefiber are provided in Table 1A and B). Normal chow, high fat diet, high fat diet modified with NutriKane and high fat diet modified with Benefiber will be referred to as NC, HF, HF-NK and HF-BF respectively, here after.

Sample collection

Individual body weight and food intake per cage were measured weekly. Weekly energy intake per mouse was calculated by multiplying the average weekly feed intake per mouse by the total energy/g of each feed. Fecal samples were collected aseptically before introducing the HF (week 0), before addition of fibre products to HF (week 17) and at two-time points after fibre addition (week 23 and 32). Fecal samples were stored at -80oC prior to subsequent microbiota and metabolites analyses.

Blood (100 µL approx.) was collected after mandibular vein piercing at 0, 17 and 32 weeks and gently mixed with EDTA at a final concentration of 4 mM, pH 7.0. These samples were centrifuged at 1000 x g for 10 min at 4 °C, plasma samples were collected, aliquoted and stored at -80 °C before analysis. Mice were euthanised at week 32 by cervical dislocation. Prior to liver excision, mice were perfused with ice cold PBS to remove blood content. Right atria vein was cut, and PBS was pumped through the heart's left ventricle. Livers were removed after a few minutes once they turned pale, immediately snap frozen in dry ice and stored at -80 °C before analysis.

Intraperitoneal glucose tolerance test (IPGTT)

Intraperitoneal glucose tolerance tests (IPGTT) were performed at week 17 and 23. Mice were fasted for six hours during the light cycle. Blood glucose levels were measured from the tail vein using a Freestyle Lite blood glucose monitoring system (Abbott Pty Ltd, Australia) prior to

injection of glucose (2.0 gkg-1, intraperitoneally) and at 15, 30, 45, 60, 90 and 120 minutes after injection.

Quantification of circulating markers of inflammation in plasma

Cytokine marker (8-plex: GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-10 and TNFα, catalogue number: M60000007A) and diabetes marker (8-plex: ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1 and resistin, catalogue number: 171F7001M) (Bio-rad, Australia) quantitation was determined according to the manufacturer's protocol. Plasma samples were diluted (1:4) in sample diluent and analysed using the Bio-Plex 200 instrument (Bio-rad, Australia). The fluorescence values were log2 normalised using the random effects model which corrects for subject, time and plate to plate variation [239]. Differences between groups were analysed by Student's t test and ANOVA (Wald Chi square test). P-value correction was performed according to the Holm's method [240].

Mass spectrometry-based proteomics analysis.

Approximately 10 mg of liver was prepared for proteomics analysis from 40 samples: NC n=9, HF-BF n=10, HF-NK n=11 and BF=10. Briefly, tissue was resuspended in 500 µL of 1% (w/v) sodium deoxycholate, 0.1 M triethylammonium bicarbonate (Sigma Aldrich) supplemented with protease inhibitor cocktail (Roche). Lysis was performed using a probe sonicator for 2 min. Lysates were boiled at 95 °C for 2 min. After centrifugation at 5000 rpm for 10 min, supernatants were collected, and protein concentration was determined by the bicinchonininc acid assay. Protein (15 µg) was reduced with 10 mM DTT for 30 min at 60 °C, alkylated with 20 mM iodoacetamide for 20 min in the dark, trypsin digested (Sigma Aldrich) in 1:50 ratio and incubated at 37 °C overnight. Peptides were purified using a C-18 mini column, recovered in 70% (v/v) acetonitrile (ACN), dried, and resuspended in 1% (v/v) TFA for LC-MS/MS.

SWATH reference spectral library was generated out of a pooled liver lysate made from representative samples of the four different diets. Such pooled lysate was fractionated in a high pH reverse phase high performance liquid chromatography (HPLC) using a C18 column 2.1 mm x 150 mm, 3.5 µm (Zorbax). After sample injection, elution was performed in a buffer A of 5 mM of ammonia pH 10.4 – buffer B of 90% (v/v) acetonitrile, 5 mM of ammonia gradient. Library and SWATH-MS data were acquired in a 6600 Triple TOF mass spectrometer coupled with an Ekspert 415 LC system (Sciex). For each sample 10 µL (0.1 µg/µL) was transferred onto a peptide trap using a 2% (v/v) ACN, 0.1% (v/v) formic acid isocratic flow at 5 µL/min for 3 min. Sample was eluted with a gradient of 5-35% ACN using a buffer B of 99.9% (v/v) ACN, 0.1% (v/v) formic acid. Library data was acquired in a data dependent acquisition mode by the selection of ion precursors of the 20 most intense ions in a 120 min run. SWATH data were acquired in a data independent mode with a mass range of 400-1250 m/z, 100 variable windows in a 60 min run per sample.

Database searches utilised the murine SwissProt database and selecting the following parameters: trypsin as digestion enzyme and carbamidomethylation as fixed cysteine modification. Biological modifications were allowed, and the reversed database search was enabled to allow false discovery rate (FDR) calculation, protein global FDR was stablished at 1%. Information extracted from SWATH MS peak areas was performed using Peak View V2.1 with SWATH MicroApp 2.0 (Sciex) using the library search output file. Extraction of ion chromatograms (XICs) and determination of peak areas for the entire data set was performed using the following parameters: 6 transitions per peptide, exclusion of shared peptides, 100 as maximum number of peptides per protein, 99% confidence, <1% extraction FDR, 75ppm fragment accuracy, and 5 min extraction time window. Shared peptides, non-tryptic and semi-tryptic peptides were excluded. The ion peak areas exported by Peak View for each sample were normalised by total area normalisation as described previously [9]. Quantitation was achieved based on XICs of MS/MS spectra. Perseus 1.5.5 was employed for statistical analysis. Decoy peptides were removed prior to log2

transformation of peptide intensity. Statistically significant hits were identified by group comparison using the Student's t-test (p-value<0.05).

16S rRNA gene amplicons sequencing and bioinformatics analysis

Total community DNA was isolated from faecal samples collected at week 0, 17, 23 and 32 (n=200) using a FastDNA spin kit (MP Biomedicals, Australia) according to the manufacturer's instructions. The lysing matrix in the kit was replaced by lysing matrix E (MP Biomedicals) according to previously published protocols (1). The V4 region of 16S rRNA gene was amplified using 515 forward (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806 reverse (5′-GGACTACHVGGGTWTCTAAT-3′) primers with custom barcodes (2, 3). Amplification was conducted using a Five prime hot master mix (5 prime, VWR, Australia) with a final primer concentration at 0.2 µM in a final volume of 25 µL. The PCR was performed with 30 cycles at 94°C for 45 seconds, 50°C for 60 seconds and 72°C for 90 seconds. The resulting amplicons were quantified using a Quant-iT™ PicoGreen® (Invitrogen, Australia) and equal molar amounts of barcoded amplicons from each sample were pooled. Pooled barcoded amplicons were gel purified using a Wizard® SV gel and PCR clean up system (Promega, Australia) and sequenced using an Illumina MiSeq platform at the Ramaciotti Centre for Genomics, Sydney, Australia.

Demultiplexed raw sequence data was processed using Quantitative Insights Into Microbial Ecology (QIIME) software (version 1.9.1) using default parameters. (4). Reads with high quality (-q 19) and full length were used to determine Operational Taxonomic Units (OTUs) at 97% similarity using an open-reference protocol against the Greengenes (version 13_8) database (5). A total of 18,527,820 reads were sequenced from the 200 samples (mean 89,997 ± 28,063). OTUs with less than 0.005% reads were filtered out and the reads per sample were rarefied at 44,361 reads prior to further statistical analyses.

Statistical analysis of the 16S rRNA gene amplicon sequencing data

Statistical analysis of the gut microbiota sequencing data was conducted using PRIMER-7 software package (6, 7). Non-metric multi-dimensional scale (nMDS) plots were constructed based on Bray-Curtis similarity matrices of Log (x+1) transformed relative abundance of the OTUs. Permutational multivariate analysis of variance (PERMANOVA) was performed with 9999 permutations. PERMANOVA was conducted with pairwise tests and type III sums of squares. The Shannon diversity index and Simpson's evenness index for each sample were also determined using PRIMER-7 software package.

Distinct phylotypes (families and OTUs) between dietary groups were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method (online Galaxy version 1.0) (8). The dietary groups were used as the classes of subjects (no subclasses). LEfSe analysis was performed under the following conditions: alpha value for the factorial Kruskal-Wallis test among classes < 0.05, alpha value for the pairwise Wilcoxon test between classes < 0.01, the threshold on the logarithmetic LDA score for discriminative features > 3.0 and with all other default parameters.

Quantification of SCFAs

The concentration of SCFAs (acetate, propionate and butyrate) was quantified using faecal samples collected at week 17, 23 and 32. Approximately 20-50 mg of faces was weighed and extracted with 500 µL of 70% (v/v) ethanol and 0.1% (v/v) trifluoroacetic acid (TFA) solution spiked with an internal standard (4-methyl valeric acid) at a final concentration of 100 ppm. The solution was mixed thoroughly, then centrifuged at 14,000 x g at 4°C for 30 minutes to pellet the faecal material. The top 200 µL was removed and analysed using a Shimadzu GC-17A gas chromatograph with a flame ionisation detector (GC-FID, Shimadzu GC-17A). Samples were separated on a 30 m x 0.25 x 0.5 µm i.d. HP-INNOWax fused silica column (Hewlett-Packard, Australia) as per the manufacturer's instructions. GC-FID analysis for each sample was performed in three technical replicates (n=450). All measurements were normalised for the weight of faecal samples used for SCFA quantification.

Mucins extraction and glycan release

Lyophilised mucus from mouse colon were extracted with 200 µL aliquots of extraction buffer (6 M guanidinium hydrochloride, 10 mM Na2PO4, and 50 mM EDTA) while kept on ice. After centrifugation at 13 000 rcf at 4° C for 20 mins, the supernatant was carefully removed by pipette and the residual pellet was extracted four more times. The residual pellet was washed with 1 mL of ethanol, then centrifuged at 13 000 rcf at 4° C for 20 mins to remove residual extraction buffer and left to air dry.

Glycans were chemically released by reductive β-elimination. Approximately 1 mg of partially purified Muc2 (obtained from 4.2.3.1) was incubated at 50° C for 16 hours with 100 µL of 1 M sodium borohydride (NaBH4) dissolved in 100 mM KOH in sealed tubes in a hot water bath. The reaction was quenched with 5 µL of glacial acetic acid then desalted over Dowex AG-50W-X8 (Biorad) strong cation exchange resin prepared in a mini column format prepared in Bond Elut OMIX C18 100 µL tips (Agilent Technologies). Samples were eluted with MilliQ water and dried in a SpeedVac rotary evaporator. Dried samples were resuspended three times in acidified methanol (0.05% w/w acetic acid) and dried by SpeedVac to remove excess borate. As an additional purification step, dried samples were resuspended in MilliQ water and loaded onto porous graphitised carbon (PGC) mini columns prepared in Bond Elut OMIX C18 100 µL tips (Agilent Technologies). Mini columns were washed with MilliQ water and PGC bound glycans were eluted with 50% (v/v) acetonitrile acidified with 0.05% (v/v) trifluoroacetic acid. Samples were dried and resuspended in 10 µL 10 mM ammonium bicarbonate pH 7.6 prior to PGC-LC-MS/MS analysis.

Glycan analysis by PGC-liquid chromatography electrospray ionisation tandem mass spectrometry (PGC-LC-ESI-MS/MS)

PGC-LC-ESI-MS/MS was performed using an established method according to Jensen et al. (2012). O-glycans (obtained from 4.2.3.2) were separated using a porous graphitised carbon column (Hypercarb, 5 µm particle size, 0.18 mm I.D. x 100 mm). Separations were performed over a 45 min gradient of 0 – 90% (v/v) acetonitrile in 10 mM ammonium bicarbonate. The flow-rate was maintained at 2 µL/min using an online high-performance liquid chromatography system (Agilent 1100, Agilent Technologies, Inc., CA, USA) coupled to an electrospray ionisation ion trap mass spectrometer (ESI-MS, Agilent 6330, Agilent Technologies, Inc., CA, USA). The LC eluate was introduced directly into the ESI source. The capillary voltage was set to 3 kV. Dry gas was maintained at 300 °C. The MS spectra were obtained in the negative-ion mode with a scan range between m/z 300 and m/z 2200.

Data analysis was performed in ESI 1.3 Compass (Version 4.0, Bruker Daltonik, GmbH, Germany). Glycan compositions were calculated using Glycomod (https://web.expasy.org/glycomod/) and glycan structure was assigned by manual interpretation of the tandem MS fragmentation spectra. Glycan peaks were quantified by relative abundance using Skyline (Version 3.7.0.11317, MacCoss Lab, UW) for assisted peak peaking, generation of extracted ion chromatograms (EICs) and integration of EIC peak area. Glycan structures were drawn using Glyco Workbench 2 (Version 2.1).

Statistical analysis

Significant differences in the Shannon diversity indices, concentration of SCFAs and physiological parameters were determined by a Mann-Whitney test using GraphPad Prism (version 7) software (GraphPad Software, USA). Bonferroni's multiple comparisons tests were used to analyse data with multiple levels such as the feed intake, energy intake and physiological parameters.

Correlation network analysis

Correlation networks were constructed to examine interactions between the gut microbiota and glycans. Pairwise correlations were determined through a Pearson correlation coefficient analysis using Hmisc R package (9). The OTUs and glycans with significantly different abundances between dietary groups were used for this analysis. OTU-OTU correlations and correlations with a P value > 0.05 were excluded from further analysis. Correlation networks were constructed using Cytoscape software (Version 3.6.1).

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4.3.3 Study 2: Effect of *Citrobacter rodentium* infection on MUC2 glycosylation

To further investigate the interactions between the gut microbiota and the colonic mucus glycosylation, we were given the opportunity to analyse colonic mucus from mice infected with *Citrobacter rodentium* in collaboration with the Vallance group at the University of British Columbia in Vancouver, Canada. *C. rodentium* is a natural mouse pathogen that shares several pathogenic mechanisms with human enteropathogenic and enterohaemorrhagic *E. coli* and is widely utilised as a model for studying the intestinal response to enteric pathogens (Mundy et al., 2005). Additionally, mice that were genetically deficient in myeloid differentiation factor 88 (Myd88), were included to determine if a key component of innate immunity was involved in MUC2 glycosylation. Myd88 is the most well characterised adaptor protein of the Toll-like receptors (TLRs), an important family of receptors that participate in the recognition of bacterial antigens and that activates the IL-1R associated kinases (IRAK), making it a central node in inflammatory pathways (Deguine and Barton, 2014; Loures et al., 2011).

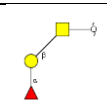
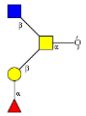
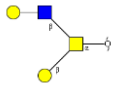
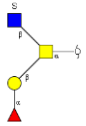
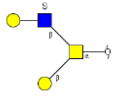
In total, we characterised the glycosylation of MUC2 collected from four groups of mice: 1) C57BL6 mice uninfected, 2) C57BL6 mice infected with *C. rodentium*, 3) Myd88 knockout mice uninfected, and 4) Myd88 knockout mice infected with *C. rodentium*. Mouse rearing, handling, infection and sacrifice were performed by the Vallance group in Vancouver according to the University of British Columbia protocol A15-0206 (Citrobacter infection and Intestinal Innate Immunity) attached as supplementary material. C57BL6 mice (Charles River, Laboratories USA) were housed in a temperature-controlled room on a 12hrs day/12hrs night light cycle with 4 mice per cage and free access to food and water. Mice were given a PicoLab Rodent Diet 20, 20% protein diet (LabDiet, MO, USA) that was autoclaved before use. Prior to infection, a streptomycin resistant strain of *C. rodentium* was grown overnight in 3ml of LB (Luria-broth with no streptomycin in the culture media) from 2-3 colonies found on a fresh LB-agar plate with streptomycin.

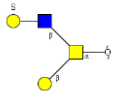
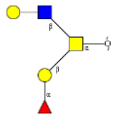
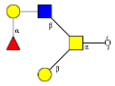
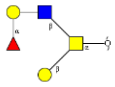
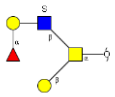
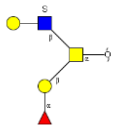
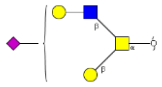
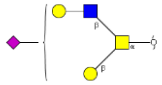
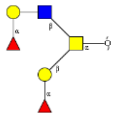
Mice between 12 to 15 weeks old were infected by oral gavage of 100 µL of the overnight culture containing between 2 to 5 x 10^9 bacteria (CFU) per ml. Mice were observed for 6 days post-infection then euthanised by isoflurane and spinal dislocation. Infection was confirmed by plating stool and colon/cecum tissue on LB-streptomycin agar plate to determine the number of bacteria infected in the mice (CFU). Colons were surgically removed, and mucus collected by vacuum suction (as described in 4.2.1) then lyophilised for shipping to Macquarie University. A total of sixteen mucus samples were received, four from each group (Control, Control infected with *C. rodentium*, Myd88 KO, Myd88 KO infected with *C. rodentium*). Extraction, release and characterisation of MUC2 *O*-glycans was performed in an identical manner to the previous study.

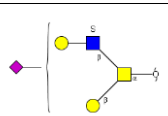
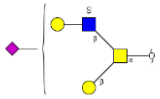
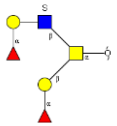
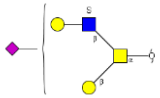
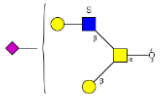
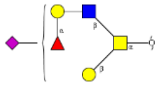
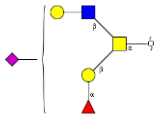
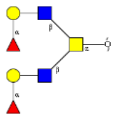
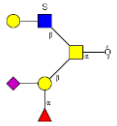
4.3.3.1 MUC2 contains predominantly Core 1 and 2 type *O*-glycans

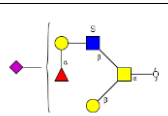
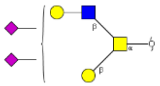
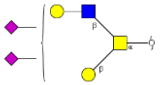
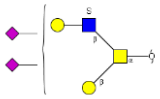
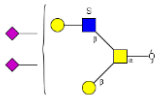
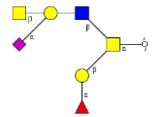
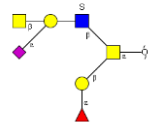
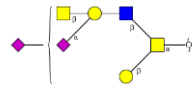
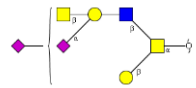
A total of 28 compositions were detected and 40 unique structures were assigned accounting for multiple isomeric structures that are summarised in **Table 4-3.** In comparison with our previous study, we detected a few different compositions and isomers. For example, the 734b and 790 glycans were not detected while additional isomers of 896 and 1120, and the larger glycans 1567 and 1713 were present. Since these samples were analysed using the same protocols and PGC-LC-MS equipment, the difference in total number *O*-glycans and presence of isomers are likely due to variations in the gut microbiota or differences in experimental diets. Another source of variation is the treatment of samples prior to glycan analysis. These samples were lyophilised immediately following survival removal of colons and vacuum collection of mucus while colons in the previous study were subjected to a freeze-thaw cycle before vacuum collection of mucus. Freeze-thaw cycles are known to degrade glycoproteins (Jumel et al., 1996). As with the previous study, a high degree of individual variation between samples was observed for specific glycans, though the overall the glycosylation profile of MUC2 was more heterogeneous and less consistent within groups. Consistent with Study 1 in this Chapter (4.3.1) and previous studies, we found a mixture of neutral and acidic structures with the prevalence of Core 1 and 2 type *O*-glycans that is characteristic of the mouse MUC2 glycome (Arike et al., 2017; Thomsson et al., 2012).

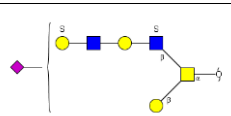
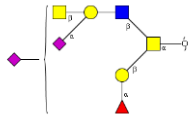
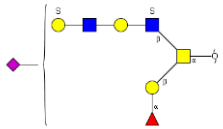
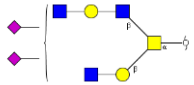
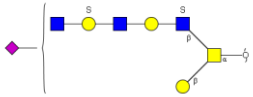
Table 4-4. Proposed structures of *O*-glycans released from MUC2 obtained from the colon of C57BL6 and Myd88 knockout mice with and without infection with *Citrobacter rodentium*. Structures were assigned by manual interpretation of fragmentation patterns and the presence of diagnostic ions in the tandem MS spectra. Values provided are % relative abundance.

Mass (m/z)			Composition	Core	Proposed sequence	B6 Control	B6 Infected	Myd88 KO Control	Myd88 KO Infected
[M-H]-	[M-H]2-	[M]				Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
530		531	(Hex)1 (HexNAc)1 (Deoxyhexose)1	1		7.6 ± 0.8	11.0 ± 2.8	6.6 ± 5.6	10.9 ± 7.4
587		588	(Hex)1 (HexNAc)2	2		9.7 ± 3.4	5.5 ± 6.7	10.3 ± 1.4	1.8 ± 0.9
667		668	(Hex)1 (HexNAc)2 (Sulph)1	2		2.2 ± 0.7	3.7 ± 1.1	4.2 ± 3.2	2.0 ± 3.5
733		734	(Hex)1 (HexNAc)2 (Deoxyhexose)1	2		6.1 ± 1.6	9.6 ± 6.0	7.4 ± 1.7	8.6 ± 4.8
749		750	(Hex)2 (HexNAc)2	2		1.5 ± 0.8	3.2 ± 4.4	1.3 ± 1.5	0.9 ± 0.4
813		814	(Hex)1 (HexNAc)2 (Deoxyhexose)1 (Sulph)1	2		14.6 ± 4.7	9.5 ± 5.4	13.3 ± 2.2	12.4 ± 8.2
829a		830a	(Hex)2 (HexNAc)2 (Sulph)1	2		4.3 ± 2.1	1.6 ± 2.0	4.8 ± 2.6	3.7 ± 2.8

829b	830b	(Hex)2 (HexNAc)2 (Sulph)1	2		1.7 ± 1.5	5.0 ± 1.4	3.3 ± 0.7	3.1 ± 2.5
895a	896a	(Hex)2 (HexNAc)2 (Deoxyhexose)1	2		2.4 ± 1.6	0.8 ± 0.1	1.2 ± 0.4	2.0 ± 0.9
895b	896b	(Hex)2 (HexNAc)2 (Deoxyhexose)1	2		1.4 ± 0.9	0.8 ± 0.6	1.5 ± 0.7	1.1 ± 1.0
895c	896c	(Hex)2 (HexNAc)2 (Deoxyhexose)1	2		4.3 ± 3.4	1.5 ± 0.9	5.6 ± 1.1	4.0 ± 2.9
975a	976a	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (Sulph)1	2		5.1 ± 1.7	2.6 ± 1.0	3.9 ± 1.7	5.8 ± 2.6
975b	976b	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (Sulph)1	2		1.1 ± 0.8	1.2 ± 0.5	1.5 ± 0.4	0.6 ± 0.5
1040a	1041a	(Hex)2 (HexNAc)2 (NeuAc)1	2		0.2 ± 0.3	1.9 ± 0.9	0.8 ± 0.5	0.2 ± 0.3
1040b	1041b	(Hex)2 (HexNAc)2 (NeuAc)1	2		4.2 ± 2.2	5.1 ± 1.2	3.1 ± 0.8	4.6 ± 1.4
1041a	1042a	(Hex)2 (HexNAc)2 (Deoxyhexose)2	2		5.0 ± 2.9	4.2 ± 2.3	2.8 ± 2.5	9.6 ± 6.7

1120a		1121a	(Hex)2 (HexNAc)2 (NeuAc)1 (Sulph)1	2		2.3 ± 2.8	4.3 ± 3.7	3.2 ± 1.9	1.1 ± 1.3
1120b		1121b	(Hex)2 (HexNAc)2 (NeuAc)1 (Sulph)1	2		1.9 ± 1.5	2.1 ± 2.1	2.6 ± 0.8	1.1 ± 1.7
1121		1122	(Hex)2 (HexNAc)2 (Deoxyhexose)2 (Sulph)1	2		4.5 ± 1.1	6.9 ± 3.2	5.2 ± 1.2	6.5 ± 3.6
	560a	1121a	(Hex)2 (HexNAc)2 (NeuAc)1 (Sulph)1	2		0.2 ± 0.4	0.8 ± 0.7	1.9 ± 1.1	0.4 ± 0.2
	560b	1121b	(Hex)2 (HexNAc)2 (NeuAc)1 (Sulph)1	2		0.1 ± 0.2	1.3 ± 1.1	1.0 ± 0.5	0.2 ± 0.2
1187a		1188a	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1	2		0.0 ± 0.0	1.6 ± 1.1	0.2 ± 0.4	0.3 ± 0.5
1187b		1188b	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1	2		0.9 ± 1.2	1.4 ± 1.7	0.7 ± 0.8	1.0 ± 1.1
1245		1246	(Hex)2 (HexNAc)3 (Deoxyhexose)2	4		0.5 ± 0.6	1.1 ± 1.7	0.0 ± 0.0	0.7 ± 0.6
1266a		1267a	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 (Sulph)1	2		1.3 ± 1.1	1.3 ± 0.9	1.4 ± 1.1	1.0 ± 0.6

1266b		1267b	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 (Sulph)1	2		0.1 ± 0.2	0.9 ± 1.1	1.0 ± 1.1	0.9 ± 1.2
1332a		1333a	(Hex)2 (HexNAc)2 (NeuAc)2	2		0.0 ± 0.0	0.5 ± 0.4	0.4 ± 0.5	1.4 ± 0.6
1332b		1333b	(Hex)2 (HexNAc)2 (NeuAc)2	2		0.4 ± 0.4	0.7 ± 0.6	0.6 ± 0.4	0.5 ± 0.6
	665a	1333a	(Hex)2 (HexNAc)2 (NeuAc)2	2		1.4 ± 1.0	1.1 ± 1.1	0.3 ± 0.2	2.3 ± 0.4
	665b	1333b	(Hex)2 (HexNAc)2 (NeuAc)2	2		2.4 ± 2.0	0.7 ± 0.5	0.6 ± 0.3	1.1 ± 1.7
1390		1391	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1	2		1.4 ± 1.0	0.8 ± 0.8	0.2 ± 0.4	0.9 ± 0.8
1470		1471	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 (Sulph)1	2		0.1 ± 0.2	0.4 ± 0.4	0.3 ± 0.3	0.3 ± 0.3
	766a	1535a	(Hex)2 (HexNAc)3 (NeuAc)2	2		1.2 ± 0.9	1.4 ± 0.6	1.2 ± 0.9	1.5 ± 1.5
	766b	1535b	(Hex)2 (HexNAc)3 (NeuAc)2	2		2.8 ± 1.3	1.1 ± 0.4	1.9 ± 0.5	3.1 ± 1.4

782	1567	(Hex)3 (HexNAc)3 (NeuAc)1 (Sulph)2	2		1.9 ± 0.9	0.6 ± 0.9	1.1 ± 0.7	0.2 ± 0.1
840	1682	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2	2		0.7 ± 0.2	0.8 ± 0.5	0.7 ± 0.5	0.3 ± 0.3
855	1713	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 (Sulph)2	2		3.8 ± 0.4	1.8 ± 1.7	2.7 ± 1.9	3.4 ± 3.3
868	1739	(Hex)2 (HexNAc)4 (NeuAc)2	2		0.2 ± 0.4	0.2 ± 0.3	0.2 ± 0.2	0.7 ± 1.2
884	1770	(Hex)3 (HexNAc)4 (NeuAc)1 (Sulph)2	2		0.4 ± 0.5	0.6 ± 0.9	1.1 ± 0.5	0.0 ± 0.0

*Proposed structures were based on the structures reported in previous studies (Arike et al., 2017; Thomsson et al., 2012).

4.3.3.2 Infection with *C. rodentium* induces minor changes in MUC2 glycosylation

To assess the effect of infection with *C. rodentium* we first compared the MUC2 glycosylation profile of C57BL6 mice with (B6 Infection) and without (B6 Control) infection (**Figure 4-14**). We performed two-way analysis of variance (ANOVA) comparing each glycan to determine which glycans were significantly altered by infection. We found that infection resulted in a 4.2 and 5.1% decrease in the relative abundance of the 588 Galβ1-3(GlcNAcβ1-6)GalNAcol and 814 Fucα1-2Galβ1-3(SO_3-6GlcNAcβ1-6)GalNAcol glycans respectively. To assess the likeness of glycosylation patterns, we used two-dimensional principal component analysis (PCA) to reduce the dimensionality of data (**Figure 4-15**). Consistent with our two-way ANOVA, the 2D-PCA plot showed only slight separation between the control (red) and infected (green) groups suggesting there were only small differences as a result of oral infection with *C. rodentium*.

We next compared the MUC2 glycosylation profile of Myd88 knockout mice with and without infection with *C. rodentium*. Two-way ANOVA and comparing each glycan between groups found that the abundance of the 531 Fucα1-2Galβ1-3GalNAcol and 1042a Fucα1-2Galβ1-3(Fucα1-2Galβ1-3/4GlcNAcβ1-6)GalNAcol glycans increased by 4.3 and 1.5% respectively, while the abundance of the 588 Galβ1-3(GlcNAcβ1-6)GalNAcol glycan decreased significantly by 8.5%. The 2D-PCA plot comparing the control (M C) and infected (Min) Myd88 knockout mice showed slight overlap between the two groups supporting the earlier observation that infection with *C. rodentium* had minimal impact on MUC2 glycosylation.

We also compared the uninfected C57BL6 and Myd88 knockout mice to determine if Myd88 loss influenced MUC2 glycosylation. Two-way ANOVA found no significant differences in the abundance of any *O*-glycans indicating that deleting Myd88 did not change MUC2 glycosylation. This was further supported by the 2D-PCA which showed overlap between the B C and M C groups.

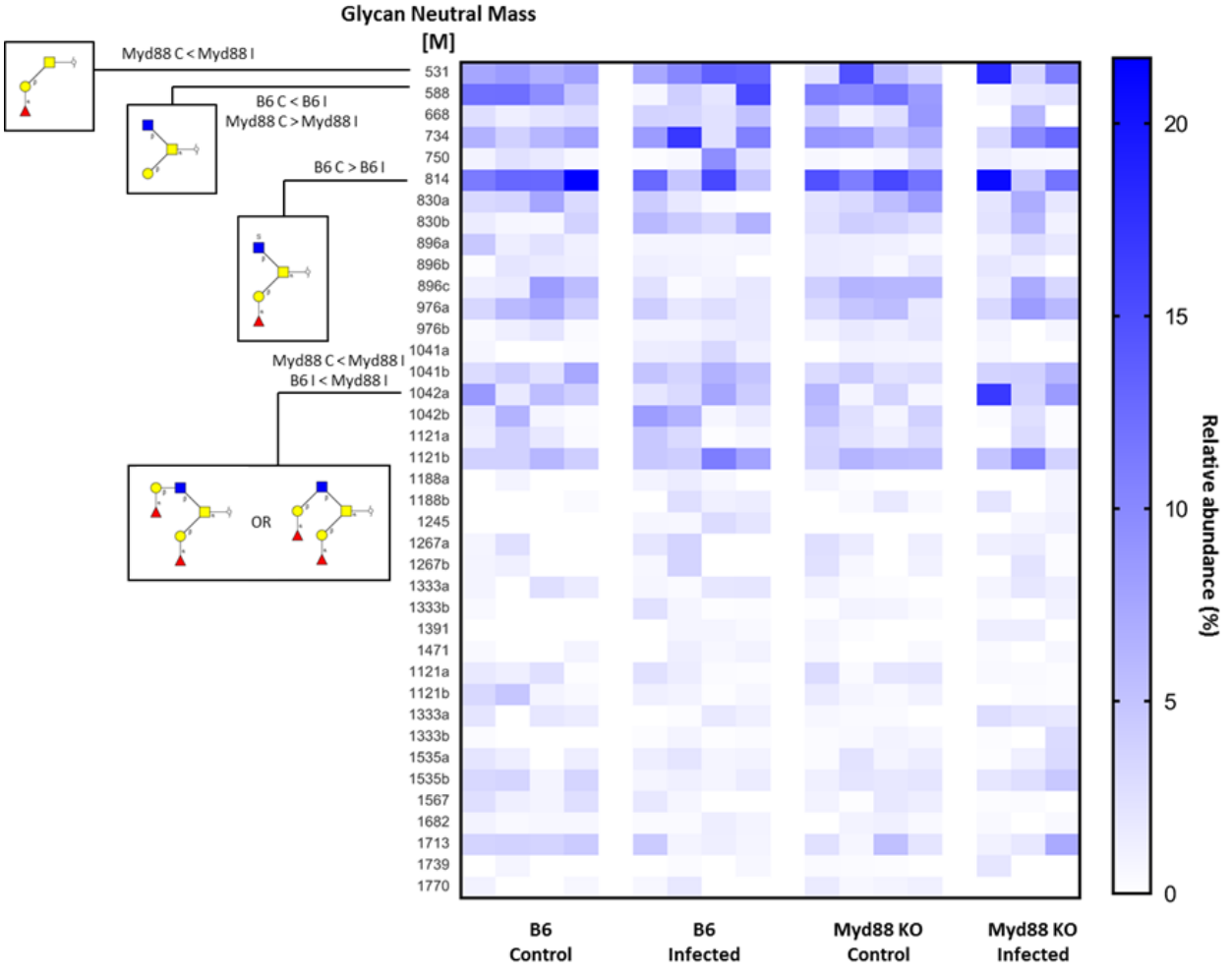


Figure 4-14. Un-clustered heat map illustrating the relative abundance of MUC2 *O*-glycans from the colon of C57BL6 or Myd88 knock out mice with and without infection with *C. rodentium*. Each column represents an individual mouse. Glycans are listed according to their neutral mass [M] with isomers labelled alphabetically according to their retention time order (a elutes before b). Relative abundance is represented by shading with darker shades representing higher abundance. The structures of *O*-glycans that were found to be significantly different between any of the groups by two-way ANOVA and multiple comparison with Tukeys tests are shown on the left.

Our results indicate that MUC2 glycosylation remains relatively unchanged following infection with *C. rodentium*. These results were somewhat surprising since previous studies have reported that pathogenic infection with other organisms can drastically alter the glycosylation of mucins. For example, chronic infection with *Helicobacter pylori* has been shown to increase the expression of *B3GNT5*, *B3GalT5* and *Fut3* genes that control the biosynthesis of the sialyl Lewis antigens on human MUC2 (Larsson et al., 2011). However, mice do not possess the *Fut3* gene or express Lewis antigens thus these changes would not appear in our study. Studies in mice however have

shown that infection with *C. rodentium* stimulates interleukin-22 (IL-22) (Zheng et al., 2008), an epithelial cell associated cytokine, that has been shown to induce the expression of *Fut2* on the intestinal epithelium and promotes fucosylation (Pham et al., 2014).

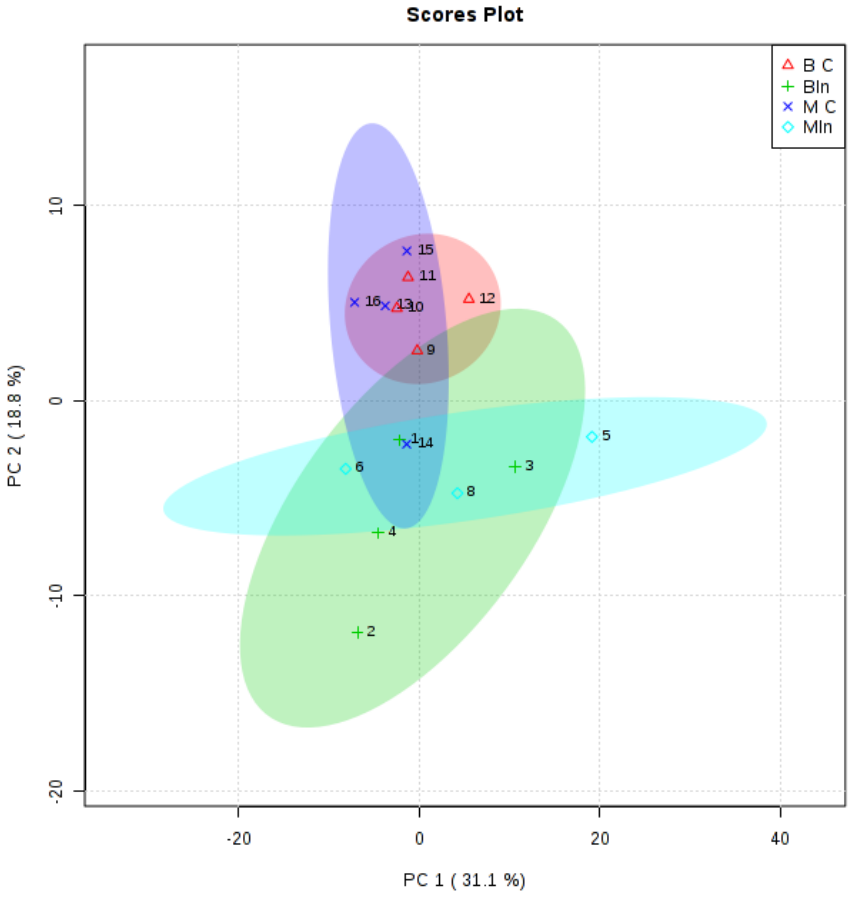


Figure 4-15. Two-dimensional principal component analysis (2D-PCA) plot of the *O*-glycosylation pattern of Muc2 from the colon of mice with and without infection with *C. rodentium*. B C: C57BL6 mice Control *n* = 4, Bin: C57BL6 infected *n* = 4, M C: Myd88 knockout Control *n* = 4, Min: Myd88 infected *n* = 3.

To determine if *C. rodentium* infection caused an increase in MUC2 fucosylation, we grouped the identified *O*-glycans into classes based on the type and number of terminal residues. A small non-statistically significant increase (~8%) in fucosylation could be observed following infection when comparing B6 control with B6 infected (**Figure 4-16**). This is consistent with previous studies that have shown increased fucosylation of intestinal mucins in response to infection (Pickard et al., 2014). Intriguingly, a significant increase in fucosylated *O*-glycans was also seen when comparing the Myd88 knockout control to the Myd88 knockout infected group (**Figure 4-16**).

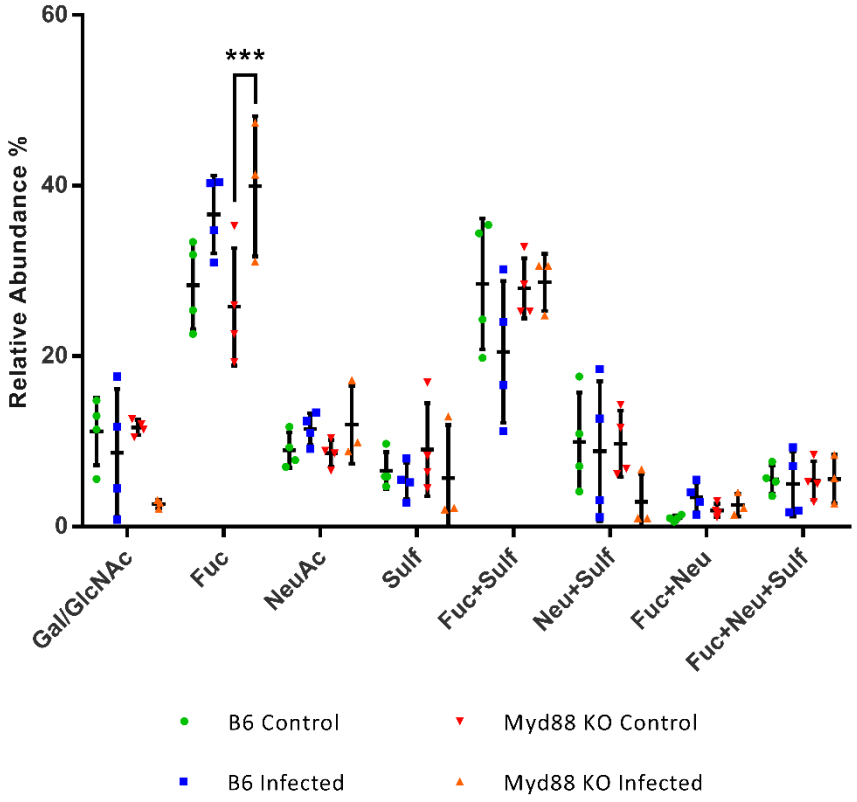


Figure 4-16. *O*-glycan groups based on the type of terminal residues. Gal/GlcNAc represent *O*-glycans that terminate in either Gal or GlcNAc and do not contains Fuc, NeuAc or Sulf. Each point represents a single mouse. Bars represent the mean ± the SD.

Separating the fucosylated glycans by the number of Fuc residues revealed that the increase in fucosylation was reflected in the abundance of both mono-and di-fucosylated glycans. Our results were unexpected since Myd88 has previously been implicated in the inducible fucosylation of the entire small intestine after infection with *C. rodentium* (Pickard and Chervonsky, 2015). This fucosylation occurs via MyD88-dependent sensing of microbial products by dendritic cells, which produce IL-23, triggering IL-22 production that causes upregulation of *Fut2* in epithelial cells of the small intestine (Pickard et al., 2014). The increased fucosylation in the Myd88 KO mice infected with *C. rodentium* as shown above may indicate that Myd88-dependent fucosylation is limited to intestinal epithelial cells and has no influence over the glycosylation of goblet cell derived MUC2 as analysed here which showed an increase in fucosylation in the absence of Myd88. Alternatively, an undefined Myd88 independent mechanism that induces fucosylation may also be responsible.

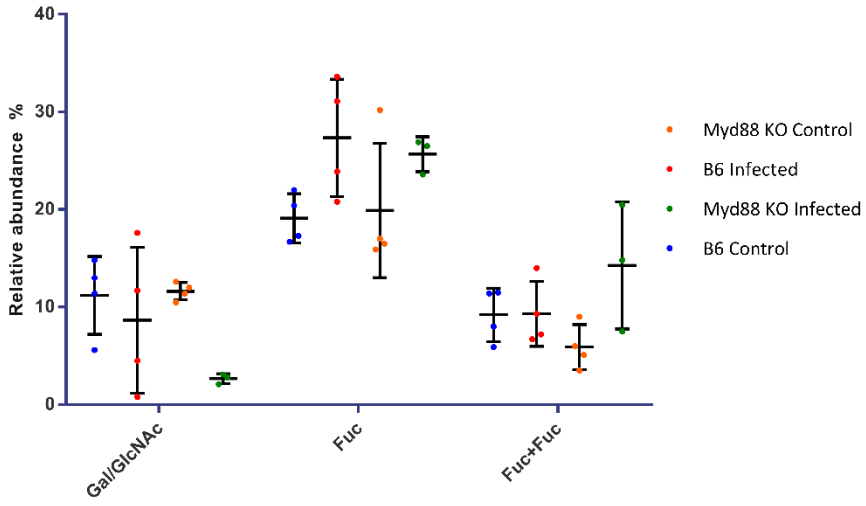


Figure 4-17. Comparison of *O*-glycans containing terminal Gal/GlcNAc, one fucose (Fuc) or two fucose (Fuc+Fuc) residues. *O*-glycan structures that belong to each group are shown below the graph. Each point represents a single mouse. Bars represent the mean ± the SD.

Due to the small sample size of the study, there were no other statistically significant differences in the abundance of *O*-glycans containing NeuAc, Sulf or combinations of NeuAc, Sulf and Fuc. We noticed there was less individual variation in NeuAc and Sulf containing glycans in the C57BL6 samples (infected and uninfected) while the Myd88 KO mice had lower variation in the abundance of Fuc+Sulf and Neu+Sulf which was high in the C57BL6 mice (**Figure 4-17**).

In summary, our results demonstrate that infection with *C. rodentium* has minimal effect on the *O*-glycosylation pattern of MUC2 suggesting that this pathogen does not induce immediate changes to the host *O*-glycome. Additionally, our results indicate that Myd88 loss induces increased fucosylation of MUC2. This contradicts previous results that have demonstrated that IL-22 induced fucosylation is controlled by Myd88, suggesting an alternative pathway that may be independent of Myd88 or even specific to goblet cells that produce and secrete MUC2. These findings warrant further investigation into the role of fucose in the innate immune response in the gastrointestinal tract. Our results are preliminary but point to the complexity of interactions between bacterial pathogens and the host mucus layer.

4.4 Chapter summary

The maintenance of intestinal health is a delicate balance between the quality of the diet, the composition of the microbiota, and the integrity of the gastrointestinal mucosa (Bischoff, 2011). Previous work has established that the gut microbiota performs multiple functions that contribute to both intestinal and overall health including energy salvage (Blaut and Clavel, 2007; Cummings, 1981), immune stimulation (Macfarlane and Macfarlane, 1997), and the prevention of pathogenic infection (Bäckhed et al., 2005). As such, diets considered unhealthy such as a high fat diet have the potential to cause microbial imbalance and dysbiosis of the gut which have been associated with conditions such as obesity (Duca et al., 2014; Shen et al., 2013).

In this work, we sought to investigate the effect of high fat diet and dietary fibre on SCFA production regarding their effects on the gut microbiota. While our body weight results showed no prevention of HF diet-induced obesity with the inclusion Benefiber, our results showed increased production of SCFAs. We found that production of SCFAs was significantly reduced in the high fat diet groups. Considering that SCFA production was restored upon reintroduction of dietary fibre incorporated into a high fat diet, it is likely that the impairment SCFA production was due to large reductions in dietary fibre content. Our results support the findings of Murphy et al. (2010) who reported similar decreases in SCFA production in response to HF diet. Introduction of Benefiber, a soluble dietary fibre, into the HF diet resulted in a significant increase in acetate and propionate concentrations while introduction of NutriKane, an insoluble dietary fibre, was not as effective in increasing acetate and propionate concentration.

The mucus layer performs a critical role in mediating the interactions between the host and the gut microbiota. In addition to preventing physical contact between the host epithelium and the luminal contents (Johansson et al., 2011b; Johansson et al., 2008), the mucus layer has also been implicated in the modulating the composition of the gut microbiota through selection and promotion of commensal microbes (Johansson et al., 2015; Kashyap et al., 2013; Koropatkin et al., 2012).

MUC2 and its proper glycosylation are critical to mucus layer function and integrity and studies have shown that absence of the MUC2 encoding gene results in complete loss of the mucus layer, increased susceptibility to pathogenic infection and the spontaneous development of colitis (Bergstrom et al., 2010; Zarepour et al., 2013). Furthermore, deletion of enzymes responsible for the formation of Core 1 and 3 type *O*-glycans results in the loss of an intact mucus layer and the spontaneous development of severe ulcerative colitis (Fu et al., 2011). The MUC2 mucin is 80% carbohydrate by mass and is primarily decorated by *O*-glycans (Allen et al., 1998), which provide complexity and are essential to mucus layer function. Our understanding of the role, function and importance of these oligosaccharides however is limited.

In this work, we present the first studies to apply glycomic analytical techniques to examine potential changes in the glycosylation of MUC2 in the colon of mice. Two studies were performed to assess the ability of the host to alter MUC2 glycosylation *in vivo* in response to diet and infection. The mucus layer of the colon is particularly exposed to changes in diet and microbial composition due to the slow movement of luminal contents and the high density of microbes.

Our first study compared a standard diet, a high fat, and two high fat diets modified with dietary fibre to examine if dietary composition could induce changes in the glycosylation of MUC2. In our second study, we obtained mucus samples from mice with and without infection with *C. rodentium* to determine if infection caused changes in MUC2 glycosylation. Overall, Core 1 and 2 type *O*-glycans were the prevalent scaffold regardless of diet composition or infection. This corroborates previous studies that showed the abundance of the same core structures in the colon of mice (Arike et al., 2017; Thomsson et al., 2012). Our results indicate that even under stressful conditions such as high fat diet and bacterial infection, the production of Core 1 and 2 type *O*-glycan scaffolds is unchanged suggesting that these are highly conserved. This is supported by previous studies that have shown that deletion of *C1gal1T*, the Core 1 producing transferase, results in a defective mucus layer (Bergstrom et al., 2016; Fu et al., 2011).

We found evidence that α1-2 fucose substitution can be induced by the inclusion of dietary fibre in a high fat (60%) diet but not by high fat alone. The replacement of 4.7% (w/w) cellulose in a high fat diet with either a soluble or insoluble dietary fibre was enough to increase the abundance of monofucosylated glycans and decrease the abundance of difucosylated glycans with both fibres. Considering our precipitation of mucus isolated the inner mucus layer, these changes may not be the result of enzymatic degradation and suggest possible induction of fucosyltransferase pathways by the gut microbiota. Fucose is an important mediator of host-commensal symbiosis (Pickard et al., 2014) and is known to protect against enteric pathogens such as *C. rodentium* and *S. typhimurium* (Pham et al., 2014; Pickard et al., 2014). Recent work has shown that fucose released by commensal microbes such as *Bacteroides thetaiotaomicron* is sensed by enterohaemorrhagic *Escherichia coli* and activates virulence-factor gene expression (Pacheco et al., 2012). While such activity would appear counterproductive to the host it helps to explains how terminal glycans epitopes can shape the gut microbiota.

We also found that without the TLR adaptor Myd88, infection with *C. rodentium* also induced an increase in the abundance of fucosylated glycans. This was contrary to previous studies since rapid fucosylation of the small intestine in response to infection is thought to be mediated by IL22 RA-1 that is controlled by Myd88 (Pham et al., 2014). However, this has only been shown in the epithelium of the small intestine and may indicate an alternative pathway that is independent of Myd88 in the mucus layer. Due to the small sample size, our results warrant further investigation to confirm our observations. One factor that may have influenced our results was the collection of mucus from the entire length of the colon since the work of Holmén et al. (2013) demonstrated that MUC2 glycosylation changes along the length of the gastrointestinal tract revealing two completely different sets of *O*-glycans observed between the proximal and distal colon (Holmén et al., 2013).

Based on previous studies that showed mucin glycosylation patterns were altered in disease states including colorectal cancer (Brockhausen, 2006) and ulcerative colitis (Larsson et al., 2011), we investigated whether environmental stressors such as high fat and infection could induce immediate changes in MUC2 glycosylation. Studies in knockout mice lacking the genes encoding for MUC2 or specific glycosyltransferases have also been used to elucidate the biological significance of MUC2 glycosylation (Bergstrom et al., 2010; Burger-van Paassen et al., 2011; Kashyap et al., 2013; Thomsson et al., 2012; Van der Sluis et al., 2006; Velcich et al., 2002). Our results can be seen to complement these studies by providing detailed information on the glycan structures in the colon and their abundance, that provides another approach to study mucus-gut microbiota interactions. Overall these findings highlight the complex interaction between diet, mucus glycosylation and the gut microbiota. Future studies will clarify the specific mechanisms by which diet regulates host glycosylation and gut-microbiota interactions and may assist in the development of novel functional food therapies that promote maintenance of the mucus layer and the proliferation of beneficial bacteria.

4.5 Supplementary

Specialty Feeds
3150 Great Eastern Hwy
Glen Forrest
Western Australia 6071
p: +61 8 9298 8111
F: +61 8 9298 8700
Email: info@specialtyfeeds.com

Diet
SF02-006

60% Fat Modification of AIN93G

A very high fat semi-pure diet formulation for laboratory rats and mice based on AIN-93G.

- The high fat content has resulted in a 74% increase in calculated energy. To allow for the high fat inclusion, the carbohydrate content has been reduced.
- The fatty acid profile has an increased proportion of saturated and mono-unsaturated fats.
- Changes in all other nutritional parameters have been kept to a minimum.
- In one research facility this formulation induced clear evidence of insulin resistance after six weeks feeding.
- The high fat content has necessitated a change in the diet from away from a pellet to a small block. The block contains around 25 grams of diet and can be fed "as is" or cut into smaller sections for feeding.

Calculated Nutritional Parameters	
Protein	19.40%
Total Fat	60.00%
Crude Fibre	4.70%
AD Fibre	4.70%
Digestible Energy	24 MJ / Kg
% Total calculated digestible energy from lipids	81.00%
% Total calculated digestible energy from protein	13.00%

Diet Form and Features

- Semi pure diet. 15mm x 20mm block to mimic similar size of pellet.
- Packed in plastic trays. Trays packed in groups of five (5). with layer of glad wrap between each to protect diet.
- Vacuum packed under nitrogen in oxygen impermeable bags. Packed in cardboard cartons for protection during transit.
- Diet must be stored under 20ºC
- Diet not suitable for irradiation or autoclave
- Lead time 2 weeks

Ingredients	
Casein (Acid)	200 g/Kg
Sucrose	106 g/Kg
Canola Oil	100 g/Kg
Cocoa Butter	400 g/Kg
Clarified Butter Fat (Ghee)	100 g/Kg
Cellulose	50 g/Kg
DL Methionine	3.0 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
AIN93 Trace Minerals	1.4 g/Kg
Potassium Citrate	2.5 g/Kg
Potassium Dihydrogen Phosphate	6.9 g/Kg
Potassium Sulphate	1.6 g/Kg
Choline Chloride (75%)	2.5 g/Kg
AIN93 Vitamins	10 g/Kg

Calculated Amino Acids	
Valine	1.30%
Leucine	1.80%
Isoleucine	0.90%
Threonine	0.80%
Methionine	0.80%
Cystine	0.06%
Lysine	1.50%
Phenylanine	1.00%
Tyrosine	1.00%
Tryptophan	0.30%
Histidine	0.60%

Calculated Total Minerals	
Calcium	0.46%
Phosphorous	0.30%
Magnesium	0.08%
Sodium	0.11%
Chloride	0.16%
Potassium	0.38%
Sulphur	0.22%
Iron	70 mg/Kg
Copper	6.7 mg/Kg
Iodine	0.2 mg/Kg
Manganese	16.5 mg/Kg
Cobalt	No data
Zinc	50 mg/Kg
Molybdenum	0.15 mg/Kg
Selenium	0.3 mg/Kg
Cadmium	No data
Chromium	1.0 mg/Kg
Fluoride	1.0 mg/Kg
Lithium	0.1 mg/Kg
Boron	2.1 mg/Kg
Nickel	0.5 mg/Kg
Vanadium	0.1 mg/Kg

Calculated Total Vitamins	
Vitamin A (Retinol)	4 790 IU/Kg
Vitamin D (Cholecalciferol)	1 000 IU/Kg
Vitamin E (a Tocopherol acetate)	94 mg/Kg
Vitamin K (Menadione)	1 mg/Kg
Vitamin C (Ascorbic acid)	None added
Vitamin B1 (Thiamine)	6.1 mg/Kg
Vitamin B2 (Riboflavin)	6.3 mg/Kg
Niacin (Nicotinic acid)	30 mg/Kg
Vitamin B6 (Pryridoxine)	7 mg/Kg
Pantothenic Acid	16.5 mg/Kg
Biotin	200 ug/Kg
Folic Acid	2 mg/Kg
Inositol	None added
Vitamin B12 (Cyancobalamin)	103 ug/Kg
Choline	1670 mg/Kg

Calculated Fatty Acid Composition	
Saturated Fats C12:0 or less	1.10%
Myristic Acid 14:0	1.30%
Palmitic Acid 16:0	14.00%
Stearic Acid 18:0	16.00%
Arachidic Acid 20:0	0.50%
Palmitoleic Acid 16:1	0.30%
Oleic Acid 18:1	22.00%
Gadoleic Acid 20:1	0.20%
Linoleic Acid 18:2 n6	3.40%
a Linolenic Acid 18:3 n3	1.20%
Arachadonic Acid 20:4 n6	Trace
EPA 20:5 n3	Trace
DHA 22:6 n3	No data
Total n3	1.39%
Total n6	3.41%
Total Mono Unsaturated Fats	22.22%
Total Polyunsaturated Fats	4.80%
Total Saturated Fats	32.84%

Calculated data uses information from typical raw material composition. It could be expected that individual batches of diet will vary from this figure. **Diet post treatment by irradiation or auto clave could change these parameters**.
We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.

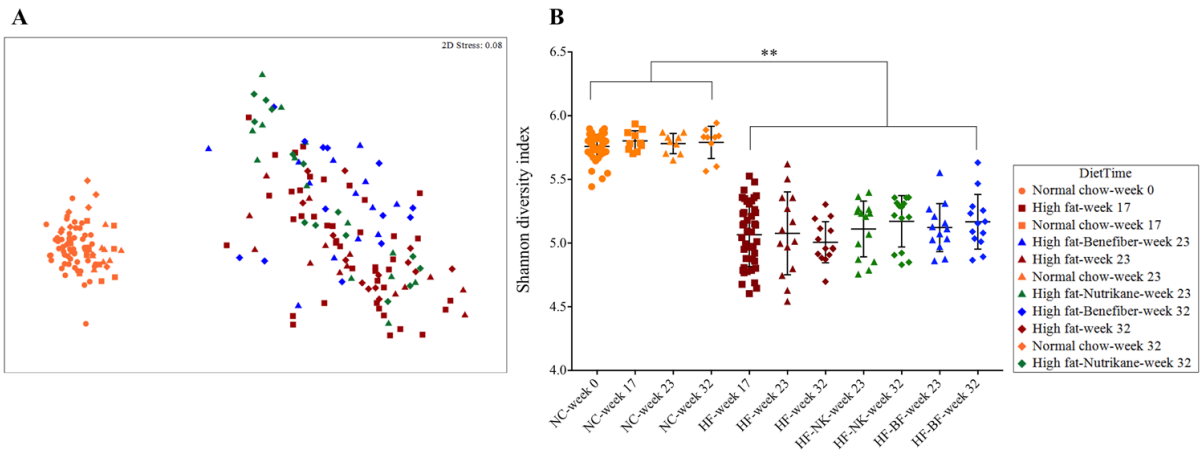


Figure 4-18. Microbial diversity of the gut microbiota in mice fed each of the four diets at weeks 0, 17, 23 and 32. **(A)** Ordination of the gut microbiota shown as a Bray-Curtis similarity of the relative abundance based nMDS plot. **(B)** Gut microbial diversity shown as Shannon diversity indices. Significance was determined using Mann-Whitney test. ** ($P < 0.01$) denotes significantly different Shannon diversity indices in the three HF diet groups compared to the normal chow group at week 0, 17, 23 and 32. Mean values with ± SD are shown. Data reproduced with permission from Hasinika K. A. H. Gamage.

Chapter 5: Summary and future directions

5.1 Thesis summary

Poor diet is now the leading risk factor for disease related death and disability in the world and new strategies are critical for combatting the increasing burden of diet related death and disease (Bauer et al., 2014; World Health Organization, 2003). Observational studies have shown that increased consumption of dietary fibre is associated with reduced risk of certain chronic diseases, such as heart disease, diabetes, and obesity, and these outcomes have prompted growing interest in the development of novel dietary fibre products and their effects on health and disease (Dahl and Stewart, 2015).

Our investigation began with the characterisation of three dietary fibres to compare their chemical compositions in relation to their unique physical properties. Dietary fibres comprise a large and heterogeneous group of molecules and have been categorised in many contexts due to their chemical complexity. The chemical characterisation of a novel dietary fibre is thus an important step to further investigation. We compared NutriKane, a dietary fibre derived from fresh whole sugarcane stalk, with Benefiber, a modified wheat dextrin, and psyllium husk, a fibre derived from the seed husk of the *Plantago ovata* plant. We found significant differences in the chemical composition of each with the most distinct differences seen in the cell wall associated components. We also observed significant differences in monosaccharide composition that correlated well with the physical properties of each fibre. Benefiber, which was completely water soluble, was primarily composed of glucose and mannose while psyllium husk, a gel-forming fibre capable of holding many times its own weight in water, was comprised of arabinose and xylose. In contrast, NutriKane, which was completely insoluble, contained mainly xylose and glucose suggesting the predominance of polysaccharides such as cellulose and hemicellulose. While the two soluble fibres, Benefiber and psyllium husk, were almost entirely composed of carbohydrates, NutriKane

was shown to also contain approximately 20% lignin and significant quantities of xylose that likely contributed to its insoluble nature. In summary, our study investigated the contribution that dietary fibres with different chemical compositions have on their functional properties.

Sugarcane is an abundantly grown agricultural crop that generates large amounts of lignocellulosic biomass primarily left over from the production of sucrose (bagasse). A key difference between the present and previous studies involving sugarcane fibres was that NutriKane was prepared from whole sugarcane stalk rather than bagasse waste, a diversion from the current paradigm of repurposing agricultural waste into potentially useful health food products (Elleuch et al., 2011). Many of the beneficial effects of dietary fibre, such as reductions in blood glucose and serum cholesterol levels, are mainly associated with soluble fibres (Brownlee, 2011). In contrast, insoluble fibres are generally believed to solely contribute to faecal bulking and have largely been ignored by the food industry (Macagnan et al., 2016). Our characterisation of NutriKane, an insoluble fibre, identified the presence of nutritionally valuable components such as chromium and manganese, which are rarely found in foods, and phenolic compounds that possess antioxidant activity. Our results suggest that insoluble dietary fibres with high lignin content may act as vehicles that transport nutrients and polyphenolic compounds to the colon and could be more important than previously thought.

It has become increasingly clear that the maintenance of intestinal health is determined by the functional state of 1) the gut microbiota (Blaut and Clavel, 2007; Chung and Kasper, 2010), and 2) the intestinal barrier (Fasano and Shea-Donohue, 2005; Meddings, 2008). To test the effect of dietary fibre on the gut microbiota, we used an *in vitro* gut mimicking system to incubate NutriKane, Benefiber, and psyllium husk with human faecal samples. We found that incubation of the faecal microbiota with dietary fibres altered the microbiome composition and the production of short chain fatty acids (SCFAs). Benefiber and psyllium husk were readily fermented by the faecal microbiota and induced significant increases in the production of the major SCFAs, acetate,

propionate, and butyrate. While soluble fibres are known to undergo fermentation to produce SCFAs (Noack et al., 2013; Timm et al., 2010), the fermentation of NutriKane or any type of fibre derived from sugarcane has never been reported. NutriKane was markedly less fermentable as the soluble fibres and only slightly increased the production of SCFAs over the no fibre added control. These results were consistent with the understanding that soluble fibres are readily fermented while insoluble fibres are resistant to fermentation. Our results indicate that certain polysaccharides, mainly the cellulosic and hemicellulosic polysaccharides, undergo minimal fermentation by the gut microbiota.

While our investigation was mainly focused on the comparison between NutriKane, Benefiber and psyllium husk, we also had the opportunity to measure the effect of different cereal products on the infant gut microbiota using the *in vitro* gut mimicking system. Incubation with wheat, sorghum, oats and rice induced changes in the infant microbiome and increased the production of acetate while propionate and butyrate production varied based on the cereal product and infant faecal sample. Since all four cereal products had similar carbohydrate compositions, our results indicate that SCFA production was highly dependent on the initial composition of the gut microbiota where there was little variation in the chemical composition of dietary fibres. The findings of both of our *in vitro* studies contribute to a deeper understanding of diet-microbiota interactions. We have demonstrated that the fermentation of dietary fibre is dependent on both carbohydrate content and the demographics of gut microbiota. Since the rate of fermentation can be partly controlled by polysaccharide composition, different fibres can serve a variety of purposes depending on the level of fermentation required. These findings have important implications in the use of dietary fibres to treat intestinal diseases such as irritable bowel syndrome where the use of a readily fermented fibre such as Benefiber may be detrimental due to excessive gas production resulting in bloating and causing discomfort (McRorie, 2015). From our collaboration with Hasinika W. Gamage, we have also shown that dietary fibres can alter the composition of the gut microbiota in a variety of

ways, including the diversification of microbes or the over representation of a single phylum, meaning that dietary fibre interventions should be tailored to individuals.

To investigate the effect of high fat and dietary fibre on intestinal health we performed an *in vivo* study in mice focussing on the three main underlying mechanisms contributing to intestinal health; the gut microbiota and their production of SCFAs, and the gastrointestinal mucosa. Since *in vitro* models of intestinal health are difficult to reproduce, we conducted a feeding study in mice then measured the effects of high fat and dietary fibres on 1) the faecal concentration of SCFAs, and 2) the carbohydrate composition of MUC2, the major component of the mucus layer and a key mediator in host-gut microbiota interactions (Hansson, 2012). This study was performed in collaboration with two other PhD projects that measured the microbiome and cytokine production in the same experiments - thus combining proteomics, genomics, metabolomics and glycomics data.

In comparison to mice given a normal high plant polysaccharide rodent diet, feeding with a high fat diet significantly reduced the faecal concentration of SCFAs. The introduction of a soluble dietary fibre (Benefiber) resulted in a gradual increase in acetate and propionate with concentrations returning to control levels. In contrast, the introduction of an insoluble dietary fibre (NutriKane) had minimal effect on SCFA concentrations in comparison with the HF diet. These results confirmed the conclusions of our *in vitro* study that linked the fermentability of dietary fibres with SCFA production. Considering that previous studies have shown that dietary supplementation of SCFAs protects against high fat diet-induced obesity (den Besten et al., 2015; Schwiertz et al., 2010; Turnbaugh et al., 2006), our work presents conflicting results.

In our assessment of MUC2, we found that high fat diets modified with NutriKane and Benefiber also altered the glycosylation patterns resulting in the increased abundance of shorter *O*-glycans, decreased terminal addition to the glycans with NeuAc and Sulfate, and di-fucosylated *O*-glycans. This study was the first to characterise the changes in the glycosylation of MUC2 from the mouse

colon in response to different diets. Our investigation has demonstrated that dietary factors such as increased fibre intake has a direct impact on the composition of the mucus layer through the changed glycosylation of MUC2. Changes in MUC2 glycosylation have the potential to alter mucus layer integrity, which is critical in managing intestinal inflammation (Larsson et al., 2011), and influences the composition of the commensal gut microbiota (Arike and Hansson, 2016). The implications of these findings can assist in the development of novel functional foods that could potentially target the mucus layer to alleviate chronic inflammation and ulcerative colitis (Larsson et al., 2011). These findings also suggest that diet influences the host physiology in addition to nutrition and interaction with the gut microbiota. To further investigate the function of MUC2 glycosylation we collaborated with the Vallance group from the University of British Columbia to determine the effect of infection with *C. rodentium* on the colonic MUC2 glycome of the mouse. We also measured if genetic knockout of Myd88, which plays a key role in controlling the inflammatory immune response, could alter MUC2 glycosylation following *C. rodentium* infection. This study was the first to characterise changes in MUC2 glycosylation in response to infection. While our study was small and results preliminary, we found evidence that infection of Myd88 KO mice with *C. rodentium* increased the fucosylation of MUC2, which is contrary to the belief that Myd88 controls intestinal fucosylation via production of IL-23 (Pickard et al., 2014). The terminal attachment of fucose as part of specific glycan structures, such as the blood group H epitope, is known to contribute to the adhesion of gut microbes (Pickard and Chervonsky, 2015) and glycosylation of MUC2 has been proposed to act as a decoy binding target to sequester both commensal and pathogenic microbes and prevent their direct contact with the host epithelium (Johansson et al., 2014). The ability of the host to alter MUC2 glycosylation in response to a state of infection thus represents an important mechanism for adaptation and this is reflected in the variability of glycan structures. Both studies were novel in characterising changes in MUC2 glycosylation at the glycan level in response to either diet or infection and provide an important step towards understanding the functional role of glycan structures in the colonic mucus layer.

Overall in this work, this thesis explored the influence of dietary fibre on intestinal health by bringing together technical expertise from the fields of analytical chemistry, metabolomics, and glycomics. In addition, the interdisciplinary approach and collaborative effort between three PhD projects was a major strength of the overarching project and permitted a more wholistic investigation of how dietary fibre influences overall intestinal health. Our studies have been important in establishing that dietary fibre as a classification encompasses a substantial variety of molecules with a range of physical properties and physiological activities. My work has shown that increased dietary fibre consumption directly impacts the production of SCFAs through the provision of fermentable carbohydrates to gut microbiota. Our findings also identified a novel interaction between diet and the colonic mucus layer through changes in MUC2 glycosylation in response to dietary fibre inclusion. The results of this study highlight the complex relationship between diet, the gut microbiota and mucus layer and support the use of dietary fibres in maintaining intestinal health.

5.2 Future directions

The work presented in this thesis contributes to the understanding of the complex relationship between diet, the gut microbiota and the host through investigating the effect of dietary fibres on intestinal health. However, our work also highlighted our limited understanding of these complex interactions. The following sections identify some questions raised by our research and the experimental approaches that could be explored in the future.

Functional characterisation of NutriKane

A fundamental aim of this project was to characterise NutriKane as a potential dietary fibre. While our study was successful in identifying the chemical components responsible for its classification as an insoluble dietary fibre, mainly its lignin and carbohydrate content, further analysis of its polysaccharide content to determine linkage type and structural arrangement could provide a greater understanding of its physical properties and resistance to digestion. Traditional methods of carbohydrate analysis have employed extraction and fractionation with a combination of acid and alkaline conditions, solubilization with detergent and treatment with enzymes to isolate different polysaccharide fractions (Knudsen, 1997). Isolated polysaccharides could then be characterised by ^{1}H and ^{13}C NMR spectroscopy to provide information on linkage type and chain sequence such as by the methods of Fischer et al. (2004). The information gathered will be essential in identifying the bacterial enzymes necessary for the digestion of insoluble dietary fibres like NutriKane and could prove invaluable in the development of novel dietary fibres or food products that resist both human and bacterial digestion. These products could serve a purely nutritional purpose or could conceivably be used as transport vectors to deliver therapeutics to specific regions of the intestinal tract.

NutriKane, which is a new commercial product that we investigated in this thesis, was also shown to contain some interesting components in the form of rare minerals like chromium and manganese, polyphenolic compounds with antioxidant activity, and potential salt binding

complexes. Each of these components have previously been implicated in nutritional deficiency (Kaur and Henry, 2014), the prevention of inflammation and tumorigenesis (Abbas et al., 2013; Abbas et al., 2014), and sequestration of dietary sodium (He et al., 1999). Further studies into the mineral content could involve speciation to determine the oxidation state of minerals to ensure fitness for consumption and nutritional value. For example, chromium exists in two oxidation states, Cr(III), which is necessary for proper physiological functions, and Cr(VI), which is carcinogenic to humans (Costa and Klein, 2006). Data from this study also found that an ethanol extract of NutriKane exerted anti-inflammatory activity by down regulating the NFκB signaling pathway (Bucio-Noble et al., 2018). Salt binding activity was thought to indicate the presence of bile salt binding complexes that could be potentially useful for reducing dietary sodium and cholesterol intake, both of which are major risk factors for cardiovascular disease (Cook et al., 2007; Scott, 2004). To confirm these observations, binding studies by incubating NutriKane with solutions of sodium salts of cholic acid, chenodeoxycholic acid, deoxycholic acid, taurocholic acid, taurocheno-deoxycholic acid, taurodeoxycholic acid, glycocholic acid, glycocheno-deoxycholic acid, and glycodeoxycholic acid could be performed to calculate the percentage bound to the NutriKane fibre.

Dietary intervention studies in humans

While *in vitro and in vivo* models are incredibly useful, they lack the complexity of human physiology. Our studies using *in vitro* and *in vivo* models were valuable in laying the foundation for further observational studies in human patients. While many dietary fibre supplements are commercially available, established and conclusive recommendations for dietary fibre interventions are still controversial. This is largely due to the plethora of compounds that fall under the classification of “dietary fibre” in addition to wide range of diseases and disorders for which dietary fibre has been indicated. Though the amount of the research required to come to any such conclusions is vast and encompasses many fields and disciplines, we believe investigation into the

usage of NutriKane or sugarcane derived fibres and other dietary fibres should be pursued considering the paucity of insoluble dietary fibre products in the market. Longitudinal studies should measure the effect of supplementation of the diet with various dietary fibres of differing composition on weight gain/loss, fasting and post-prandial glucose and insulin levels, changes in appetite, mood, and other indicators of intestinal health such as stool transit time, faecal bulk, inflammatory state, and gut microbiota composition and metabolism. In addition to the effects of the insoluble dietary fibre, the mineral content, antioxidant and salt binding activity of the various dietary fibres should also be measured in these studies to link the presence of nutritionally valuable compounds to tangible physiological effects. These studies would be conducted as randomized clinical trials with participants provided with enough dietary fibre for the duration of the study. A review of dietary fibre interventions found that most studies had small sample sizes (<100) and few studies had durations longer than 12 weeks (Hartley et al., 2016). Focus should be placed on increasing the number of participants. Two cohorts, such as diabetic and non-diabetic, could be monitored in parallel to measure prevention and treatment of type 2 diabetes. Specific phenotypes, such as obesity, could also be targeted.

Investigating the complexity of MUC2 glycosylation

Our studies on MUC2 indicated that dietary fibre could induce distinct and different changes in MUC2 glycosylation. As a highly glycosylated protein and the major component of the mucus layer, MUC2 and its glycosylation perform an important role in host-gut microbiota interactions by acting as the interface between the luminal contents and the underlying gastrointestinal mucosa (Johansson et al., 2011a). Mechanisms that alter MUC2 glycosylation are thus incredibly important to understand the complex relationship between diet, the host, and the gut microbiota. A key finding of our feeding studies was that dietary fibre in a high fat diet, regardless of being soluble or insoluble in nature, noticeably and similarly changed the glycosylation of MUC2. To confirm our previous findings, we would suggest performing quantitative reverse transcriptase

polymerase chain reaction (RT-PCR) on colon tissue samples to detect changes in RNA expression of glycosyltransferases responsible for the alterations in MUC2 glycosylation, such as the fucosyltransferase *Fut2*. This would confirm our hypothesis that the observed changes in MUC2 glycosylation were from the host and not due to trimming by bacterial enzymes. While we found no indication that the faecal microbiota were responsible for these changes in glycosylation, we cannot rule out the involvement of the mucus adhered microbiota. Mucus microbial "specialists" such as *Akkermansia muciniphila* and *Bacteroides thetaiotaomicron* are known inhabitants of the mucus layer that selectively degrade mucin glycans and may contribute to trimming of mucus layer glycosylation by bacterial GHs (Tailford et al., 2015). To differentiate whether the changes in MUC2 glycosylation are occurring at the glycosylation synthesis level and not a result only of bacterial trimming we suggest performing mucin-bacterial binding assays following the protocol of McNamara et al. (2000) before and after the removal of bacterial binding epitopes. Alternatively, samples of MUC2 could be incubated with cultures of faecal and mucus adhered bacteria to determine the extent of bacterial induced changes in glycosylation. Using the glycomic approach of our study, it would also be of interest to characterise the *O*-glycosylation of the surface of the intestinal tract epithelium that lies just beneath the mucus layer. Similarities in the glycosylation of the intestinal epithelium and MUC2 would support the hypothesis that secreted mucins act as binding decoys to prevent contact between microbes and the epithelium since similar glycan binding targets would be found on both.

5.3 Concluding remarks

In summary, the work presented in this thesis has contributed to a deeper understanding of the influence of dietary fibre on intestinal health through collaborative investigation of the effect of dietary fibre supplements on the microbial production of SCFAs and the glycosylation of the colon mucus layer. Our analysis of dietary fibres was combined with *in vitro* and *in vivo* studies to make correlations between the chemical composition of the fibres and physiological activity. The results

indicate that different dietary fibres exert different influence on the gut microbiota and their production of microbial metabolites and the host intestinal mucus layer, highlighting the complexity of diet, gut microbiota and host interactions. Improving ones' diet may be one of the most effective ways of ensuring good intestinal health and our findings support the use of dietary fibres to this end.

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Appendix

Macquarie University Animal Ethics Approval for Chapter 4, Study 1

Animal Ethics Application

Application ID :	5201500129
Application Title :	(Collaborative) Effect of Nutrikane on immunosystem and gut microbiome in lean, obese and diabetic mice
Date of Submission :	08/09/2015
Primary Investigator :	Dr Liisa Kautto

1. Administration

Project Overview

Your Application ID

5201500129

1.1 **Title of Proposed Project***

(Collaborative) Effect of Nutrikane on immunosystem and gut microbiome in lean, obese and diabetic mice

1.2 **Please indicate the purpose(s) of this application**
*Tick all applicable**

- [x] Research
- [ ] Teaching
- [ ] Survey
- [ ] Breeding/Monitoring
- [ ] Holding

1.3 **Reason for Application***

- ( ) New Project
- ( ) Resubmission
- ( ) Continuation of Expired, or soon to expire, Animal Research Authority (ARA) at MQ/other institution
- (•) Notification of Approved Project by External AEC

1.3.2 Please upload a copy of your
- Application
- Approval letter
Please go to the attachment page and upload the documents.

1.4 **Clearance Purpose code** Select the most appropriate Purpose Number(1-10) from those listed below to describe the primary purpose of the project *(only one purpose may be entered for each project)**

5 - Research: human or animal health and welfare

1.5 **Proposed start date**
Projects can only commence after they have been approved by the AEC and a valid Animal Research Authority (ARA) has been issued.

25/09/2015

1.6 **Proposed end date:**
Maximum initial approval duration is 36 months. After that time, a new application is required.
*Note that annual (or less than) reporting is a legislative requirement (so, for example a 13 months project would require two annual reports)**

01/03/2017

Collaborations

1.7 **Does this project involve collaboration with other institution?**
*You will be required to provide a copy of the Institution approved ARA and application form **

- (•) Yes
- ( ) No

Please select all Collaboration applicable *

- [ ] Project will be carried out at more than one institution
- [x] Collaborating investigator external to Macquarie University
- [ ] Animal housed or being moved to and from another institution during project
- [ ] Similar projects being conducted simultaneously at more than one institution by collaborators
- [x] MQ Staff/Student performing research at another institution
- [ ] Other (describe below)

1.7.2 **List all institutions involved, the contact person (PI at other institution), and the AEC who is responsible for overseeing the project***

Institutions: University of Sydney, Medical School, Diabetes and Islet Biology/ NHMRC Clinical Trial Centre
Contact person: AProf Anandwardhan A Hardikar
AEC responsible: Prof David Allen, Chief of University of Sydney Animal Ethics Committee

Please upload a copy of your MOU between MQ and the said Institution if any.
Please go to the attachment page and upload the documents.

I confirm that, for the Institutions listed above where Macquarie University's AEC is overseeing the project:
(i) The Institutions have been informed of this arrangement.
(ii) The Institutions agree to delegate the responsibility for decision making to, and support the necessary action of, the Macquarie University AEC.
(iii) The Institutions agree to abide by the requirements set out in the Macquarie University's AEC Terms of Reference; regarding the Consideration of Protocols and Non-Compliance with the Code

Signed: (Print full name)*

Dr Liisa Kautto

Date:*

03/08/2015

Location

1.8 **Identify all locations where research / teaching involving animals will be conducted**
(including where experimental procedures will be performed, where animals will be collected, and where animals will be housed). *

- ☐ Central Animal Facility (CAF)
- ☐ Fauna Park (FP)
- ☐ Australian School of Advanced Medicine (ASAM)
- ☐ In-Situ / Wildlife
- ☑ Other facilities

1.8.1 **For sites other than CAF, FP or ASAM, please provide a list of all locations where Research will be performed including full street address, state and country***

Medical Foundation Building, 92-94 Parramatta Road, Camperdown, NSW 2050 Australia

1.11 **Does the facility have its own Animal Ethics Committee?** *If housing of animal is taken care by this facility, it is suggested that you apply to the relevant AEC and provide us with a copy of your approval* *

Yes

2. Financial Support

Funding

2.1 **Is this project being funded by an internal or external grant?***

- ◉ Yes
- ○ Intend to apply for funding
- ○ No

Approved Grant details:
(Enter all grants associated with this project)

Name of Funding Body : ARC Training Centre for Molecular Technology in Food Industry, Subproject: Nutraceuticals from sugar cane - New treatments for diabetes

Grant ID/Reference: IC300100009

Name of Funding Body:
Grant ID/Reference:

Name of Funding Body:
Grant ID/Reference:

Name of Funding Body:
Grant ID/Reference:

4. Personnel Details

Investigator details

IMPORTANT:

- Personnel must only be named below if they have a specific role in the animal component(s) of this project N.B. in an emergency, a person who is not listed on the Animal Research Authority (ARA) may still step in to act if it is in the interest of animal welfare, without being in breach of the ARA. As such, students and/or researchers should NOT be listed on the protocol merely for `back-up'.
- Animal Technicians/Animal Care Staff do not need to be listed on an AEC protocol, provided that:
  - they are only performing routine animal care/husbandry (e.g. feeding, cleaning, general maintenance, monitoring)
  - they are competent to carry out the required procedures.
  If however personnel are involved in specific research procedures (e.g. anaesthesia, blood collection etc.) additional to routine animal care, then they must be listed on the AEC protocol.
- Fieldwork volunteers do not need to be listed on an AEC protocol, provided that:
  - they are only performing a logistical and/or administrative function (e.g. driving the boat)
  - they are competent to carry out the required procedures
  If however personnel are involved in specific research procedures (e.g. anaesthesia, blood collection etc.), then they must be listed on the AEC protocol.
- Each person listed on the project is accountable for the welfare of the animals involved in the project
- All personal details provided are strictly confidential

List all personnel who will be working on this project and will have contact with the animals
Note: Please make sure that all questions have been completed for each personnel before proceeding.
Click on investigator's name to enter details

1	Given Name	Liisa
	Surname	Kautto
	Full name	Dr Liisa Kautto
	MQ ID	MQ20034694
	Department:	4301
	Position in this project	Associate Investigator
	Primary Contact?	Yes
	Work number:	+61-2-9850-9257
	Mobile number:	
	Email address:	liisa.kautto@mq.edu.au
	Is your mobile number current? (for external personnel, please make sure all of your details are correct above).	Yes
	Please outline your relevant qualifications	We'll be getting only samples from other institute, if need to handle the animal we'll be trained in Sydney University.
	Has the Personnel attended an Animal ethics or Welfare Course?	No
	Please register to next available Animal Research Ethics Course here	
	Please select reasons applicable to why you have not attended an Animal Ethics or Welfare course	I will be attending the next available course
	Have you ever been, or are you currently, suspended from working with animals by Macquarie AEC or at any other institutions?	No

4.2 **Primary Contact**
This person will be the primary contact for this protocol

Dr Liisa Kaarina Kautto

7. Attachment

Documents

Please attach any other documents related to your application

*Attachments may include, for example, personnel expertise (Appendix 1), monitoring sheet, etc. Please note that the max size limit is **40MB** for an attachment. If any of your attachments is bigger than 40MB then split it into two or more parts and attach, just clearly mark those as part1, part2 etc...*

To begin attaching items:

1. Click **Add New Document**
2. Place the cursor in the textbox and **type the name of the attachment** (as listed above)
3. Click on the **green tick** to confirm the name
4. Click on the **Soft copy icon** to open the browsing window and select a file
5. Press **OK** to attach (and repeat the process as necessary).

Documents: *(maximum limit per document: 40MB)*

Description	Reference	Soft copy	Hard copy
Biohazard Risk assesment non-GMO IAP310314BHA2	IAP310314BHA2-3-3 for BHA for Human feces samples.pdf	✓	
Biohazard Risk assessment non-GMO NIP041214BHA	NIP041214BHA_all cells and tissue at PC2 E8C323.pdf	✓	
Ethics Application Sydney University	Application_Anand Hardikar_160414-1.pdf	✓	
Ethics approval Sydney University	Ethics approval Sydney University.pdf	✓	
Flowchart	Flowcharts_Anand Hardikar.pdf	✓	
Mouse trial_LKautto_2015-19-08	Mouse trial_LKautto_2015-19-08.docx	✓	

Checklist

Based on your answer to the questions previously listed, here is a list of required documents to include with your application.
Please check that all documents are attached.

External notification related documents
Please upload the following documents, tick when completed.*

- ☑ Application
- ☐ Approval letter or Animal Research Authority (ARA) letter

8. SignOff

Investigators

Declaration of Responsibility

I confirm that:
(i) I have read and understood this application.
(ii) I agree to comply with the procedures described.
(iii) I agree to comply with those conditions set out in the Macquarie University Animal Ethics Committee's Terms of Reference; regarding the Consideration of Protocols and Non-Compliance with The Code and any other conditions imposed by the Macquarie University Animal Ethics Committee.
(iv) I am familiar with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th edition, 2013 (NHMRC) ("The Code") and the NSW Animal Research Act 1985 and Regulation 2010.
(v) I have read and understood the University OH&S guideline entitled "Working with Research Animals".

(For the Principal investigator/Supervisor only):
I confirm that:
(i) In my opinion the proposed work described in this application is of scientific and/or educational merit.
(ii) The use of animals in this project will conform to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th edition, 2013 (NHMRC) ("The Code") and the NSW Animal Research Act 1985 and Regulation 2010.
(iii) I accept responsibility for the conduct of all procedures detailed in this application and for the supervision of all personnel delegated to perform any such procedures.
(iv)I accept personal responsibility for all matters relating to the welfare of all animals used in the project. I understand that I have an obligation to treat the animals with respect and to consider their welfare as an essential factor when planning or conducting projects (see The Code Section 2.4.1).
(v) I agree to comply with those conditions set out in the Macquarie University Animal Ethics Committee's Terms of Reference; regarding the Consideration of Protocols and Non-Compliance with The Code and any other conditions imposed by the Macquarie University Animal Ethics Committee.
(vi) I have read and understood the University OH&S guideline entitled "Working with Research Animals".
(vii) Adequate resources (financial, human, etc) are available to undertake this project.

Conflict of Interest
Please indicate if there are any Conflict of Interest that is likely to occur:
(This question relates to *all applicants listed on this application)* *

- ☐ I am also a member of MQ AEC
- ☐ I am also the Facility Manager
- ☐ I am also the Head of Department
- ☐ I am/have been a supervisor for / supervised by a member of MQ AEC
- ☐ I am/have been supervised by my Head of Department / other relevant signoff parties
- ☐ I am related to/in spousal relationship with the relevant signoff party / MQ AEC member
- ☐ Other (Not listed above)
- ☑ I foresee no potential conflict of interest in submitting this research proposal to MQ

Individual investigator signoff.
Please click on your name below to open the signoff page.

1	Full Name	Dr Liisa Kautto
	Position	Associate Investigator
	ID	MQ20034694
	Type	Internal
	I accept the above Declaration of Responsibility	Yes
	Signoff Date	03/08/2015

Email other investigators to signoff this application

Principal investigator are allowed to signoff on behalf of external applicants

Send Email Notification to Signoff

Notify principal investigator of your signoff

Click the button below to notify your principal investigator (or any other investigators) that you have review the application, completed/listed your expertise and provide signoff.

Send Email Notification to PI

Other Parties Signoff

Head of Department Signoff

Note: This page is only editable by the relevant Head of Department after the application is being submitted by applicant or forwarded by Facility Manager.

I have reviewed this application and I confirm that sufficient financial and other resources are available to enable the research to occur in compliance with the regulations. *

- (•) I agree
- ( ) I disagree

Print full name*

Mark Molloy

To complete signoff:
1. Click the Save button on the top right hand corner.
2. Go to the Action tab on the left panel and click **SignOff Application and Forward to Research Office**. This will forward the application to the Research Office to be prepared for the next AEC meeting available. Clicking this will also notified the relevant applicant that you have approved their applications.

Office Use Only

Secretary

Committee Secretary minutes for this application from the AEC Meeting.

This question is not answered.

MACQUARIE University

WEI CHONG <wei.chong@students.mq.edu.au>

Fwd: Animal Ethics Application - Outcome of AEC Meeting

Liisa Kautto <liisa.kautto@mq.edu.au> Thu, Sep 24, 2015 at 2:36 PM
To: DANIEL BUCIO NOBLE <daniel.bucio-noble@students.mq.edu.au>, Wei Chong <wei.chong@students.mq.edu.au>, HASINIKA KALHARI ARIYARATNE HEWAWASAM GAMAGE <hasinika-kalhari-ariyarat.h@students.mq.edu.au>

FYI, please save the number for your thesis submission.
Cheers Liisa

Liisa Kautto
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MACQUARIE University

---------- Forwarded message ----------
From: <animal.ethics@mq.edu.au>
Date: Wed, Sep 23, 2015 at 9:49 AM
Subject: Animal Ethics Application - Outcome of AEC Meeting
To: Animal.Ethics@mq.edu.au, liisa.kautto@mq.edu.au

Dear Dr Kautto

Your new application was considered and approved by the Animal Ethics Committee on 23/09/2015
RE: 5201500129 - Dr Kautto - (Collaborative) Effect of Nutrikane on immunosystem and gut microbiome in lean, obese and diabetic mice

Decision
The Committee agreed to approve the application and to issue an Animal Research Authority for work to commence.

Animal Research Authority (ARA) is attached to your online application. Please carefully note the approval dates and read the conditions of approval (if any) outlined in the ARA.

Grants:
If you have applied for funding for the above project, you will need to advise the Research Office Grants Team of your Ethics Reference Number: 5201500129

Please note the following standard conditions of approval (mandatory under The Animal Research Act 1985 NSW and Australian code for the care and use of animals for scientific purposes 8th edition (2013) :

1. A Progress Report must be submitted before the end of each 12-month (or less) approval period while the project is still current. The date of expiry of approval is shown clearly on the ARA.

Progress reports must be submitted to the AEC Secretariat in time for review at an AEC meeting before the ARA expires. Any animal work carried out during the period after expiry of an ARA, and before issuance of a new ARA, is in breach of the NSW Animal Research Act. If reports are submitted after the required closing date and cannot be reviewed by the AEC before expiry of the ARA, researchers will be expected to cease their animal work until such time as the AEC issues approval for the work to continue.

The Progress Report form (along with instructions for submission of the form) is available at http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/animal_ethics/forms

Please note that although the Research Office may issue a report reminder notice, timely submission of reports remains the responsibility of the ARA holder.

2. A Final Report must be submitted within one month of expiration of the full duration of approval or within one month of completion or abandonment of the work, whichever occurs sooner. If the Final Report is not submitted within three months of expiry of the final ARA, no further AEC approvals will be issued until the report is submitted. The full duration of approval is shown clearly on the attached ARA. Researchers are highly encouraged to make contact with the Animal Ethics Secretariat regarding any difficulty with submitting reports on time.

The Final Report form (along with instructions for submission of the form) is available at website

3. An amendment request must be submitted to the AEC for approval should you wish to make any changes to the approved protocol, including the addition of new research personnel, prior to the changes occurring.
Amendment request forms (along with instructions for submission of the forms) are available from website.

4. Any unexpected adverse events, including illnesses of animal(s), unexpected animal deaths or any event that may affect animal welfare and/or the continued ethical acceptability of the project must be notified to the Animal Welfare Officer within 72 hours of occurrence. The Unexpected Adverse Events form is available

5. At all times you are responsible for the ethical conduct of your research in accordance with the guidelines established by Commonwealth and State bodies and

the University. If you have any queries regarding such guidelines, they are accessible online, or you may direct your queries to the AEC Secretariat at animal.ethics@mq.edu.au

All forms available at: http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/animal_ethics/forms

Please retain a copy of this email and the attached ARA as proof of approval by the Animal Ethics Committee.

Regards,

Professor Mark Connor
Chair, Animal Ethics Committee

Ethics Application.pdf
227K

Animal Ethics for samples used in Chapter 4. Study 2

Re: Animal Ethics enquiry

Animal Ethics

Thu 12/01/2017 9:33 AM

To:Raymond Wei Wern Chong (HDR) <wei.chong@hdr.mq.edu.au>;

Hi Raymond,

As you are working on samples only, and not live animals, and as long as the samples were collected under approval from the collaborating institution, you do not require ethics approval from Macquarie University to work with these samples.

Justin

Dr Justin A Clarke BVSc MWIdMgt
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From: Raymond Wei Wern Chong (HDR)
Sent: Wednesday, 11 January 2017 3:46:07 PM
To: Animal Ethics
Cc: Nicki Packer; Arun Everest Dass
Subject: Animal Ethics enquiry

To Justin Clarke,

This is Raymond Chong from CBMS. I have just spoken with Michelle Keeling in regards to the animal ethics requirements for some samples we have received from an external collaboration and

The samples we have received are lyophilized mucosal scrapings from mice, which were housed and sacrificed externally at the BC Children's Hospital Research Institute in Vancouver.

Michelle stated that since the samples were obtained offsite as part of a larger project, we do not require animal ethics approval to begin work on these samples.
Could you please confirm in writing that this is the case.

Best regards,

Raymond Wei Wern Chong
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Faculty of Science and Engineering
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