Investigating the impact of dietary fibre on the gut microbiota

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This thesis is presented as a partial fulfilment to the requirements for a Doctorate of Philosophy

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Table of Contents

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of tables

List of supplementary figures

Chapter 5

List of supplementary tables (provided on the CD)

Chapter 2

Table S2.1 Metadata of the six biological samples (sample 1-6).

Table S2.2 The relative abundance of specific OTUs that were found to contribute to fibre-specific microbiota alterations, based on SIMPER analysis.

Table S2.3 The OTUs that were significantly differentially abundant between each product and the no added fibre control at 48 hours.

Table S2.4 The abundance of the OTUs in cultures with each of the fibre additions.

Table S2.5 The OTUs that were significantly differentially abundant between the fibre adherent and liquid fraction microbiota in cultures with each fibre product at 48 hours.

Table S2.6 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.

Table S2.7 SCFA concentrations of each sample.

Table S2.8 Antioxidant potential (mmolL-1) and Polyphenolic content (mgL-1) measurements for each biological sample

Chapter 3

Table S3.1 The relative abundance of bacterial families that were found to be significantly differentially abundant between dietary groups at week 32.

Table S3.2 The relative abundance of bacterial OTUs that were found to be significantly differentially abundant between dietary groups at week 32.

Table S3.3 Concentration of acetate, butyrate and propionate in each treatment at week 17, 23 and 32.

Table S3.4 The predicted relative abundance of KEGG Orthology pathways of each mouse gut microbiota inferred using PICRUSt.

Table S3.5 The Spearman's correlation (*r*) and the significance of the correlations.

Table S3.6 The abundance of the OTUs identified in microbial communities.

Chapter 4

Table S4.1 The relative abundance of the OTUs that were found to be significantly responding to fasting in each of the four dietary groups.

Table S4.2 The relative abundance of the OTUs that were significantly differentially abundant between dietary groups based on LEfSe analyses.

Table S4.3 The inferred relative abundance of the gut microbiota functional pathways predicted using PICRUSt.

Table S4.4 The abundance of the OTUs identified in the gut microbial communities.

Chapter 5

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.

Table S2 The relative abundance of bacterial families that were found to be significantly differentially abundant in at least three biological samples.

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.

Table S4 Concentration of acetate, butyrate and propionate in each treatment at 0, 24 and 48 hours.

Table S5 The predicted relative abundance of KEGG Orthology pathways inferred using PICRUSt for each biological sample (sample 1-6) with different cereal additions.

Declaration

I declare that the work in this thesis was conducted by me under the primary supervision of Prof. Ian T. Paulsen. The contributions and assistance of others have been appropriately acknowledged. The work presented here has not been previously submitted for any other degree.

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Conference proceedings

Oral presentations

- Hasinika K A H Gamage, Sasha G Tetu, Carly Rosewarne, Raymond W W Chong, Liisa Kautto and Ian T Paulsen. Fibre products derived from sugarcane, wheat dextrin and psyllium husk influence the gut microbiota *in vitro.* Keystone symposia-Gut microbiota, metabolic health and beyond, USA (2016).
- Hasinika K A H Gamage, Sasha G Tetu, Carly Rosewarne, Raymond W W Chong, Liisa Kautto and Ian T Paulsen. Fibre products derived from sugarcane, wheat dextrin and psyllium husk influence the gut microbiota *in vitro.* Sydney Micro meeting-ASM, Australia (2016).
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- Hasinika K A H Gamage, Sasha G Tetu, Carly Rosewarne, Raymond W W Chong, Liisa Kautto and Ian T Paulsen. Fibre products impact the gut microbiota *in vitro.* Joint Academic Microbiology seminars (JAMS) symposium, Australia (2016).
- Hasinika K A H Gamage, Sasha G Tetu, Carly Rosewarne, Raymond W W Chong, Liisa Kautto and Ian T Paulsen. Fibre supplements derived from sugarcane stem, wheat dextrin and psyllium husk have different *in vitro* effects on the gut microbiota. Advances in biotechnology for food and medical applications workshop, Australia (2016).
- Hasinika K A H Gamage, Sasha G Tetu, Carly Rosewarne, Liisa Kautto, Ian T Paulsen. A fibre supplement changes cultured gut microbiota. Islet society & Australian Islet study group 8th annual scientific meeting, Australia (2015).

Achievements

- Jeff Powell perpetual student award for the best poster at Joint Academic Microbiology Seminars (JAMS) symposium, Australia (2016).
- John Christian Young Food Microbiologist award, 48th Australian AIFST annual convention & 15th Australian Food microbiology conference, Australia (2015).
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Contributions

Chapter 2: Fibre supplements derived from sugarcane stem, wheat dextrin and psyllium husk have different *in vitro* effects on the gut microbiota.

(Manuscript submitted to *Frontiers in Microbiology*)

HKAHG, SGT, ITP and CPR designed the study. HKAHG conducted *in vitro* digestion, culturing, DNA extraction, bioinformatics and all statistical analyses. RWWC quantified SCFA concentrations and determined the chemical composition of the products. DBN performed TPC and FRAP quantifications. HKAHG, SGT, ITP. LK, MSB, MM and NP interpreted the results. HKAHG drafted the manuscript with contributions of DBN, RWWC, SGT and ITP.

Chapter 3: Effect of fibre supplementation on high fat diet-induced changes in the mouse gut microbiota

(Manuscript in preparation)

HKAHG, LK, RWWC, AH, DBN, ITP, NP, MM and MSB designed the study. HKAHG, LK, RWWC, DBN and AH maintained the mice, obtained all measurements. HKAHG performed the 16S rRNA amplicon library preparation and bioinformatics analysis. RWWC prepared the samples and quantified SCFAs using a GC-FID. All statistical analyses were performed by HKAHG. HKAHG, SGT, ITP, LK and NP interpreted the results. HKAHG drafted the manuscript with contributions of SGT and IPT.

Chapter 4: Effect of weekly overnight fasting and fibre supplementation on the gut microbiota of mice fed a high fat diet

HKAHG, LK, RWWC, AH, DBN, ITP, NP, MM and MSB designed the study. HKAHG, LK, RWWC, DBN and AH maintained the mice, obtained all measurements. HKAHG performed

the 16S rRNA amplicon library preparation and bioinformatics analysis. All statistical analyses were performed by HKAHG. HKAHG, SGT and ITP interpreted the results. HKAHG drafted the chapter with contributions of SGT and IPT.

Chapter 5: Cereal products derived from wheat, sorghum, rice and oats alter the infant gut microbiota *in vitro*

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HKAHG, SGT, ITP and JA designed the study. HKAHG prepared samples for the GC-FID and conducted all experiments including *in vitro* digestion, culturing, DNA extraction and bioinformatics analysis unless otherwise indicated. RWWC performed the GC-FID quantification of SCFAs. All statistical analyses were performed by HKAHG. HKAHG, SGT, ITP, NP and JA interpreted the results. HKAHG drafted the manuscript with contributions of SGT and ITP.

Abstract

Diets low in complex polysaccharides have been shown to perturb the gut microbiota-host relationship, and thus impact host health. As evidence supporting this hypothesis continues to grow, therapeutic modulation of the gut microbiota through supplementation of complex polysaccharides for preventing or treating diseases has gained significant scientific and commercial interest. Several supplements in the form of dietary fibre or prebiotics are marketed commercially for this purpose. However, only limited work has been conducted to scientifically evaluate the ability of these products to alter the gut microbiota and improve host health.

In this work, we investigated the impact of commercially available dietary fibre and cereal products on the gut microbiota and metabolite production. We used an *in vitro* adult digestive and gut microbiota model system and a high fat diet fed mouse model to examine the effect of dietary fibre supplementation on the gut microbiota, metabolites and host physiology. Using an *in vitro* infant digestive and gut microbiota model system we investigated the impact of cereal products on the infant gut microbiota and metabolites.

Our results demonstrated significant shifts in the overall gut microbiota community structure upon addition of each product. The abundance of various bacterial taxa associated with fibre digestion and anti-inflammatory capabilities increased with fibre additions. However, the specific nature of the alterations was product-dependent. Fibre supplementation in mice ameliorated high fat diet-induced changes in the abundance of specific gut bacteria, whilst no significant changes in the glucose clearance or body weight were observed. Further, we demonstrated significant differences in the gut microbiota response to a high fat diet and fibre supplementation upon weekly overnight fasting in mice. The product-dependent impact on the gut microbiota and metabolites highlights the need for stringent scientific evaluation of commercial fibre products for their effect on the gut microbiota and host physiology.

List of abbreviations

16S rRNA	16S Ribosomal Ribonucleic Acid
AMPs	Antimicrobial Peptides
ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
ARISA	Automated Ribosomal Intergenic Spacer Analysis
AUC	Area Under Curve
BCFA	Branched Chain Fatty Acids
BF	Benefiber®
CA	Cholic Acid
CAZymes	Carbohydrate-Active Enzymes
CBMs	Carbohydrate-Binding Molecules
CDCA	Chenodeoxycholic Acid
CRC	Colorectal Cancer
DCA	Deoxycholic Acids
DNA	Deoxyribonucleic Acid
FISH	Fluorescent *In Situ* Hybridisation
FMO3	Flavin-Containing Monooxygenase 3
FMTs	Fecal Microbiota Transplants
FODMAPs	Fermentable Oligosaccharides, Disaccharides, Monosaccharides And Polyols
FOS	Fructo-Oligosaccharides
FRAP	Ferric Reducing Antioxidant Power
FXR	Farnesoid X Receptor
GC	Gas Chromatography
GC-FID	Gas Chromatograph-Flame Ionisation Detector
GH	Glycoside Hydrolases
GH13	Glycoside Hydrolases 13
GI tract	Gastrointestinal Tract
GLP1	Glucagon-Like Peptide 1
GOS	Galacto-Oligosaccharides
GPCR	G-Protein-Coupled Receptors
HDAC	Histone Deacetylase

HF	High Fat Diet
HF-BF	High Fat Diet Supplemented with Benefiber®
HF-NK	High Fat Diet Supplemented with NutriKane™
HLA	Human Leukocyte Antigen
HLPC	High-Performance Liquid Chromatography
HMI	Host-Microbiota Interaction
HMOs	Human Milk Oligosaccharides
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IM	Inner Membrane
IPGTT	Intraperitoneal Glucose Tolerance Test
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCA	Lithocholic Acid
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis Effect Size
LPS	Lipopolysaccharide
MACs	Microbiota-Accessible Carbohydrates
MWCO	Molecular Weight Cut-off
NAC	No Added Cereal
NAF	No Added Fibre
NAFLD	Non-Alcoholic Fatty Liver Disease
NC	Normal Chow
NK	NutriKane™
nMDS	Non-Metric Multi-Dimensional Scale
OM	Outer Membrane
OTU	Operational Taxonomic Unit
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PH	Psyllium Husk
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PL	Polysaccharide Lysases
PolyFermS	Polyfermentor Intestinal Model

Ppm	Parts Per Million
PTS	Phosphotransferase System
PULs	Polysaccharide Utilisation Loci
PYY	Peptide YY
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative PCR
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
Rpm	Revolutions per minute
RS	Resistant Starch
SCFA	Short Chain Fatty Acid
SCG	Single-Cell Genomics
SHIME	Simulator of Human Intestinal Microbial Ecosystem
sIgA	Secretory Immunoglobulin A
SIMPER	Similarity Percentages
SSU rRNA	Small Subunit Ribosomal RNA
Sus	Starch Utilisation Systems
T/DGGE	Temperature Denaturing Gradient Gel Electrophoresis
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TBDT	TonB-Dependent Transporter
TFA	Trifluroacetic Acid
tGOS	Trans Galacto-Oligosaccharides
TIM	TNO Intestinal Model
TMA	Trimethylamine
TMAO	Trimethylamine N-Oxide
TPC	Total Polyphenolic Content
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TβMCA	Tauro-B-Muricholic Acid
XOS	Xylo-Oligosaccharides

CHAPTER 1

Introduction

1.1 Compositional and functional overview of the gut microbiota

The human body is in symbiosis with trillions of microorganisms (bacteria, archaea, viruses and microscopic eukaryotes), these are collectively referred as the human microbiota. The microbiota inhabits various human body parts such as the oral cavity, skin, vagina and gastrointestinal tract. Within the human microbiota are an estimated 40,000 bacterial strains from at least 1,800 bacterial genera [1]. These microbes collectively contribute around 9.9 million non-human genes [2]. An accumulating amount of data demonstrates the impact of the microbiota on normal host physiology and susceptibility to diseases through its metabolic activities and host interactions [3, 4]. In particular, the microorganisms that inhabit the human gastrointestinal tracts and their link with host health are increasingly gaining scientific and public attention and awareness.

The human gastrointestinal tract, one of the densest microbial habitats on the planet, constitutes an estimated mass of 1-2 kg microorganisms [3, 5]. These microbial communities are collectively known as the gastrointestinal tract (GI tract) microbiota. The GI tract microbiota is distributed in the oral cavity, stomach, small intestine and large intestine with varying densities and diversities (Figure 1.1), owing to the unique physiochemical properties of these anatomical sites [6]. The oral cavity contains a complex microbial ecosystem of more than 700 bacterial species from around 12 bacterial phyla. The diversity of the oral microbiota varies on different surfaces such as saliva, mucosa, tongue and teeth [7, 8]. Surface attachment of the microorganisms forming multispecies biofilms is a key feature of this complex environment [7]. Bacterial species common to all surfaces of the oral cavity belong to the genera *Gemella*, *Granulicatella*, *Streptococcus*, and *Veillonella*, all of which are members of the phylum *Firmicutes* [9].

Until recently, the stomach was believed sterile or very low in microorganisms due to acid production, and therefore has very low pH levels. However, recent studies have confirmed the presence of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Bacteroidetes* and

Gemmatimonadetes in the stomach. At a genus level *Prevotella*, *Veillonella*, *Rothia*, *Streptococcus*, *Helicobacter*, *Lactobacillus* and *Haemophilus* dominate the stomach microbiota [10, 11].

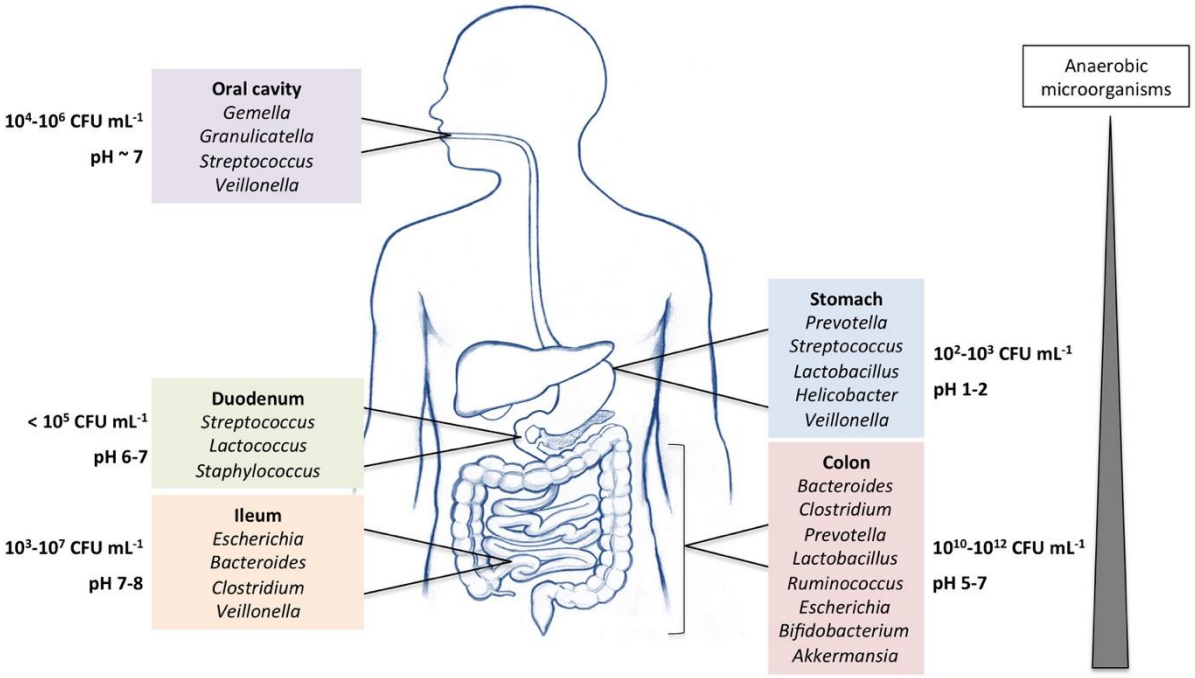

Figure 1.1 The dominant bacterial genera typically found in different sites of the gastrointestinal tract. Bacterial density (CFU mL^{-1}) and pH level in each site are indicated. The percentage of anaerobic microorganisms is lower in the upper gastrointestinal tract compared to the colon.

The small intestine microbiota is usually dominated by the genera *Streptococcus*, *Veillonella*, *Clostridium*, *Escherichia*, *Bacteroides*, *Lactococcus* and *Turicibacter* [12]. Microbial populations in the distal small intestine is denser (~10^7 CFU mL^{-1}) than in the proximal small intestine (~10^5 CFU mL^{-1}), possibly due to slower transition times, increasing populations of anaerobic bacteria and lower concentrations of acid, bile and pancreatic secretions towards the colon [7, 13]. The colon microbiota or large intestine microbiota is commonly known as the gut microbiota. This is the most diverse and densest population of microorganisms residing in the gastrointestinal tract and even in the human body. Microbial densities in different sections of the large intestine vary from 10^{10}-10^{12} CFU mL^{-1} of the colonic content [13, 14]. Various anaerobic bacteria usually dominate the gut microbiota, which outnumber the aerobic and facultative anaerobic bacteria by 100 to 1,000-fold [15].

1.1.1 Composition of the gut microbiota

Each individual hosts around 160 bacterial species in the gut, whilst a total of around 1,200 different gut bacterial species have been identified [16, 17]. However, most of these bacterial species belong to a few known bacterial phyla [18]. *Bacteroidetes* and *Firmicutes* are the most dominant phyla in the adult gut microbiota, while phyla *Actinobacteria, Proteobacteria, Fusobacteria, Cyanobacteria* and *Verrucomicrobia* are generally minor constituents [18, 19]. Key members within the phylum *Firmicutes* are the genera *Clostridium, Lactobacillus* and *Ruminococcus* and butyrate producers *Eubacterium, Faecalibacterium* and *Roseburia*. Members of the phylum *Bacteroidetes* are known dietary fibre degraders including the genera *Bacteroides, Prevotella* and *Xylanibacter*. *Bifidobacterium* is an important genus in the gut microbiota from the phylum *Actinobacteria.* The gut genera of *Proteobacteria* include *Escherichia* and *Desulfovibrio*, whereas the mucus-degrading genus *Akkermansia* belongs to the phylum *Verrucomicrobia.* The human gut microbiota also contains methanogenic archaea (mainly *Methanobrevibacter smithii*), various eukaryotes (mainly yeasts) and viruses (mainly phage) [4].

Several studies have attempted to determine core members of the gut microbiota based on the functional and compositional characteristics. Turnbaugh *et al* 2009 identified key players such as *Faecalibacterium prausnitzii, Roseburia intestinalis* and *Bacteroides uniformis* as the core members of the gut microbiota [20]. However, these attempts on defining a core gut microbiota have been unsuccessful due to continuously growing sample size and increasing variations in the gut microbiota of individuals from different geographical locations, age groups, disease states etc [4].

1.1.2 Functions of the gut microbiota

The combined genome of the gut microbiota (known as the gut microbiome) exceeds human genetic potential at least by two-order of magnitude [17, 21]. The metabolic capacity of this extensive array of gene products in the gut microbiome equals to that of the liver, and

therefore, can be considered as an organ [18, 22]. The diverse range of biochemical and metabolic activities of the gut microbiota are essential for various aspects of the host biology. Functions of the gut microbiota include, (a) Metabolism and energy harvest, (b) Host-microbiota signalling and (c) Regulation of gut permeability and immunity.

(a) Metabolism and energy harvest

One of the major outcomes of the host-microbiota symbiosis is the production of metabolites that contribute to the host physiology and immunity. As defined by Nicholson *et al* 2012, host-microbe metabolic axis is a multidirectional interactive chemical communicational highway between specific host cellular pathways and a series of microbial species, sub-ecologies and activities [23]. Substrates metabolised by the gut microbiota include dietary compounds (carbohydrates, amino acids, lipids and phytochemicals), mucosal macromolecules (mucins), endogenous metabolites (bile acids) and xenobiotic chemicals [24]. Metabolism of these substrates results in the production of bacterial metabolites (Figure 1.2) such as short chain fatty acids (SCFAs), secondary bile acids, choline, certain vitamins, phenol, benzoyl, indole and phenyl derivatives, polyamines and specific lipids [23].

The human ability to digest carbohydrates is limited to starch, lactose and sucrose, therefore, humans largely depend on the gut microbiota to digest otherwise non-digestible complex carbohydrates such as cellulose, xylans, resistant starch and inulin [25, 26]. Microbial degradation of these complex carbohydrates produces SCFAs such as acetate, butyrate and propionate. These are produced through various microbial biochemical pathways, for example acetate is mainly produced from acetyl CoA using pyruvate or the Wood-Ljungdahl pathway, while propionate is produced via the Carboxylation pathway or Acrylate pathway and butyrate is generally produced through condensation of two molecules of acetyl CoA or utilising acetate, respectively [27].

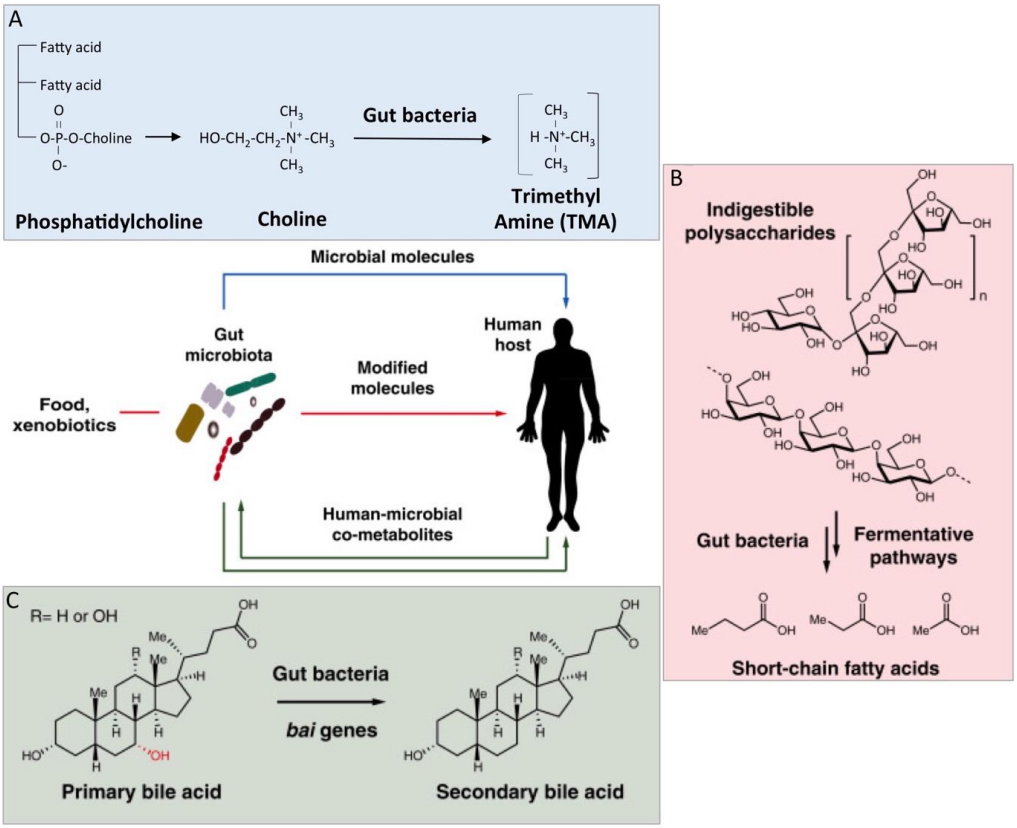

Figure 1.2 Examples of metabolites produced by the gut microbiota. **(A)** Dietary phosphatidylcholine is digested to choline in the small intestine, and the gut microbiota metabolises choline into Trimethyl amines (TMAs). **(B)** Gut microbial fermentation of dietary fibre produces short chain fatty acids. **(C)** Gut microbiota deconjugates bile acids and synthesises secondary bile acids. Image adopted from Koppel and Balskus 2016 [3] and modified (licence number 4277980504688).

SCFAs are absorbed in the colon and have established roles in the host physiology. A large part of these bacterial metabolites is used as an energy source and provides up to 10% of daily caloric requirements [27]. Butyrate is generally utilised as an energy source in the colonic epithelial cells. Acetate and propionate reach the liver and other peripheral organs and function as precursors and regulators of gluconeogenesis and lipogenesis [25]. SCFAs are also crucial for proliferation, differentiation and modulation of gene expression in colonic epithelial cells through the activity of butyrate as a potent histone deacetylase (HDAC) inhibitor [28]. SCFAs also regulate colonic gene expression through binding and activating the G-protein-coupled receptors (GPCR) such as GPR41 or GPR43 [24].

Microbial regulation of bile acid metabolism occurs through deconjugating bile acids (which are conjugated to glycine by the host) and transforming into secondary bile acids in the caecum and colon [24]. Secondary bile acids function as signalling molecules and cellular receptors that modulate glucose and lipid metabolism, energy metabolism and therefore, protect against diet-induced obesity and diabetes [25]. Gut microbial metabolism of choline also contributes in modulating glucose and lipid metabolism in the host. Transformation of choline to toxic methylamines decreases bioavailable choline, which could trigger non-alcoholic fatty liver disease, cardiovascular diseases, diet-induced obesity and diabetes [23].

(b) Host-microbiota signalling

The gut microbiota also synthesises bioactive metabolites that signal the host through various mechanisms (Figure 1.3). Bacterial metabolite SCFAs are not only used as an energy source but also are involved in signalling the host through inhibiting HDAC and activating GPCR (such as GPR41 and GPR43). GPR41 and GPR43 contribute in several different functions such as suppression of inflammation and tumour growth and modulation of hormone secretion. Examples of secreted hormones influenced by the gut microbiota include the Glucagon-like peptide 1 (GLP1, effects pancreatic function, insulin release and controls appetite) [25, 29] and peptide YY (PYY, inhibits gut mortality and increases energy harvest from diet). Metabolism of primary bile acids such as cholic acid (CA) and chenodeoxycholic acid (CDCA) to secondary bile acids such as deoxycholic acids (DCA) and lithocholic acid (LCA) also mediates GLP-1 production by signalling the host though G-protein-coupled bile acid receptor 1 (also known as TGR5). Microbial metabolism of other abundant primary bile acids such as, tauro-β-muricholic acid (TβMCA) inhibits the farnesoid X receptor (FXR, also known as the bile acid receptor), which in turn regulates bile acid homeostasis.

Microbial lipopolysaccharides (LPS) are taken up into chylomicrons (lipoprotein particles formed from dietary saturated fat) and these then promote the host signalling to induce insulin resistance [30]. Gut microbial metabolism of choline and L-Carnitine (mostly obtained from

red meat and eggs) produce trimethylamine (TMA). These are further oxidised by the enzyme Flavin-containing monooxygenase 3 (FMO3) into TMA N-oxide (TMAO), which is associated with poor cardiovascular health [30].

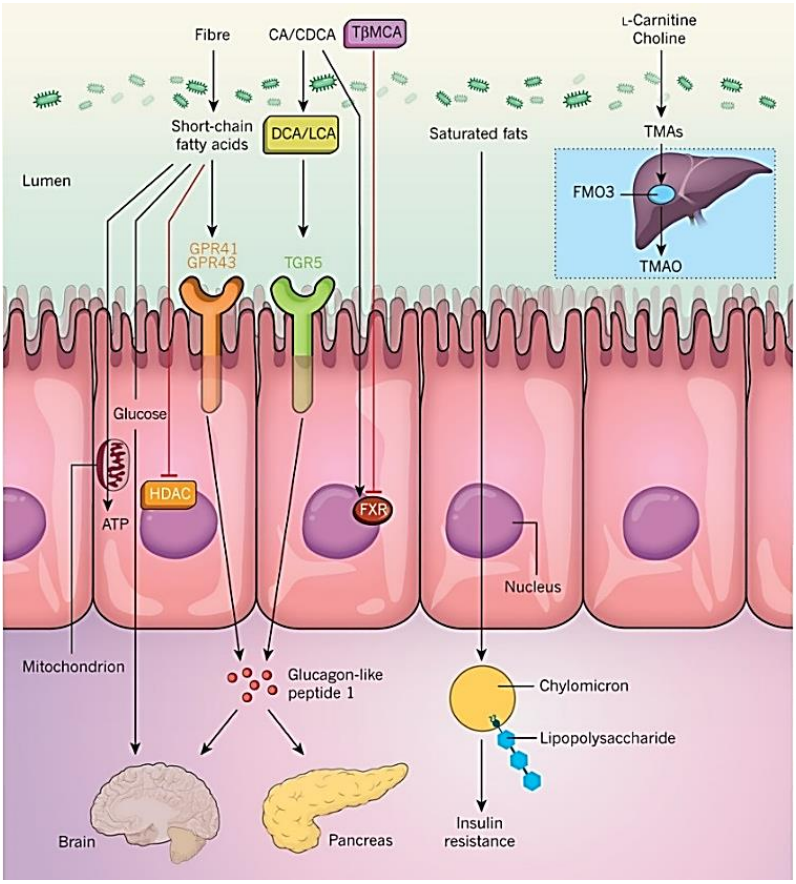

Figure 1.3 Mechanisms of the host-microbiota signalling. Short chain fatty acids are used as a source of energy (ATP), substrate for gluconeogenesis, and are involved in signalling the host by inhibiting histone deacetylase (HDAC) or by activating G-protein coupled receptors (GPR41 and GPR43), which releases the glucagon-like peptide 1. The gut microbiota metabolises cholic acid (CA) and chenodeoxycholic acid (CDCA) into deoxycholic acid (DCA) and lithocholic acid (LCA), these activate the signalling to the host through G-protein-coupled bile acid receptor 1 (TGR5). Tauro-β-muricholic acid (TβMCA) inhibits the Farnesoid X receptor (FXR). Uptake of microbial lipopolysaccharides into chylomicrons induces insulin resistance in the host. Microbiota metabolises choline and L-Carnitine into trimethylamine (TMA), TMAs are oxidised into TMA N-oxide (TMAO) by Flavin-containing monooxygenase 3 (FMO3) in the liver. Image reprinted from Sonnenburg and Bäckhed 2016 [30] (licence number 4277990603164).

(c) Regulation of gut permeability and immunity

The gut microbiota is separated from the host intestinal epithelial cell lining by a thin layer of mucus, this is only a few micrometres thick in the small intestine and up to several hundred

micrometres in the colon [31]. The mucosal immune system is tolerant to mutualistic bacteria in the gut microbiota and responsive to pathogens. The mucus layer influences the gut microbiota community, as specific bacteria can use lectins to adhere to glycosidases [18]. In turn, the gut microbiota has profound effects on the villus architecture, crypt depth, stem cell proliferation, blood vessel density, mucus layer properties (the density and the composition) and maturation of mucosa-associated lymphoid tissues [18]. In addition to regulation of lymphoid structure, the gut microbiota is also associated with the modulation of immune cell subsets such as lymphoid tissue inducer cells, natural killer cells, T helper 1, 2, and 17 cells, regulatory T cells and B cells [18]. Furthermore, the gut microbiota regulates the production of immune mediators such as cytokines, chemokines, secretory immunoglobulin A (sIgA) and antimicrobial peptides (AMPs).

The gut microbiota provides protection against enteropathogens through competition for colonisation. Commensal gut bacteria directly inhibit the invading pathogens through production of toxic compounds, maintenance of the mucus barrier and epithelial cells, regulation of immune responses and efficient utilisation of host nutrients, which might limit the expansion of the less well-adapted invaders [32]. Additionally, the gut microbiota associated signalling molecules such as lipopolysaccharides (LPS), peptidoglycan and flagellin and metabolites such as SCFAs and secondary bile acids hold the potential to regulate inflammation.

1.2 Impact of the gut microbiota on host health

Due to the link between the gut microbiota and host physiology, alterations to the gut microbiota trigger various physiological disorders in the host (Figure 1.4). Changes in the gut microbial composition, diversity and metabolites are associated with disease development. The diseases that have been linked to altered composition and functions of the gut microbiota can be grouped into, (1) Metabolic diseases, (2) Inflammatory diseases and (3) Neurological diseases (not discussed here in detail). While a literature review on commonly associated

changes in the gut microbiota with specific diseases have been provided, it is important to note there is a significant degree of variation between studies in linking disease association with microbiota changes.

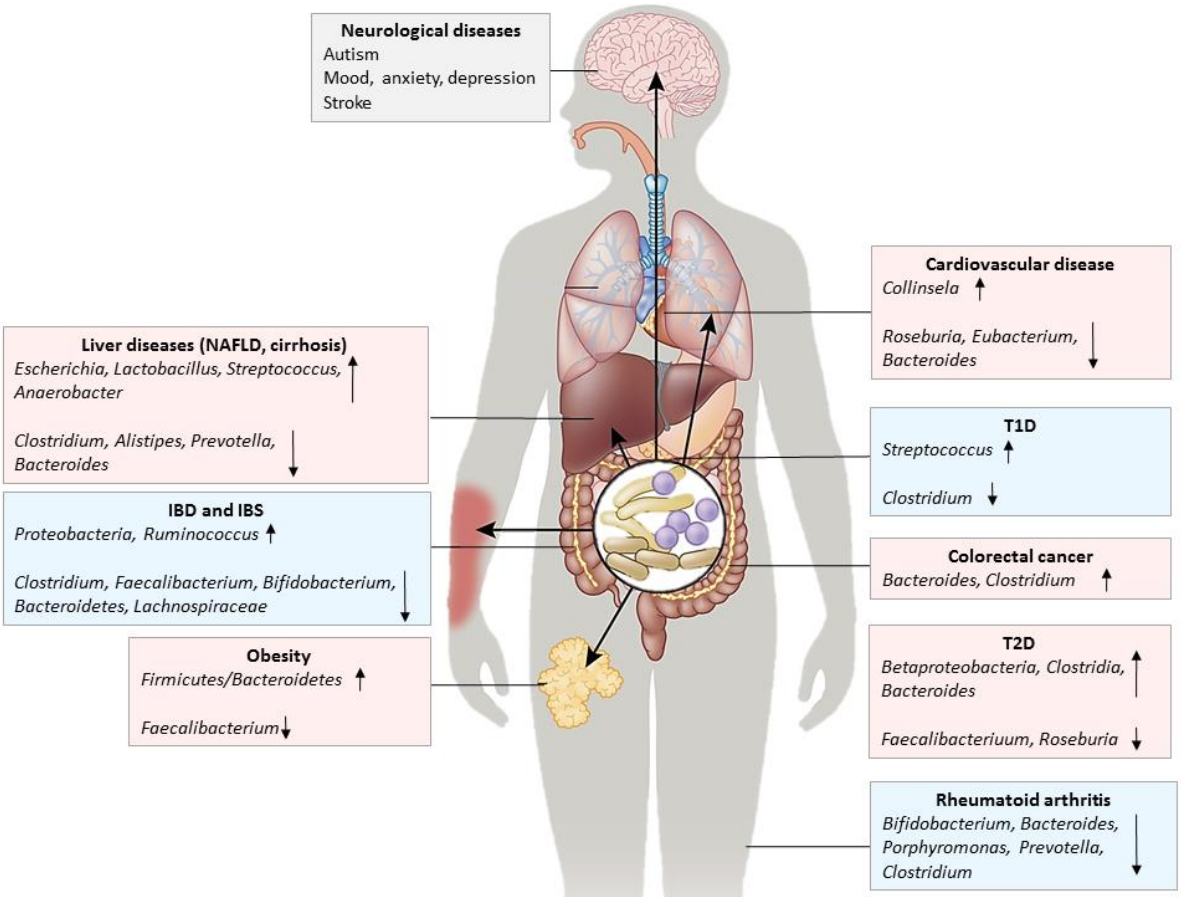

Figure 1.4 Diseases associated with the gut microbiota. Metabolic diseases (pink) and inflammatory diseases (blue) are shown with the changes in gut microbiota commonly associated with the disease. An increase or a decrease in the relative abundance is indicated by up or down arrows, respectively. Image adopted from Schroeder and Bäckhed 2016 [33] and modified (licence number 4277990880236). See sections 1.2.1, 1.2.2 and 1.2.3 for more detailed discussions.

1.2.1 Metabolic diseases

Gut microbial composition, diversity and function effect energy harvest and metabolism in the host. Therefore, alterations in the gut microbiota are linked to metabolic disease development. These alterations impact the gut physiology and motility through increasing the density of small intestinal villi capillaries, therefore, increase caloric extraction. Gut microbiota associated changes in polysaccharide digestion have been shown to promote fat deposition in adipocytes and impact the SCFA, bile acid and choline metabolism, which also

contributes to metabolic disease development [33-35]. These gut microbiota-associated changes in host physiology increase the risk of metabolic diseases such as (a) Obesity, (b) Type-2 diabetes and (c) Other diseases such as cardiovascular disease, non-alcoholic fatty liver disease, colorectal cancer etc.

(a) Obesity

Dysregulation of energy balance between the intake and expenditure causes an excess of adipose tissue, therefore, results in obesity. Onset of obesity is a complex process that has various chronic complications such as hyperglycaemia, hypertriglyceridemia, dyslipidaemia and hypertension, most of which also increase the risk of developing type 2 diabetes and cardiovascular diseases [35]. Interestingly, many studies have demonstrated a relationship between altered gut microbiota and obesity. An increase in the ratio between the abundance of *Firmicutes* and *Bacteroidetes* has been commonly reported in mice with genetically modified (*ob/ob)* and diet induced obesity [36-38]. Altered ratio of *Firmicutes*:*Bacteroidetes* is also linked to more efficient hydrolysis of complex polysaccharides, therefore, extraction of more energy from food [35, 39]. Although several studies have reported a link between higher *Firmicutes*:*Bacteroidetes* ratio and obesity in rodent models, many human studies and meta-analyses have not supported this association [40-45]. Obesity is also associated with lower concentrations of SCFAs and a lower bacterial gene count, especially in species associated with an anti-inflammatory status such as *Faecalibacterium prausnitzii* [20, 46, 47].

(b) Type 2 diabetes

The main driver of type 2 diabetes (T2D) is long-term insulin resistance, which is associated with constant high blood glucose concentrations. An increased relative abundance of *Betaproteobacteria* and a decrease in *Clostridia* have been observed in individuals with T2D compared to the healthy controls [48]. The ratios of *Bacteroidetes* to *Firmicutes* and *Bacteroides-Prevotella* groups to *Lachnospiraceae* family (formerly *Clostridium coccoides-*

Eubacteria rectale groups) positively correlated with the plasma blood glucose levels in T2D patients [48]. T2D is also associated with lower abundance of butyrate-producing bacteria such as *Faecalibacterium prausnitzii, Roseburia intestinalis* and *Roseburia inulinivorans* and higher abundance of opportunistic pathogens such as *Clostridium symbiosum*, *Eggerthella lenta* and *Escherichia coli* [49].

An enrichment of genes associated with oxidative stress resistance, methane metabolism, sulphate reduction, starch and glucose metabolism, fructose and mannose metabolism, ABC transporters and glutathione synthesis has been observed in the gut microbiome of individuals with T2D compared to the healthy controls [49, 50]. Downregulation of flagellar assembly and flavin metabolism pathways has also been observed in T2D patients compared to the healthy controls [50].

(c) Other diseases (cardiovascular disease, liver diseases and colorectal cancer)

The gut microbiota of atherosclerosis patients is associated with higher abundance of *Collinsella* and lower abundance of *Roseburia, Eubacterium* and specific species of *Bacteroides* compared to the healthy controls [51]. At a functional level, the gut microbiome of these patients is enriched in genes responsible for peptidoglycan biosynthesis, while phytoene dehydrogenase was downregulated [51]. A gut microbiota associated toxic metabolite TMAO has been linked to elevating the risk of cardiovascular disease [52, 53].

Liver diseases such as non-alcoholic fatty liver disease (NAFLD), hepatic encephalopathy and cirrhosis have also been linked with altered gut microbial composition. Patients with NAFLD have demonstrated an increase in the abundance of *Escherichia*, *Anaerobacter*, *Lactobacillus* and *Streptococcus*, whilst demonstrated a reduction of *Alistipes* and *Prevotella* compared to the healthy subjects [54]. Patients with hepatitis B liver cirrhosis had lower abundance of the genera *Bacteroides* and *Clostridium*, which are associated with colonic bile acid metabolism [55].

The composition of the gut microbiota has also been associated with colorectal cancer (CRC). Gut microbial metabolites, SCFAs have been related to the suppression of CRC, whilst secondary bile acids have been reported to promote the onset of CRC [56]. High risk of CRC has been associated with higher abundance of the genera *Bacteroides* and *Clostridium* [57].

1.2.2 Inflammatory diseases

The crosstalk between the gut microbiota and host innate and adaptive immune systems through the mucosal interface influences the functions of the immune system and the gut microbiota. Gut microbiota generally induces anti-inflammatory effects that contribute to the protection of the epithelial cells against pathogens through various signalling pathways [58]. This host-microbiota link also has a major role in the development and constant reshaping of the immune system [57]. Hence, alterations in the gut microbiota are linked with the development of immunological diseases such as, (a) Inflammatory bowel disease, (b) Type 1 diabetes and (c) Other diseases such as irritable bowel syndrome and rheumatoid arthritis.

(a) Inflammatory bowel disease

Inflammatory bowel disease (IBD) is characterised by chronic and relapsing inflammation in the gut and is mainly defined as either ulcerative colitis or Crohn's disease. One of the common features of Crohn's disease is the reduction in the gut microbial diversity, with a greater reduced abundance of *Clostridium leptum* [59, 60]. Temporal variations in the gut microbiota has been reported to be higher in IBD patients compared to the healthy controls, indicating the instability of the microbiota [61]. For example, Joossens *et al* reported an increase in the abundance of *Dialister invisus, Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis*, and a decrease in the abundance of *Ruminococcus gnavus* in the gut microbiota of IBD patients [62]. Another study has reported an increase in the abundance of *Proteobacteria* and a decrease in the abundance of *Bacteroidetes* and *Lachnospiraceae* in the gut microbiota of IBD patients [59]. While changes in the gut

microbiota composition have been observed in patients with IBD, the specific bacteria differ between studies.

(b) Type 1 diabetes

Type 1 diabetes (T1D) is an autoimmune disorder caused by T cell-mediated destruction of pancreatic β-cells. A study on the gut microbiota of children with T1D reported an increase in the abundance of *Veillonella, Clostridium* and *Bacteroides,* whilst the abundance of *Lactobacillus, Bifidobacterium* and *Prevotella* were lower [63]. Another study on adult T1D patients has demonstrated an increase in the abundance of *Bacilli* (*Streptococcus)* and decrease in butyrate producing groups of *Clostridium* [64]. Functionally, the gut microbiota of T1D patients is typically enriched in genes involved in stress responses, virulence factors and sulfur metabolism [65].

(c) Other diseases (Irritable bowel syndrome and rheumatoid arthritis)

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder that has been associated with high abundance of the genera *Dorea, Ruminococcus* and *Clostridium,* whilst the abundance of *Faecalibacterium, Bifidobacterium* and methanogens are lower [66]. Dysbiosis in the gut microbiota has also been related to rheumatoid arthritis (RA). Wu *et al* has reported a decrease in the abundance of *Bifidobacterium, Bacteroides-Porphyromonas-Prevotella* group and *Lachnospiraceae* (formerly *Eubacterium rectale* and *Clostridium coccoides*) in RA patients [67]. In contrast, recent studies have observed an increase in the abundance of specific members of *Prevotella* in patients with RA [68], indicating the complexity in associating specific gut microorganisms with the onset of diseases.

1.2.3 Neurological diseases

Another area of accumulating interest is the bidirectional communication of the gut microbiota with the brain development and functions. Homeostasis between the gut microbiota and brain contributes in the development of the central and enteric nervous

systems, maintains normal circadian function and intestinal immune response. However, altered gut microbiota has been related to conditions such as anxiety, stress response, behaviour, depression, autism and multiple sclerosis [69]. An increasing number of studies also suggest a link between the gut microbial composition and ischemic brain injury [70].

1.3 Development of the gut microbiota

The composition and physiology of the gut microbiota co-develop with the host and are shaped by various endogenous and exogenous factors. The infant gut microbiota is species poor and unstable, this diversifies and stabilises to an adult-like composition during the first few years of life (Figure 1.5). This complex process is influenced by factors such as the *in utero* environment, mode of birth delivery, host genetics, gestational age, environment, mucin glycosylation, use of antibiotics and diet (breast milk, formula milk or solid food) [71].

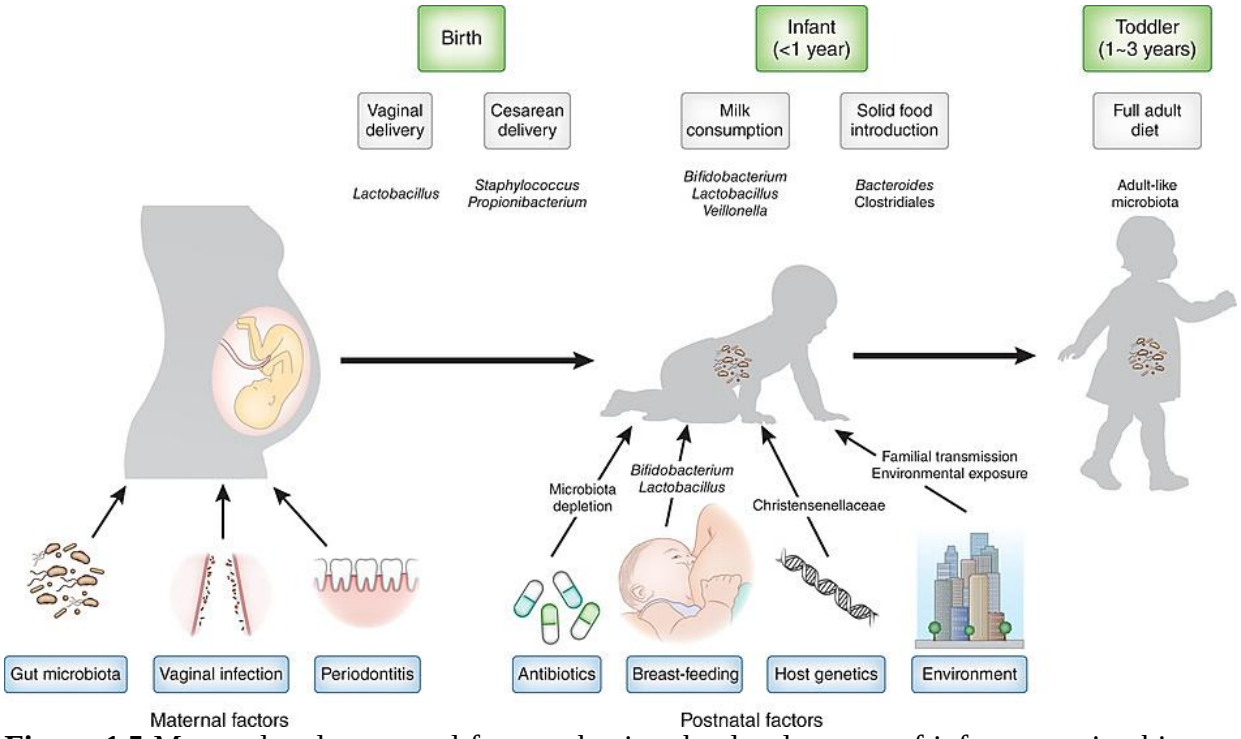

Figure 1.5 Maternal and post-natal factors shaping the development of infant gut microbiota. The bacterial genera typically associated with the differences in the mode of birth delivery at birth and diet during infancy are indicated. An adult-like gut microbiota composition is developed at 1-3 years of age. Image reprinted from Tamburini *et al* 2016 [72] (licence number 4277991130237).

1.3.1 Determinants of the gut microbiota development

(a) *In utero* environment

An increasing amount of research suggests that development of the gut microbiota is initiated even before birth [72, 73]. Microorganisms have been detected in the placenta, amniotic fluid, fetal membrane, umbilical cord blood and meconium of healthy cohorts without any infection or inflammation [72, 74]. Bacteria such as *Enterobacteriaceae*, enterococci, streptococci, staphylococci and bifidobacteria have been found in the meconium, supporting the notion the microbes can be transmitted to the unborn fetus through the amniotic fluid [73]. Therefore, seeding of the gut microbial colonisation in the fetus could occur through translocation of the maternal gut microbiota via the blood stream [73, 75]. This is in agreement with higher physiological gut bacterial translocation through dendritic cells during pregnancy and lactation in rodents [76].

However, *in utero* colonization of the gut microbiota is a highly controversial notion, as the existence of microbiomes within the healthy fetal milieu has been continuously challenged [77-79]. A recent review by Perez-Muňoz *et al* has highlighted that many studies supporting the *in utero* colonization hypothesis lack a higher detection limit to study low microbial biomass samples, appropriate controls for contamination and tests for bacterial viability [78].

(b) Mode of delivery

The neonatal gut microbiota is heavily influenced by the mode of delivery (vaginal vs caesarean section). Vaginally delivered infants have a higher resemblance to the maternal vaginal microbiota (*Lactobacillus, Prevotella* and *Sneathia*) [80, 81], whilst caesarean sections births have enriched populations of bacteria found in the maternal skin microbiota (*Propionibacterium, Corynebacterium, Streptococcus* and *Staphylococcus*) [81, 82]. Caesarean section births have also been associated with delayed colonisation of the genera *Bacteroides* and *Bifidobacterium* [83] and lower species diversity in the gut microbiota

compared to vaginal births [84, 85]. These differences in the gut microbiota between vaginal and caesarean section births have been reported to persist for at least one year of life [71]. However, a recent study by Chu *et al* 2017 suggests that these variations in the gut microbiota between the two delivery modes are insignificant at birth and any mode of delivery driven differences in the oral, nasal and skin microbiota become less evident within the first six weeks of life [86].

(c) Environment

Environmental factors such as family contacts (siblings, pets), geographical location and ethno-geography (distinct genetic backgrounds, regional diets and cultural practices) also strongly effect the early colonisation of the gut microbiota. The gut microbial diversity of the firstborn children is lower than that of children with older siblings, which may be due the transfer of bacteria from close contact or through changes in parental hygiene practices [87]. Several studies have investigated the effect of geographical location on the colonisation of the gut microbiota and have observed distinct variations between geographical locations possibly also due to the differences in the ethno-geography [88-91].

(d) Antibiotics

Use of antibiotics is a major determinant of the gut microbiota development. Although the adult gut microbiota is more resilient to disruptive factors such as antibiotics, the delicate ecosystem of the infant gut microbiota can be highly affected. Other than conferring antibiotic resistance, over usage or exposure early in the infancy can be disruptive to the overall gut microbial ecology and have long lasting negative health impacts [92]. Infants exposed to ampicillin and gentamicin shortly after birth tend to have a microbiota dominated by organisms within the phyla *Proteobacteria, Actinobacteria* and genus *Lactobacillus* compared to infants exposed to these antibiotics later in life [93]. Disrupted microbiota is also prone to infection with enteric pathogens such as *Clostridium difficile* [94].

(e) Diet (Breast or formula milk)

Exposure to different nutrients and energy sources highly impacts the composition and function of the infant gut microbiota and contributes largely towards establishing an adult-like composition. Infants are primarily dependent on nutrients from breast milk in the first few months of life. Breast milk not only delivers a range of complex human milk oligosaccharides (HMOs) but also introduces an array of different bacteria through the milk microbiota [95]. The milk microbiota contains more than 700 bacterial species, which serve as some of the first colonisers of the infant gut microbiota [96]. Bacteria such as *Staphylococcus, Streptococcus, Lactobacillus* and *Bifidobacterium* are prevalent in the milk microbiota, therefore, in the gut microbiota of breast-fed infants [97]. Most HMOs are non-digestible by humans and serve as an energy source for the primary digesters in the gut microbiota such as *Bifidobacterium, Lactobacillus* and *Bacteroides* [72]. The remaining sugars are digested by the secondary digesters such as *Streptococcus, Staphylococcus* and *Enterococcus* [80]. A high abundance of *Bifidobacterium* and *Lactobacillus* results in lower pH levels in the gut through their metabolic activities (production of SCFAs) and serves as a defence mechanism against many pathogen invasions in breast-fed infants [72, 98].

On the other hand, formula-fed infants demonstrate a higher abundance of *Bacteroides, Clostridium, Streptococcus, Enterobacteria* and *Veillonella* spp. [89, 99]. Furthermore, formula-fed infants have been reported to have lower bacterial population density, but a higher bacterial species diversity within the gut microbiota compared to that of the breast-fed infants [99]. A functional analysis of the gut microbiota between breast-fed and formula-fed infants demonstrated only small differences [100]. However, the genes encoding phosphotransferase system (PTS) transporters, bile acid synthesis and methanogenesis were enriched in formula-fed infants compared to breast-fed infants [100].

(f) Introduction to solid food

Exposure to solid food during weaning introduces a range of complex plant and animal polysaccharides, which are mostly metabolised via the gut microbiota. This major transition of nutrients diversifies infant gut microbiota, broadens its metabolic capacity and plays a large contribution towards establishing a stable adult-like composition [92]. In addition, the introduction to solid food contributes to maturing the pancreatic function, small intestine absorption and colonic fermentation abilities in the host. This not only changes the physiology and functions of the gastrointestinal tract, but also changes the undigested material that reaches the gut microbiota [18, 92].

Weaning in general is linked with lowering the abundance of *Bifidobacterium, Enterobacteria* and groups of *Clostridum,* whilst increasing the abundance of some other groups of *Clostridium* (*C. coccoides, C. leptum*) [89]. Therefore, weaning reduces the abundance of bacteria prevalent in the infant gut microbiota and enriches the abundance of species dominant in the adult gut microbiota such as *Bacteroidetes* and different groups of *Firmicutes* (*Clostridium, Ruminococcus, Faecalibactrium, Roseburia* and *Anaerostipes*) [80]. Functionally, production of SCFAs increases during weaning, possibly due to high availability of complex polysaccharides [101]. Exposure to solid food and xenobiotics enriches the abundance of bacterial genes involved in carbohydrate utilisation, vitamin biosynthesis and xenobiotic degradation, which influences maturation of the gut microbiome [100].

1.3.2 Implications of early-life gut microbiota on host health

Infants delivered by caesarean section have a higher risk of developing asthma, systemic connective tissue disorders, juvenile arthritis, immune deficiencies, IBD, obesity and food allergies compared to vaginally delivered infants [102-104]. This has been suggested to be due to the gut microbial effects on the host physiology and immune system development. To improve the health outcomes of babies delivered by caesarean section, “vaginal seeding”

(transfer of maternal vaginal fluid, hence the vaginal microbiota, using a gauze swab to an infant delivered by caesarean section) has been introduced [105]. However, the long-term impact and possible health effects of such practices are yet to be discovered [71].

Prolonged use and exposure to antibiotics early in the infancy may increase the development of diseases such as asthma, allergies, eczema, obesity and IBD [106-109]. In addition, poorer hygiene and early-life contact with pets lowers the risk of developing asthma, allergy and preclinical T1D [110, 111]. The time and type of weaning is another aspect of early-life exposures with consequences on health. For example, exposing infants to wheat-based cereals in the first six months of life has been associated with reducing the risk of allergy development towards wheat, compared to infants introduced after six months of age [112]. Similar observations have been reported for other popular weaning foods such as potato, eggs and rye [113].

Therefore, early life gut microbiota homeostasis effects the host immune system development, and hence long-term health. In addition, the composition and functions of the gut microbiota contribute towards host metabolism and energy harvest, and hence modulate host metabolic health. As the infant gut microbiota develops to an adult-like composition, it becomes relatively more stable with a higher species diversity and lower inter-individual variation [100]. Pre- and post-natal factors mentioned in section 1.3.1 contribute heavily in establishing a stable adult-like composition and diversifying the functions of the gut microbiota.

1.4 Inter- and intra-individual variation in the gut microbiota

Although a healthy adult gut microbiota is relatively more resilient to changes and has a lower intra-individual variation compared to infants, various environmental and endogenous factors can cause significant intra- and inter-individual variation. The healthy adult gut microbiota is dominated by the phyla *Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria,*

Fusobacteria, Cyanobacteria and *Verrucomicrobia.* However, the abundance of these bacteria varies within and between individual(s). Various endogenous factors such as the gender, age, genetics and life events (ovarian cycle, pregnancy and menopause) and exogenous factors such as diet and dietary changes, use of antibiotics, life styles (smoking, stress, exercise), geographical location and environment shape the gut microbial communities.

1.4.1 Endogenous factors

(a) Age

The infant gut microbiota changes drastically over time due to various environmental factors. A stable adult-like gut composition is established in the first few years of life, which is relatively more resilient to changes. Another significant change in the gut microbiota occurs in later age in life [88]. A recent Japanese study investigated the difference in the gut microbiota across pre-weaning infants to 104 years old elders. They observed a higher abundance of the phyla *Proteobacteria* and *Bacteroidetes* in elders compared to adults [114]. Other studies have also observed an increase in *Proteobacteria* (especially *Enterobacteriaceae*) and *Bacteroidetes* in the elderly [115-117]. Elderly gut microbiota has also been associated with less temporal variability, a decrease in the *Firmicutes*:*Bacteroidetes* ratio and in anti-inflammatory bacteria such as *Faecalibacterium prausnitzii* [116-118].

(b) Gender and life events

The composition of the gut microbiota could also be dependent on the gender. Previous studies have shown higher abundance of *Bacteroides-Prevotella* groups, *Veillonella* and *Methanobrevibacter* in males compared to women, whilst woman had more *Bilophila* [115, 119]. These differences in the gut microbiota between male and female might have effects on gender-based prevalence of certain metabolic and inflammatory diseases [119]. The composition and functions of the gut microbiota in females can also vary depending on life events such as puberty, ovarian cycle, pregnancy and menopause [120].

(c) Genetics

The gut environment is influenced by specific host genes (for example, the genes encoding IgA and the human leukocyte antigen (HLA) system), hence host genetics plays a vital role in establishing and shaping the gut microbiota [15, 121]. The abundance of many microbial taxa is influenced by host genetics, especially the family *Christensenellaceae.* This bacterial group has been linked to reduced weight gain [122]. Therefore, host genetics not only shapes the gut microbial composition but also may indirectly impact the host metabolism [122].

1.4.2 Exogenous factors

(a) Geographical location, environment and lifestyle

Human gut microbiota demonstrates distinct variations based on the geographical location. For example, children from rural Africa have higher populations of *Prevotella* and *Xylanibacter* and higher concentrations of SCFAs compared to European children [90]. Similar studies have demonstrated differences in the gut microbial composition between children from Bangladesh and United States [123]. Children and adults from the United States had significantly different gut microbiota compositions compared children and adults in Malawi and Venezuela [88]. Differences in the gut microbiota based on the geographical location are likely linked to the differences in regional diets and cultural practices.

Apart from these factors, differences in sanitation and levels of cleanliness can also significantly impact this variation. The higher occurrence of diseases associated with altered gut microbiota (such as IBD and allergies) in industrialised Western countries compared to agrarian communities could be linked to the differences in the environment factors such as appendectomy, use of oral contraceptives, childhood infections, mycobacterial infections and vaccinations [124]. Lifestyle factors such as smoking, lack of exercise and stress can also impact the composition of the gut microbiota [120]. Interestingly, factors associated with

travelling such as changes in sanitary conditions, hygiene and circadian rhythm (also associated with shift work) could alter the gut microbiota, therefore impact health [120, 125].

(b) Use of antibiotics

Humans are exposed to antibiotics not only through direct administration but also through consumption of farm animals and crops [126]. Gut microbial resilience to antibiotics varies between individuals and even between treatments in the same individual. However, recovery following exposure to antibiotics is mostly incomplete, therefore, leading to an altered gut microbiota structure that is different to the initial composition [127]. Prolonged exposure to antibiotics can cause rapid and significant drops in the species diversity and richness of the gut microbiota [128]. Use of antibiotics is linked to altered microbial metabolism of dietary compounds, hence, reduced insulin sensitivity, increased weight gain, obesity and diabetes [126, 129]. Antibiotics also affect the homeostasis of the immune system, possibly through disrupted host-microbe signalling pathways [130]. Therefore, exposure to antibiotics in early infancy has lasting effects on the gut microbiota and immune system development and functions [93, 131]. Some antibiotics can also reduce the thickness of the mucus layer and disrupt the tight junctions [132], which increase the host susceptibility to pathogen invasion [128]. Further, indiscriminate use of antibiotics has made the gut microbiome a reservoir for antibiotic resistant genes. This has likely contributed to the emergence of multiple antibiotic resistant bacteria that are providing a major challenge to global public health [126].

(c) Diet and dietary changes

Accumulating evidence has shown that diet largely modulates the development and maintenance of the gut microbial composition, function and spatial arrangement. The gut microbiota rapidly responds to dietary changes, with changes seen within as little as a day. For example, switching individuals between completely plant- and animal-based diets changed the gut microbial composition within 24 hours [133]. Similarly, a low-fat/high-fibre

or high-fat/low-fibre diet also altered the gut microbiota within 24 hours [134]. Even though short-term dietary changes cause significant changes in the microbiota, long-term dietary habits determine the gut microbial composition in an individual. Therefore, prolonged exposure to specific types of diets (Western, vegetarian, high-fat/low-fibre, low-fat/high-fibre) can cause lasting compositional and functional changes.

Several studies have investigated the impact of long-term consumption of a high-fat/low-fibre or low-fat/high-fibre diet on the gut microbiota in both humans and animal models. Consumption of a low-fat/high-fibre diet has been associated with high levels of *Prevotella* [134]. Similarly, the gut microbiota of communities (such as Malawian, Venezuelan, African and Hazda-hunter gatherers) that consume diets rich in fibre have a higher abundance of *Prevotella* compared the Western communities that consume low-fibre diets [88, 90, 135]. Similarly, the gut microbiota of children in Africa were dominated by the phylum *Bacteroidetes* with a lower abundance of *Firmicutes* compared to European children [90]. Furthermore, gut microorganisms have co-evolved with the host to facilitate dietary variations. For example, in Japanese populations the gut bacterium *Bacteroides plebeius* has a unique gene encoding porphyranase (an enzyme that degrade seaweed) likely to facilitate the digestion of seaweed in their diet [136].

1.5 Gut microbial degradation of dietary components

Diet is a major determinant of the intra- and inter-individual compositional and functional variations in the gut microbiota. These effects are likely driven by the differences in the amount, type and balance between dietary macronutrients (carbohydrates such as dietary polysaccharides/fibre, fat, proteins etc.) and micronutrients (antioxidants, phytochemicals, vitamins etc.) in specific dietary patterns (Western, vegetarian diet etc.) and foods (whole grain, fruits, vegetables etc.).

1.5.1 Dietary macronutrients

(a) Carbohydrates

Due to the limited ability of humans to digest complex carbohydrates, their digestion is largely dependent on the gut microbiota. Carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine are defined as dietary fibre [137]. Dietary fibre/dietary polysaccharides are broadly categorised as resistant starch and non-starch polysaccharides (such as polyfructans, pectin, cellulose and hemicellulose) [138]. Utilisation of dietary fibre by specific gut bacteria depends on the chemical structure of fibre such as the source, sugar types, linkage types, chain lengths, particle size and association with other compounds [139]. Different types of resistant starch (RS) namely, physically inaccessible starch (RS1), native granules (RS2), retrograded starch (RS3) and chemically modified starch (RS4) promote the growth of different types of bacteria [140]. For example, RS4 can increase the abundance of *Actinobacteria* and *Bacteroidetes*, whilst RS2 increase the growth of the *Firmicutes* [141].

Dietary fibre that are categorised as non-starch polysaccharides are usually more complex and diverse with both soluble and insoluble varieties [138]. Galacto-oligosaccharides (GOS) are a group of non-resistant polyfructan polysaccharides that are also found in human breast milk. GOS can increase the abundance of *Bifidobacterium* and reduce the abundance of *Bacteroides* and *Clostridium* [142-144]. Similarly, other polyfructans, such as fructo-oligosaccharides (FOS) and xylo-oligosaccharides (XOS) can also increase the growth of *Bifidobacterium* and butyrate-producing bacteria [145-147].

Digestion of dietary polysaccharides largely depends on carbohydrate-active enzymes (CAZymes) such as glycoside hydrolases (GHs) and polysaccharide lysases (PLs). These enzymes have varying substrate specificity [148]. Many of these enzymes are not represented in the human genome, therefore, digestion of dietary fibre/polysaccharides heavily relies on the CAZymes encoded by the gut microbiome. The phylum *Bacteroidetes* encodes the highest

number of CAZymes in the gut microbiota, followed by the phyla *Firmicutes*, *Actinobacteria* and *Proteobacteria* (Figure 1.6). This has possibly made *Bacteroidetes* generalists for polysaccharide degradation, while *Firmicutes* are more specialised for digestion of specific polysaccharides [149, 150].

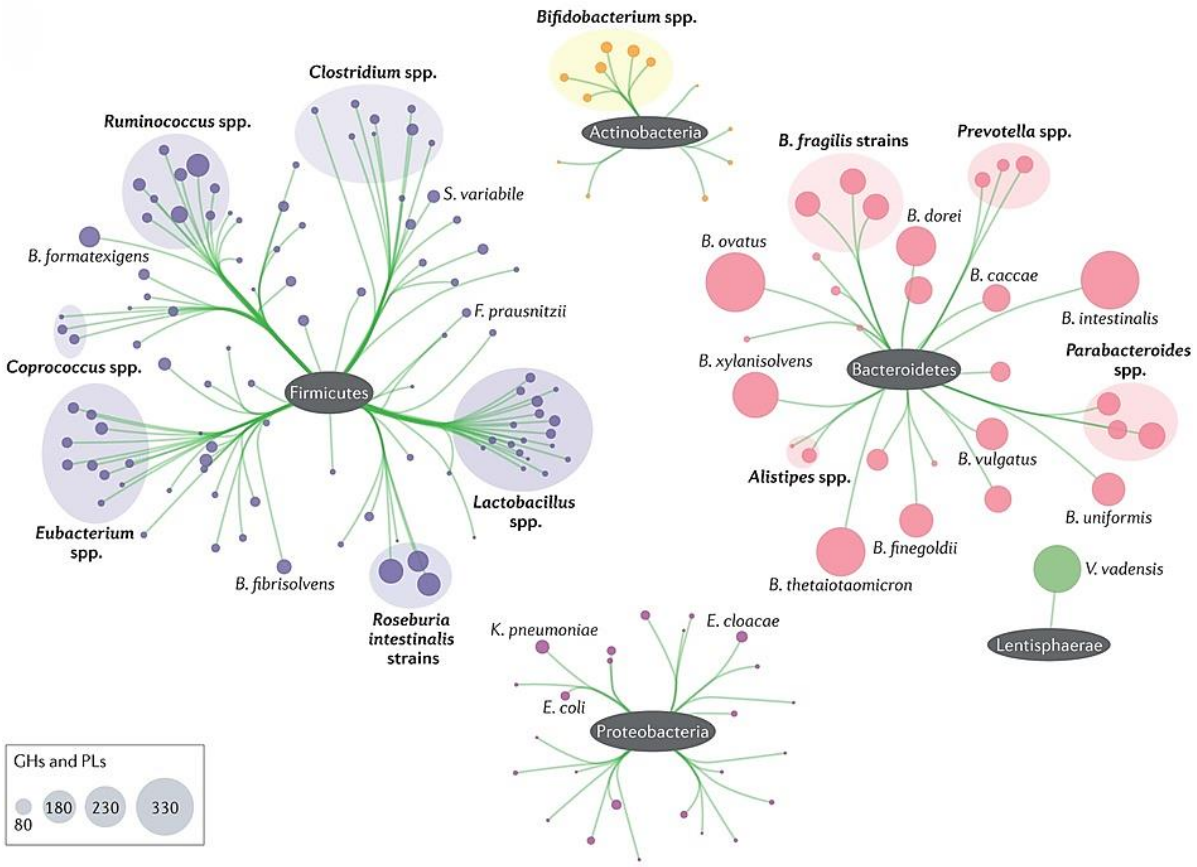

Figure 1.6 Distribution of genes encoding carbohydrate-active enzymes (glycoside hydrolases (GHs) and polysaccharide lysases (PLs)) in the gut microbiota (only a representation of the gut microbiota has been used to construct this diagram). The size of a circle denotes the number of GH and PL families produced by each taxonomic group (as shown in the legend). Image reprinted from Kaoutari *et al* 2013 [148] (licence number 4277991391599).

Specific gut bacteria digest different dietary polysaccharides with specialised mechanisms [149]. For example, *Bacteroidetes* package their diverse array of CAZyme genes into polysaccharide utilisation loci (PULs) within their genomes. PULs encode proteins for capturing, degrading and importing specific polysaccharides. The best-described PUL is an eight-gene locus in *Bacteroides thetaiotaomicron* encoding the starch utilisation systems (Suss) to metabolise starch (Figure 1.7A) [149, 150]. SusDEFG recognise and bind starch to

the cell surface, the amylase SusG hydrolyses starch to maltooligosaccharides and SusC (a TonB-dependent transporter-TBDT) transports digested products into the periplasm. In the periplasm, SusA and B depolymerise maltooligosaccharides into glucose and maltose, respectively, which are transported into the cytoplasm via an undefined transporter. SusR senses maltooligosaccharides and maltose in the periplasm and transmits signals to activate the expression of *sus* genes [149]. *B. thetaiotaomicron* harbours various other Sus-like systems to digest polysaccharides other than starch [150].

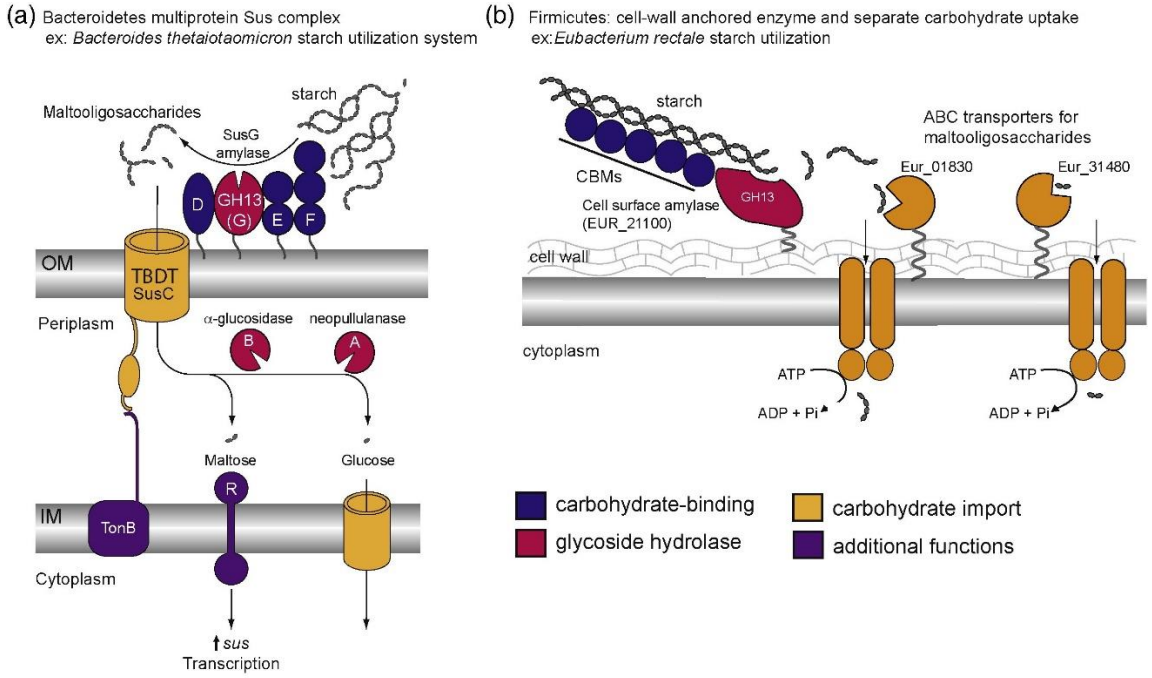

Figure 1.7 Two examples of gut bacterial polysaccharide degradation mechanisms. **(A)** Starch utilisation system (Sus) in *Bacteroides thetaiotaomicron.* The Sus is composed of three starch-binding lipoproteins SusDEF, a transporter SusC to import maltooligosaccharides liberated by SusG and maltooligosaccharides digesting Sus A and B. **(B)** Starch utilisation by *Eubacterium rectale.* This is typically initiated when a large cell-wall-anchored enzyme digests starch into maltooligosaccharides that can be recognised by transport systems such as ABC transporters. Abbreviations, OM-outer membrane and IM-inner membrane. Image adopted from Cockburn and Koropatkin 2016 [149] and modified (licence number 4278000233114).

In contrast, *Firmicutes* employ extracellular degradation less commonly than the *Bacteroidetes*, and instead use an array of transporters to import polysaccharides for intracellular degradation. Furthermore, *Firmicutes* tend to use a large polypeptide for binding and degrading polysaccharides in comparison to Sus like systems in *Bacteroidetes* [149, 151].

For example, in *Eubacterium rectale*, starch is digested to maltotetraose and maltose by glycoside hydrolases-13 (GH13) enzyme EUR_21100 that contains many putative carbohydrate-binding molecules (CBMs) (Figure 1.7B). Digested products are then imported via ABC transporters (EUR_01830 and EUR-31480).

The primary degraders (such as *Bacteroides, Eubacterium, Ruminococcus*) digest complex polysaccharides from plant, animal (meat), human-milk, microbial and host (mucin) origins. Thereafter, the secondary degraders further digest dietary polysaccharides through various cross-feeding mechanisms. Both these mechanisms produce SCFAs, which participate in previously mentioned (see section 1.1.2) regulatory functions in the host and impact host health [152].

(b) Fat

High-fat diets are generally associated with a lower intake of carbohydrates, which might lead to the gut microbial changes related to low fibre intake. Dietary fat impacts host bile acid secretion and composition, therefore, indirectly modulates the gut microbiota [140]. Consumption of a high-fat diet has been associated with high concentrations of bile acids and secondary bile acids such as deoxycholic acid (DCA). DCA can alter the composition of the gut microbiota due to their selective antimicrobial activity [153, 154]. High consumption of fat is associated with increased abundance of *Alistipes*, *Bilophila* and *Bacteroides*, whereas the abundance of *Prevotella* decreases with high fat consumption [133, 134]. Dietary fat from different sources such as olive, sunflower and palm oil have varying effects on the gut microbiota and host physiological parameters [155]. Consumption of palm oil has been linked to an increase in the ratio between *Firmicutes*:*Bacteroidetes* and abundance of specific clusters of *Clostridium* [156]. Diets high in fat interact with the gut microbiota to facilitate the translocation of bacterial LPS into chylomicrons, this contributes in triggering inflammation [138]. Hence, dietary fat can modulate bile acid metabolism and the activity of LPS, therefore, the composition and functions of the gut microbiota.

(c) Protein

About 10% of dietary protein reaches the gut microbiota and are used as substrates for proteolytic bacteria and as a nitrogen source for saccharolytic species [156]. Fermentation of proteins occurs through deamination by bacteria such as *Clostridium,* some *Bacteroides* and *Enterobacterium* [156]. High-protein diets have been associated with lower abundances of *Roseburia* and *Eubacterium,* higher concentrations of branched-chain fatty acids and lower production of butyrate [157]. However, high intake of dietary protein has also been linked to lower inflammatory markers, improved metabolism and increased bacterial diversity in professional athletes [158]. This could suggest the impact of dietary protein on the gut microbiota is dependent on other factors such as host body composition and exercise intensity.

1.5.2 Dietary micronutrients

Another emerging aspect of microbiota-diet axis is the effect of bioavailable and bioactive micronutrients (vitamins, minerals and phytochemicals such as polyphenols) on the gut microbiota. Vitamin A deficiency has been linked to the modulation of immune responses through direct or indirect interaction with the immune cells or indirect modulation of the gut microbiota [159]. Dietary iron has been positively correlated with the abundance of lactobacilli [159].

Dietary polyphenols and antioxidants also contribute in shaping the gut microbiota composition and host physiology [160]. Gut microbial activities transform polyphenols into compounds with higher bioavailability and impact the host. Polyphenols from various fruits and tea can inhibit the growth of pathogens, whilst maintaining the growth of commensal gut bacteria such as *Lactobacillus, Bifidobacterium* and *Clostridium* [138, 161, 162]. Gut microbial fermentation of soy isoflavone daidzein to equol has been linked to the regulation of endocrine functions [161]. However, some dietary polyphenols or products of polyphenol

metabolism are associated with negative impacts on host health such as mitochondrial dysfunction through directly or indirectly modulating the gut microbiota [161].

1.6 Therapeutic modulation of the gut microbiota

Gut microbiota-host symbiosis makes a large contribution towards the host immunity and physiology throughout life. However, this symbiosis is frequently jeopardised in the modern world due to the use of antibiotics, diets low in microbiota-accessible carbohydrates (MACs), excessive sanitation and caesarean-section births [163, 164]. Rather unexpected factors such as environmental temperatures and disruptions to circadian rhythms can also contribute towards this dysbiosis [165, 166]. Rapidly accumulating evidence suggests an association between disrupted gut microbiota-host symbiosis with wide spread metabolic and inflammatory diseases such as obesity, diabetes (T1D and T2D), IBD, liver-associated diseases, cardiovascular diseases, cancer and even brain functionality. Due to limitations in pharmaceutical therapies for many of these diseases, investigation of therapies through modulation of the gut microbiota is timely and important [167]. Therapeutic strategies such as (1) Dietary modulation (prebiotics and dietary restriction), (2) Probiotics and (3) Experimental therapies (fecal microbiota transplants and phage therapy) are currently studied. Use of these therapies and combinations of therapies such as synbiotics (prebiotics and probiotics) [168, 169] are rapidly growing in scientific and public popularity.

1.6.1 Dietary modulation (prebiotics and dietary restriction)

Long- and short-term diet and dietary changes heavily affect the gut microbiota and microbial metabolites [90, 133, 134, 170]. Lack of MACs contributes in altering the gut microbial composition, functions, spatial arrangement and leads to a loss of bacterial diversity [170-172]. Although, the exact features of a “healthy gut microbiota” or an “ideal diet” that promotes its growth have not yet been resolved, several studies suggest and demonstrate a strong impact of diet as a therapeutic modulator of the gut microbiota [167, 173-175].

Therefore, higher intake of MACs/dietary fibre, in line with the concept of prebiotics is growing in popularity.

A prebiotic is defined as "a non-digestible compound that, through its metabolism by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host" [175]. Prebiotics exert their benefits through selective stimulation of the growth and/or activity of gut bacteria associated with health and increased production of SCFAs [176]. Compounds that have been shown to contain prebiotic effects include, inulin, FOS, transGOS (tGOS) and human milk oligosaccharides [175, 177]. Candidate prebiotics that require additional research to prove their efficacy include resistant starch, pectin, arabinoxylan, whole grains, various dietary fibres and non-carbohydrates [175].

The health benefits of prebiotic consumption potentially include optimised colonic function (stool bulking, regularity and consistency), increased bone density through stimulating mineral absorption, regulation of appetite through stimulation of gut peptide secretion, improvement of intestinal barrier integrity, regulation of glucose and lipid metabolism and modulation of immune functions [177-179]. Consumption of prebiotics and combinations of prebiotics can change the gut microbiota and result in beneficial health outcomes. Consumption of FOS, XOS, inulin or other prebiotic candidates has been observed to increase the abundance of *Bifidobacterium,* lactobacilli and *Enterococcus* and overall microbial diversity [167, 178, 180]. FOS and XOS are also associated with elevating the production of SCFAs and reducing the damage of ulcerative colitis [178]. Intake of tGOS has been shown to reduce IBD symptoms, inflammatory markers and promote the growth of *Bifidobacterium* in the elderly [144]. A reduction in inflammatory markers has also been observed in T2D patients upon consumption of a mixture of FOS and inulin [181].

Supplementation of infant foods (such as formula-milk) with prebiotics is also gaining scientific as well as commercial interest. Consumption of mixtures of FOS and GOS has been

correlated with reduced total cholesterol levels in infants [178]. Addition of FOS/GOS to infant food has also been reported to promote the growth of *Bifidobacterium* and *Lactobacillus*, increase the production of SCFAs and reduce pH levels in the gut [182].

However, consumption of dietary fibre and prebiotics are associated with some adverse effects such as diarrhoea, bloating, abdominal cramps and flatulence [183]. These side effects are dose-dependent, and the severity varies between individuals. Intake of dietary fibre and prebiotics at moderate levels, intake of slowly fermented fibre types/low intake of fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) are associated with reduction of these adverse effects [179, 183].

In addition to dietary supplementation, dietary restriction has also been shown to alter the gut microbiota. Dietary restriction through limiting specific types of macronutrients or time-restricted feeding with little or no impact on overall caloric intake alters the gut microbiota and can potentially alleviate the symptoms of metabolic disease [184-186]. Time-restricted feeding or fasting is studied in line with evolutionary circadian rhythms of the gut microbiota and host in relation to the light/dark cycles [187, 188].

1.6.2 Probiotics

Probiotics are defined as "live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host" [189]. Probiotic strains belong to a range of bacterial species, such as members of the genera *Lactobacillus, Bifidobacterium, Enterococcus* and *Streptococcus*. Some strains of *Saccharomyces* are also used as probiotics [176]. Effects of probiotics on the host are mainly strain specific, however, wide spread effects across most of the probiotic strains include colonisation resistance, acid and SCFA production, regulation of the intestinal tract and competitive exclusion of pathogens [189, 190]. Species/strain-specific effects can include regulation of vitamin synthesis, bile metabolism, neutralisation of carcinogens, gut barrier reinforcement, immunological and endocrinological effects and the production of specific bioactives [189].

Probiotics have the potential as a therapy for diseases. Use of *Bifidobacterium*, lactobacilli, streptococci, *Saccharomyces boulardii* and *Escherichia coli* individually or in combination has been suggested to reduce the symptoms of IBS [191]. Successful probiotic treatments have also been reported for diseases such as IBD (particularly, for ulcerative colitis), atopy and allergic diseases such as atopic dermatitis and reducing the symptoms of both antibiotic-associated and infectious diarrhoea [191, 192].

Use of probiotics has many limitations, including that many probiotic bacteria are not viable after freeze-drying and processing, the limited number of microbes that can be used as ingredients or supplements, and limited data to support the efficacy of probiotic bacteria to induce changes in the gut microbiota of healthy individuals [193]. Probiotic strains should preferably be isolated from the human gut microbiota, proven to be safe for consumption, genetically stable and capable of surviving the passage through the gastrointestinal tract [167]. Although many probiotic strains are currently available in the market, many of them lack strong clinical evidence to support the specified functions. Therefore, stringent clinical studies, use of probiotics at a non-viable state, non-viable bacterial metabolites (such as SCFAs) or use of genetically modified microorganisms could be potential alternatives to overcome the limitations [193].

1.6.3 Experimental therapies

(a) Fecal microbiota transplants (FMTs)

Fecal microbiota transplants (FMTs) are successfully (~90% success rate) used in severe *Clostridium difficile* infections [194]. Even though the precise mechanism of action is currently unknown, one possibility is certain bacteria (*Bifidobacterium longum*, *Lachnospiraceae* and *Porphyromonadaceae*) in the microbiota of the healthy donor could contribute in colonisation resistance, inhibiting the growth and outcompeting the infectious pathogens for space and nutrients [167]. There are conflicting data about the utility of FMT for treatment of other disease conditions such as ulcerative colitis [195-198]. Given the

success in FMT in *C. difficile* infections and potential in reducing ulcerative colitis symptoms, there is an increasing interest in application of FMT on other gastrointestinal diseases [199]. However, regulations in preventing transmission of pathogens or other morbidity pathogens and transmission of other conditions (i.e. obesity) through FMT are important [167].

(b) Phage therapy

Emerging knowledge on the gut virome has demonstrated a possible link between alterations to the virome and diseases [200]. For example, IBD is associated with an increase in the abundance of phage independent of the decrease in the bacterial diversity [201]. Therapeutic modulation of the gut microbiota could be potentially achieved through altering the virome. Possible applications of phage therapies range from phage-bacterial-specific therapies through targeted bacterial resistance, blocking phage induction to affect the viability of some gut bacteria and inducing immune responses through utilising the ability of phage to interact with the host [167, 200].

1.7 Studying the gut microbiota

1.7.1 Models for the gut microbiota studies

Major challenges in human gut microbiota studies are inherent limitations involved in sampling the human gastrointestinal tract, ethical restrictions implicated on human clinical trials, costs and volunteer compliance [202]. Therefore, development of *in vitro, ex vivo, in silico* and animal systems to model the human gut microbiota has become crucial. Even though none of these models can be used as a complete replacement for human clinical trials, these provide useful evidence for proof of concept experiments [202].

(a) Animal models

Animals are widely used as models of the human gut microbiota studies, and commonly used models include mouse, rat, guinea pig, dog, pig, chicken, Mongolian gerbil, ferret and other

primates such as chimpanzees and macaques [202-204]. Arguably, mouse models are the most extensively used animal model in the gut microbiota research owing to factors such as similarities to human gut anatomy, physiology and genetics, competent knowledge on mouse genetics, availability of genetically modified mice for functionality analyses, low maintenance cost, high reproductive rates and short life cycle [203]. A strain of mice typically has a homogenous genetic background, as they are usually inbred. Use of murine models also provides the ability to control common sources of variation such as genetic background, diet and housing compared to that in human studies.

As observed in the human gut microbiota, the murine gut microbiota is dominated by the phyla *Firmicutes* and *Bacteroidetes* [37]. Humans and murine share significant similarities in the overall gut microbiota at higher taxonomic levels and the factors that contribute in altering the gut microbiota. However, the gut microbiota compositions between humans and murine vary significantly at lower taxonomic levels such as the genera (Figure 1.8) [205, 206].

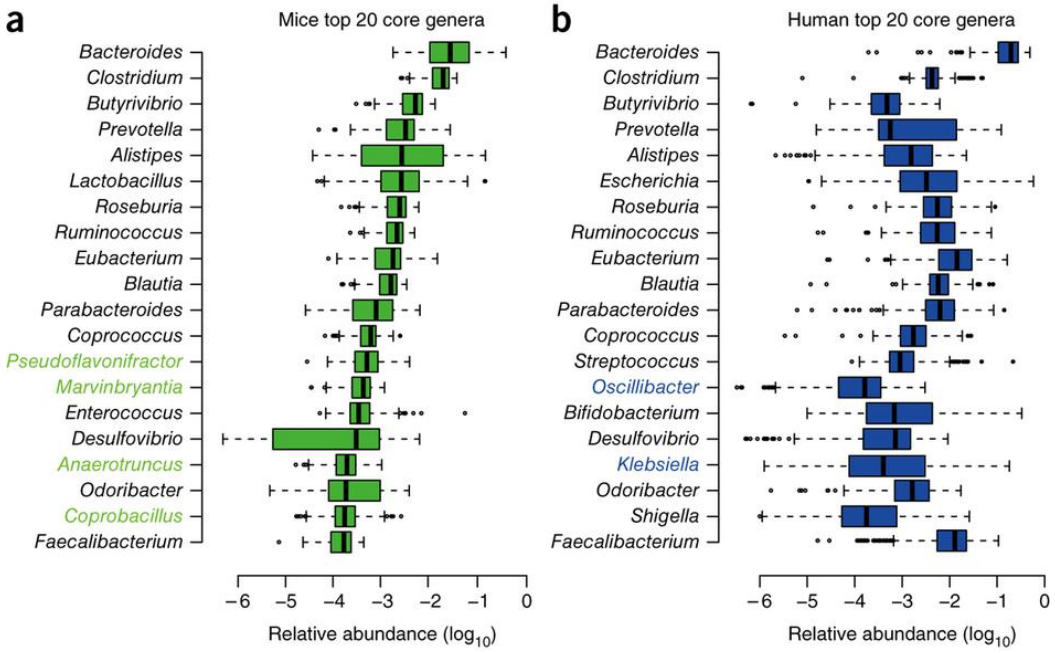

Figure 1.8 Top 20 most abundant bacterial genera in the (**A)** murine and **(B)** human gut microbiota. The genera in green and blue font exhibit higher abundances in mouse and human gut microbiota, respectively. Thirteen out of the 20 most abundant genera in mice were also present among the top 20 genera in humans. Image reprinted from Xiao *et al* 2015 [205] (licence number 4278000406143).

The murine gut microbiota can be manipulated to reduce the inherent differences compared to humans. One option is the use of germ-free or gnotobiotic mice, as they can be inoculated with any microbial strains of interest [207]. This facilitates the study of microbiota-host and microbe-microbe interactions without the interference of background complexity of a larger microbiota [208]. Another way to mitigate the differences in gut microbiota composition is through colonising murine gut with human microbiota [208]. These humanised mouse models (germ-free mice inoculated with the human microbiota) are widely used in gut microbiota research. Another advantage of murine models is the availability of a range of gene-knockout mouse lines that allows the study of host genetics-microbiota relationship. Furthermore, murine models allow interventions such as extreme dietary changes [172], regulation of light cycles [166] and extreme environmental changes (such as temperature) [165] on the gut microbiota.

Murine models are widely used in the study of gut microbiota-associated diseases such as obesity, IBD and diabetes. For example, mice fed high animal-fat diets or with genetic modifications (leptin deficient *ob/*ob) showed higher body weight gain and an increase in the ratio of *Firmicutes:Bacteroidetes* [37, 38]. The abundance of the genera *Prevotella* and *Roseburia* decreased in high fat fed mice, whilst *Barnesiella, Bacteroides* and *Alistipes* increased [209-211]. As previously mentioned in sections 1.4.2 and 1.5.1, human dietary studies have also reported similar results [88, 90, 134].

However, like any available model to date, murine models fail to completely replicate the human gut microbiota. Contributing factors may include inherent differences in the anatomy, physiology and genetics, discrepancies in results between different mouse strains and between mice of the same strain but from different vendors or breeding facilities, and failure to capture the genetic diversity of humans as inbred mouse strains are genetically homogenous [212]. Furthermore, unavoidable variations such as cohousing associated coprophagy that results in

transfer of the microbiota, stress induced by handling could also contribute to alterations in the gut microbiota [203, 208].

(b) *In vitro* models

In vitro culturing experiments can eliminate most of the limitations such as large biological variations in the gut microbiota, host interference, cost and ethical restrictions associated with animal and human studies. *In vitro* models allow high controllability thus higher reproducibility, frequent sampling and a simplified system to focus on the gut microbiota changes without host intervention [213]. Obligatory anaerobic nature and fastidious nutrient requirements have made culturing the gut bacteria challenging. Therefore, strictly anaerobic conditions and specific growth substrates (filtered stool extract or SCFAs) are typically utilised in *in vitro* models [208]. Many *in vitro* models have been developed throughout the years with varying complexity and applications. These models have been used to investigate the effect of factors such as prebiotics [214, 215], probiotics [216], diet [217] and dietary changes [218] on the gut microbiota.

Batch culture fermentation models are the simplest and most frequently used *in vitro* models, particularly, for substrate digestion assessment such as probiotics [219] and prebiotics/other dietary components [214, 220, 221]. These systems are comparatively inexpensive and convenient, therefore, allow testing many conditions and/or replicates. However, limitations of these models include changes in pH levels and redox potential, substrate depletion and accumulation of inhibitory bacterial metabolites [202, 220, 222].

Continuous culture fermentation models are designed with a constant influx of nutrients and efflux of waste products. Single stage continuous fermenters are usually used to elucidate proximal colon functions, whilst multi-stage continuous fermenters with multiple chemostats can simulate the distinct regions of the colon [220]. Multi-stage continuous models require strict control of factors such as pH levels, retention time, temperature, anaerobiosis and flow

rate of the medium for the reproducibility of the system. EnteroMix® is an example of a multi-stage semi-continuous culture model, which has been used to examine the effects of carbohydrate metabolism by the gut microbiota [223]. Fecal beads (immobilised fecal samples within a porous polysaccharide matrix) are used in multi-stage fermenters to avoid washout of less competitive planktonic bacteria due to limited-time in the liquid reaction vessels [220]. The Polyfermentor Intestinal Model (PolyFermS) is a continuous fermenter that is inoculated with such immobilised fecal samples [202].

In vitro models for stomach and small intestine digestion with varying complexity are used in combination with different *in vitro* gut microbiota models. The design of these digestion models can be static mono-compartmental, dynamic mono-compartmental or dynamic bi- and multi-compartmental [224]. Artificial digestive systems are an extension of both multi-stage continuous gut microbial fermentation modelling and stomach and small intestine digestion modelling. These can mimic physiochemical conditions along the gastrointestinal tract with an influx of enzymes, peristaltic motility, absorption capacities, high-shear force and host-microbiota interactions [202]. TNO intestinal model (TIM) 1 and 2 [225] and simulator of human intestinal microbial ecosystem (SHIME) [226] are examples of such advanced artificial digestive systems. Despite the complexity in simulating the gastrointestinal tract, even these models fail to mimic the complexity of human body (i.e. immune and neuroendocrine responses) [220]. Mucosal models incorporate the host responses using mucus or mucosal associated models. Immobilised mucus models with mucus beads/gels and M-SHIME model are examples of mucosal models. Recently developed host-microbiota interaction (HMI) model and the gut-on-a-chip model provide the complexity of the human gastrointestinal tract to a higher degree [202].

One of the biggest drawbacks of *in vitro* models is the simplicity especially, highly simplified gastrointestinal tract and lack of feedback mechanisms from the host compared to highly complex human gastrointestinal tract. However, continuing development of these *in vitro*

gastrointestinal tract model systems hold promising possibilities to better characterise and understand the gut microbiota.

1.7.2 An overview of frequently used techniques

Study of the human microbiota dates to the 17th century, with the first microscopic observation of the oral microbes by Van Leeuwenhoek. However, our knowledge on microbiota-host symbiosis was limited until recently due to technological limitations. Recent and continuing technological and methodological advancements in molecular technology, particularly, breakthroughs in genomics and DNA sequencing technologies have broadened the knowledge on gut microbiota-host linkage in a rapidly increasing rate. This highly innovative field of study continues to flourish owing to the reducing cost and increasing throughput of DNA sequencing technologies [227].

Commonly used approaches to study the gut microbiota are briefly outlined in this section (Figure 1.9). Marker gene surveys are commonly used culture-independent method to obtain an overview of the species present in a sample. Small subunit ribosomal RNA (SSU rRNA) gene (such as 16S rRNA and 18S rRNA) amplicons are commonly used in this method [228]. Metagenomics on the other hand allows simultaneous profiling of both species composition and functional potential profiles of microbial communities in an uncultured environmental sample [22]. Whole genome sequencing of a targeted organism (usually cultured) is employed to obtain information on the complete coding potential [229]. Single-cell genomics (SCG) is an emerging technique in the gut microbiota studies, which is a more targeted culture-independent sequencing technique compared to metagenomics. This method holds a number of promising opportunities through the discovery of genomic data of mostly unculturable gut microbiota, which can be used to improve databases for metagenomics analyses [208]. Metatranscriptomics or RNA-sequencing is another sequence-based technique used to obtain information on functional activity profiles of an uncultured microbial community using total community RNA [230].

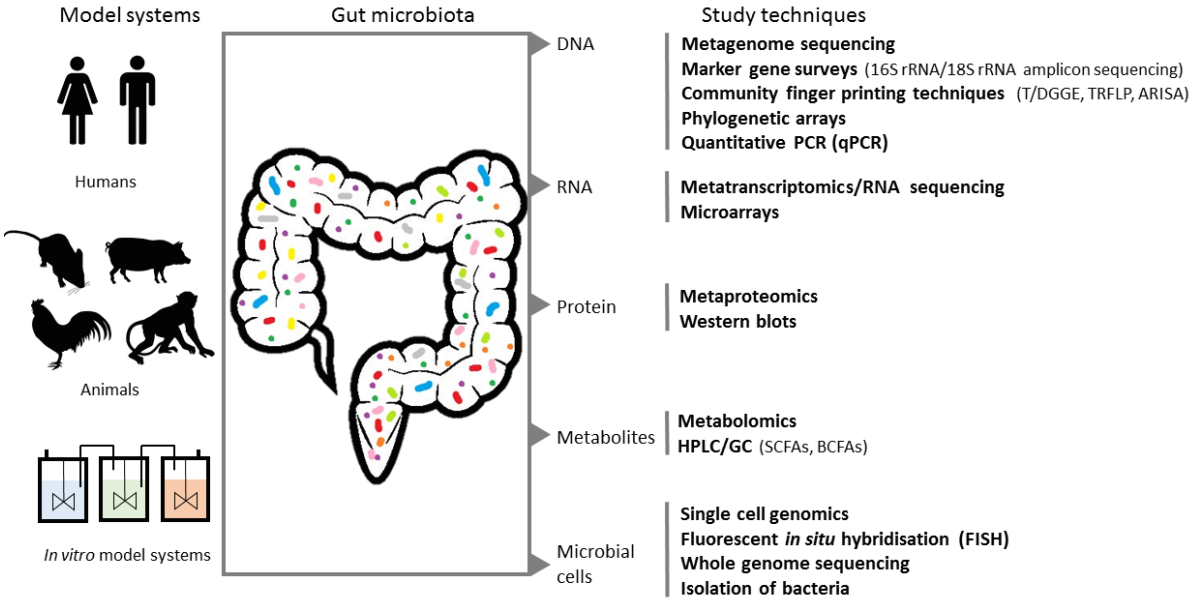

Figure 1.9 Examples of models and techniques used in the gut microbiota studies. Models include human, animal and *in vitro* human gut microbiota systems. Techniques are typically based on the gut microbial DNA, RNA, protein, metabolites and culture-independent or dependent techniques using cells.

In addition to sequence-based community profiling techniques, community fingerprinting techniques such as temperature denaturing gradient gel electrophoresis (T/DGGE) [231], terminal restriction fragment length polymorphism (T-RFLP) [232] and automated ribosomal intergenic spacer analysis (ARISA) [233] are also available. Phylogenetic microarrays/ phylochips are other community profiling techniques that have been used in the gut microbiota studies. This usually involves a custom array seeded with short oligonucleotides of the SSU rRNA genes for selectivity of a particular range of organisms present in an environmental sample [208]. However, due to reducing cost and high throughput of DNA sequencing-based community profiling methods, fingerprinting methods and microarrays are less frequently used in recent studies.

Quantitative PCR (qPCR) and fluorescent *in situ* hybridisation (FISH) techniques are usually employed for quantitative analysis of dominant groups in a microbial community. Although the types of microorganisms in the gut microbiota are well studied, the functional profiles of these communities are comparatively less known. Techniques such as metaproteomics,

functional metagenomics, metabolomics and stable isotope probing (SIP) have largely contributed in exploring more on the functionality of the gut microbiota [208].

1.8 Scope of the project

Gut microbiota-host symbiosis has co-evolved with humans and contributes to maintaining the host physiology and immunity. Accumulating evidence demonstrates a link between the dysbiosis of this relationship and disease development. Modern lifestyle changes (dietary habits, antibiotics, modern clinical practices, sanitation) have largely contributed to this dysbiosis. However, diets low in microbiota-accessible carbohydrates have been empirically shown to be the biggest contributor [234].

Dietary fibre holds some potential to restore the diversity and metabolic pathways in the gut microbiota. However, daily dietary fibre consumption in most Western communities is on average half of the recommended amount [137]. Bridging this gap in fibre intake is of utmost importance to manipulate and restore the gut microbiota to conserve its diversity and beneficial metabolic functions.

A variety of purified dietary fibre, fibre-raw material and prebiotics are already commercially available worldwide as dietary fibre supplements [234]. Although purified forms of some of these dietary fibre products have been scientifically tested with clear health and microbiota related outcomes, there is a lack of research conducted in evaluating the effect of commercially available dietary fibre products on the gut microbiota. This research was conducted to investigate the effect of commercially available dietary fibre products on the gut microbiota. The specific aims of the project were:

(a) Examining the impact of fibre products derived from sugarcane, wheat dextrin and psyllium husk on the adult human gut microbiota *in vitro* (Chapter 2).
(b) Investigating the impact of sugarcane and wheat dextrin-based fibre products on the gut microbiota of high fat diet fed mice (Chapter 3).

(c) Exploring the effect of fibre products derived from sugarcane and wheat dextrin in combination with weekly overnight fasting on the gut microbiota of high fat diet fed mice (Chapter 4).

(d) Examining the effect of wheat, sorghum, rice and oat-based cereal products on the infant gut microbiota *in vitro* (Chapter 5).

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CHAPTER 2

Fibre supplements derived from sugarcane stem, wheat dextrin and psyllium husk have different *in vitro* effects on the gut microbiota

Title

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Running title -Fibre products alter the gut microbiota

Conflict of interest

MSB is an employee of Gratuk Technologies Pty Ltd, the producer of NutriKane™. All other authors have no conflict of interest.

2.1 Abstract

There is growing public interest in the use of fibre supplements as a way of increasing dietary fibre intake and potentially improving the gut microbiota composition and digestive health. However, currently there is limited research into the effects of commercially available fibre 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 fibre products; NutriKane™, Benefiber® and Psyllium husk (Macro) on the adult gut microbiota. The 16S rRNA gene amplicon sequencing results showed dramatic fibre-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 fibre product(s), while *Enterobacteriaceae* and *Pseudomonadaceae* showed a reduction in the relative abundances upon addition of all fibre treatments compared to the no added fibre control. Fibre-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 fibre product were determined and found to be highly variable. Application of different commercially available fibre supplements to the gut microbiota *in vitro* resulted in strong fibre-dependent shifts in the microbiota and production of SCFAs. Observed product-specific variations could be linked to differences in the chemical composition of the fibre products. The general nature of the fibre-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.

Key words- Gut microbiota, dietary fibre supplementation, *in vitro* gut models, 16S rRNA gene, short chain fatty acids, polyphenols.

2.2 Introduction

Trillions of microorganisms reside in the human large intestine, which is collectively referred to as the gut microbiota [1]. The gut microbial composition is shaped by exogenous and endogenous factors and interacts with the host metabolism and physiology [2, 3]. The adult gut microbiota is usually dominated by the phyla *Firmicutes* and *Bacteroidetes* [4, 5] while *Actinobacteria, Proteobacteria* and *Verrucomicrobia* constitute minor proportions of the bacterial populations [6, 7]. Compositional and functional alterations of the gut microbiota have been associated with various inflammatory and metabolic diseases such as obesity [8], type 2 diabetes (T2D) [9, 10], type 1 diabetes (T1D) [11] and inflammatory bowel disease (IBD) [12].

Diet has been shown to impact the composition and activities of the gut microbiota [13, 14]. 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 [15]. There is also evidence that individual dietary preferences correlate to some degree with longer-term gut microbiota composition [16]. 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 fibre and carbohydrate consumers [16]. Similar observations have been made in a number of studies that looked at different communities of people who consume diets rich in fibre in comparison to diets low in fibre [17-21].

Modulation of the gut microbiota using dietary components is potentially therapeutically useful [22, 23]. 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) [24-26]. 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 [27]. 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, tumour suppression and production of the hormone leptin [28].

The most commonly studied prebiotics are dietary fibre, which include carbohydrates such as cellulose, xylan, resistant starch, pectin, inulin and mannan [26]. Fermentation of dietary fibre by the gut microbiota and concomitant effects on human health has been investigated in the context of conditions such as IBD, T2D and obesity [29]. For example, studies have shown an increase in the abundance of bifidobacteria upon consumption of inulin, short chain fructooligosaccharides (FOS) or galactooligosaccharide (GOS) [30] and the ability of these dietary fibres to reduce inflammatory markers associated with obesity and T2D [31, 32]. *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 [33]. Several recent studies have also employed *in vitro* models to compare the effects of introducing pectin, inulin [34, 35] and wheat dextrin [36] on the gut microbiota. These studies have demonstrated the potential of the fibre additions to enrich specific members of the genus *Bacteroides* and phylum *Firmicutes* [34-36].

Dietary polyphenols and antioxidants have also been studied for their ability to beneficially change gut microbial composition and functions [37, 38] 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* and *Clostridium* [37, 39]. Several other studies have indicated the ability of the antioxidant action to be delivered to the gut epithelia resulting in a reduction of inflammation [40-42] and improvement in tissue recovery in IBD patients [43].

Utilisation 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 [44, 45]. *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 [46]. Various *in vitro* models of the gut microbiota have been used to examine the effects of prebiotics [34, 47], probiotics [48], diet [49] and dietary modulations [50] on the gut microbiota and its metabolites.

The recommended daily individual intake of dietary fibre in many countries ranges from 25-30 g/day [51], however, increasing amounts of data show that this requirement is poorly met, especially in many western countries [52]. Many commercially available fibre supplements are marketed as a means of bridging this gap in dietary fibre intake. However, to date only a few studies have directly examined commercially available dietary fibre products for prebiotic potential [36, 53].

In this study, we investigated the effect of three commercially available fibre 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.

2.3 Results

Samples of three commercially available fibre products; NutriKane, Benefiber and Psyllium husk (Macro), with varying chemical composition (Table 2.1), were chosen to investigate the impact of fibre supplementation on the human gut microbiota *in vitro*. Each fibre 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 Table S2.1). For each biological sample, four treatments were applied, this included three fibre products and one 'no added fibre' control (details of the experiment design are provided in Fig. S2.1).

The anaerobic cultures for all tested fibre products with each fecal inoculum produced visually detectable gas by 24 hours, indicating that the microbiota was metabolically active. At 48 hours the pH of the culture vials with Benefiber significantly reduced ($P < 0.001$) compared to the samples with NutriKane, Psyllium husk or no added fibre control, which maintained the pH at 7.0 ± 0.5 in the buffered media (Fig. S2.2).

Samples were collected at 0, 24 and 48 hours from the liquid fraction, and at 48 hours the insoluble fibre 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 fibre fraction samples, and after filtering and rarefaction a total of 681,792 reads were used for further analyses.

Effects of fibre addition on microbial community structure and diversity

To determine the impact of different fibre 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 fibre-dependent changes in the bacterial community structure over time based on Bray-Curtis similarity non-metric multi-dimensional scale (nMDS) plots for each biological sample (Fig. 2.1) and Permutational Multivariate Analysis of Variance (PERMANOVA) tests. Fibre product-mediated shifts in the gut microbiota structure showed very similar trends upon each treatment at 24 and 48 hours across biological samples (Fig. 2.1 and Fig. S2.3). For all individuals, supplementation with NutriKane resulted in a significantly different community structure at 24 hours compared to at

0 hours ($P < 0.05$). At 48 hours this shift was more pronounced ($P < 0.005$, comparing 0 and 48 hours). Clear shifts in the microbial community structure were observed in the nMDS plots upon addition of NutriKane compared to the no added fibre samples at both 24 and 48 hours (Fig. 2.1), 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 hours compared to that of the no added fibre control and community at 0 hours ($P < 0.001$).

Ordination of the gut microbiota of all samples (n=212) showed significant fibre addition-mediated changes in the community structure common across the biological samples (Fig. S2.3). At 0 hours 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 of each sample was determined using a Shannon diversity index (Fig. 2.2). Shannon diversity indices of samples with Benefiber and Psyllium husk reduced to 3.2 ± 0.5 and 2.4 ± 0.4, respectively, at 48 hours, while samples with NutriKane showed no significant loss of diversity (3.8 ± 0.4) compared to the no added fibre control at 48 hours (3.7 ± 0.2) and all samples at 0 hours (4.0 ± 0.2).

Effects of fibre addition on microbiota composition

For all individuals the starting fecal microbiota communities (0 hours) 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 fibre product differentially altered the microbiota composition at 24 and 48 hours in each of the six biological samples (Fig. S2.4).

The OTUs that contributed most to these product-specific changes in the microbial community composition were identified using Similarity Percentage (SIMPER) analyses, and changes in the relative abundance of this set of OTUs in each treatment and time point were analysed for each biological sample (Fig. S2.5 and Table S2.2). The OTUs in the genus *Bacteroides* showed a higher relative abundance in all samples supplemented with fibre, however, the specific OTUs varied between fibre 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 fibre control, samples with Benefiber and Psyllium husk showed a reduction in the abundance of this OTU.

The complete set of bacterial families and OTUs showing significant differences in abundance between each fibre addition and the no added fibre control were identified with linear discriminant analysis (LDA) effect size (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 fibre control (Fig. 2.3). 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 fibre 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 (Fig. S2.6 and Table S2.3). While most of these trends were commonly observed across biological samples, the degree of changes varied between individuals (Table S2.4). Many specific OTUs within the *Bacteroidaceae* were significantly higher in abundance in samples with each of the three fibre products compared to the no added fibre control (15, 35 and 33 OTUs in NutriKane, Benefiber and Psyllium husk, respectively, Fig. S2.6 and Table S2.3). Among the fibre specific changes, Benefiber addition resulted in higher relative abundance of 5 and 15 OTUs in *Faecalibacterium prausnitzii* and *Porphyromonadaceae* (*Parabacteroides*), respectively, a change not observed for the other products. NutriKane supplementation promoted high relative abundance of an OTU (OTUNew_Reference723) 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 fibre 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 fibre 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 fibre supplementation

The addition of fibre 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* (Fig. 2.3, Fig. S2.4, Fig. S2.5 and Fig. S2.6).

In addition to these, we also observed individual-specific changes in some bacterial groups (Fig. S2.4). 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 fibre 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 fibre products only in biological sample 4. In biological sample 5, the relative abundance of *Enterobacteriaceae* increased at 24 hours upon addition of fibre products.

Comparison of the fibre-adherent and liquid fraction microbiota

To investigate possible differences in the microbial communities adhered to the fibre relative to the liquid fraction, we examined the microbiota detached from insoluble material in the cultures at 48 hours. While the community structure was observed to be similar between fibre and liquid fractions (Fig. S2.7), some differences in the composition were observed (Fig. S2.8). Analysis of the fibre adherent microbial community relative to the liquid fraction at the OTU level was performed using LEfSe analyses (Fig. S2.9 and Table S2.5). The relative

abundance of 13, 19 and 31 OTUs were higher in the fibre 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 (Table S2.6).

Fibre additions increased the production of SCFAs

To examine the impact of the fibre 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 ionisation detection (GC-FID, Fig. 2.4). Addition of NutriKane, Benefiber and Psyllium husk resulted in significantly higher ($P < 0.05$) concentrations of all three SCFAs at 48 hours, compared to the same treatments at 0 hours. In comparison to the no added fibre control at 48 hours, Benefiber and Psyllium husk supplementation showed significantly higher ($P < 0.005$) concentrations of all three SCFAs. Propionate was the major SCFA produced upon potential microbial degradation of Benefiber and Psyllium husk, followed by butyrate and acetate. Butyrate was the major SCFA produced upon microbial degradation of NutriKane followed by propionate and acetate.

Changes in the relative abundance of the *Porphyromonadaceae* correlated with the concentrations of all three SCFAs (Spearman's $r > 0.33$, $P < 0.0001$). Changes in the abundance of *Bacteroidaceae* correlated with the concentration of propionate (Spearman's $r = 0.43$, $P < 0.0001$). While all biological samples showed similar trends with the specific fibre additions, we observed individual-dependent differences in the concentrations of each SCFA (Table S2.7).

Comparison of the polyphenol content and antioxidant potential of fibre products

The polyphenolic content and antioxidant potential of each fibre product at time 0, 24 and 48 hours were determined using total polyphenolic content (TPC) and Ferric reducing antioxidant power (FRAP) techniques respectively (Fig. 2.5, Table S2.8). NutriKane showed significantly higher ($P < 0.0001$) antioxidant potential and polyphenolic content compared to Psyllium husk at 0 hours. The antioxidant potential of NutriKane was significantly higher ($P < 0.0001$) compared with Benefiber at 0 hours. In all fibre-supplemented samples polyphenolic content decreased across the full incubation while antioxidant potential decreased over the first 24 hours, but no further decrease was observed at 48 hours.

2.4 Discussion

This research examined the effect of three commercially available fibre products on the human gut microbiota from healthy individuals *in vitro*. Our findings demonstrated fibre product-induced strong shifts in gut microbiota community structure and composition at 24 hours, which were further pronounced at 48 hours. These changes in the relative abundance of microbial families and OTUs following fibre 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 [54].

Each fibre product resulted in distinct alterations to the microbiota composition

Fibre-specific community changes were observed, which are potentially linked to the chemical composition of each tested product. NutriKane, which is primarily derived from

dried whole sugarcane stem, is rich in cellulose, hemicellulose and lignin [55, 56]. In comparison, Psyllium husk typically contains viscous fibre such as certain hemicelluloses and arabinoxylans [57], while Benefiber, which is derived from wheat dextrin is rich in D-glucose [58]. The abundance of OTUs in *Bacteroidaceae* and *Porphyromonadaceae* 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 of the family *Porphyromonadaceae* have been shown to digest chemically modified starch, while members in *Bacteroidaceae, Ruminococcaceae* and *Lachnospiraceae* digest starch as well as more complex polysaccharides such as cellulose, hemicellulose and pectin [59, 60]. Previous studies have also investigated the effect of specific purified dietary fibres on the gut microbiota and have shown that different types of resistant starch [60], pectin [61], hemicellulose [62], cellulose [63] and inulin [64] have different effects on the gut microbiota, likely due to variations in the chemical composition of different dietary fibres [65].

Significant increases in the relative abundance of *Bifidobacteriaceae* were observed solely for NutriKane. NutriKane contained higher levels of xylose, and *Bifidobacteriaceae* has been shown to cross-feed on xylan [59]. Increased relative abundance of *Bifidobacterium* has been linked to potential prebiotic effects and has been shown to increase in IBD patients upon remission [65-68]. *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 [59], 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 [69].

The OTUs in the families *Enterobacteriaceae* and *Pseudomonadaceae* showed the highest relative abundance in samples with no added fibre. Most of the members of these families belong to the normal microbiota, while some are associated with inflammation [66, 70].

Observed reductions in the relative abundance of *Enterobacteriaceae* and *Pseudomonadaceae* upon supplementation with these fibre products might indicate the potential of the products to improve or maintain host health.

Analysis of the diversity of each community after fibre addition indicated varied capacities of the fibre products to maintain gut microbiota diversity *in vitro*. Of the tested fibres, only NutriKane treatment resulted in maintenance of the microbial diversity. Supplementation with Benefiber and Psyllium husk resulted in significant reductions in the microbial diversity. These are likely explained by the dramatic increases in fibre-digesting families such as *Porphyromonadaceae*, which constituted 30.5% of the total bacteria at 48 hours in Benefiber, and the *Bacteroideaceae*, which constituted 68.9% and 24.0% of the total bacteria at 48 hours in the samples with Psyllium husk and Benefiber, respectively. Whilst reduced gut microbiota diversity has been shown in individuals with obesity, T2D and IBD [9, 71, 72], we believe that such dramatic increases in specific groups would likely be ameliorated by host and phage controls *in vivo*, and hence the reduction in diversity observed in the *in vitro* system is unlikely to be observed *in vivo*.

Microbiota composition differed between fibre-adherent and liquid fractions

The relative abundance of bacterial groups attached to the insoluble fibre fraction, and therefore potential primary degraders, varied between each tested fibre product. The relative abundance of members of the phyla *Firmicutes* (*Lachnospiraceae* and *Ruminococcaceae*), and *Verrucomicrobia* (*Turicibacteraceae*) were higher in the fibre fraction compared to the liquid fraction. 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 [73, 74]. Even though bacterial groups among *Bacteroidetes* are known to encode the largest number of CAZymes, bacterial groups in the

phyla *Firmicutes*, *Actinobacteria* and *Verrucomicrobia* are known to be more nutritionally specialised and initiate the degradation of complex carbohydrates undigested by human [75].

Fibre additions stimulated production of acetate, propionate and butyrate

The concentration of measured SCFAs was significantly higher in all fibre-supplemented samples. Acetate, propionate and butyrate are all major bacterial fermentation products, each of which is likely to contribute to host health [76]. The degree of stimulation of SCFA production was fibre 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 *Porphyromonadaceae* and *Bacteroidaceae* following addition of these fibre products. This is potentially linked to the ability of the members of these bacterial families to digest highly available dietary fibre in the fibre products and produce SCFAs [77].

While production of all three SCFAs was higher with fibre 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* [78].

Polyphenol and antioxidant availability differed between fibre products

We observed significant differences in the availability of polyphenols and antioxidants in the tested fibre 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* [49, 79] and *in vivo* [80]. Of the tested fibre 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 fibre, 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* [37, 80]. The observed reduction of polyphenols and antioxidant potential of the fibre products over the time of incubation is likely due to metabolism of these compounds by the gut microbiota [81, 82].

Fibre supplementation-induced common and biological sample-specific microbial community shifts

Whilst each biological sample varied in the exact nature of the fibre-induced community shifts, common, broad patterns were observed regarding both the abundance changes of specific OTUs and changes in the concentrations of SCFAs. This suggests that the tested fibre 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.

Several biological sample-specific changes 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 while other biological samples did not show substantial differences. These 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.

2.5 Conclusions

As dietary supplementation grows in popularity it is important to examine how commercial fibre products impact the human gut microbial communities and host health, and the degree to which this varies between products. The three 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 fibre and its associated micronutrients, for example the antioxidant and polyphenol content in each fibre product. The observed differences in microbial community composition upon fibre supplementation may also explain the observed fibre-specific differences in acetate, propionate and butyrate production.

Utilisation 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 fibre 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 fibre 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 [83].

2.6 Materials and methods

Compositional analysis of fibre products

Fibre 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 fibre 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 [84]. Fat content was determined by Soxhlet extraction according to the AOAC Method 945.16. Dietary fibre was determined enzymatically according to the AOAC method 985.29. Insoluble and soluble dietary fibre content was determined according to AOAC 991.43. Acid insoluble lignin was measured

gravimetrically following acid hydrolysis [85]. Monosaccharides were quantified using acetylated samples on a Shimadzu 17A gas chromatograph with flame ionisation 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 2.1).

In vitro digestion of fibre 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 fibre products and a sterile water (Milli-Q, Millipore, Australia) sample as the no added fibre control were processed by a simulated oral, gastric and small intestinal digestion as described by Minekus *et al* 2014 [86]. 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 minutes at pH 7, followed by porcine pepsin (2000 UmL^{-1}) for 2 hours at pH 3. The small intestine digestion was performed for another 2 hours with the following enzymes (porcine trypsin (100 UmL^{-1}), bovine chymotrypsin (25 UmL^{-1}), porcine pancreatic lipase (2000 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. 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 [87] 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 [88] 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 aluminium lids prior to sterilisation.

Collection and preparation of fecal inocula

All experimental procedures and protocols were reviewed and approved by the Human Research Ethics Committee Macquarie University (reference number 5201400595). 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 three months, had no history of gastrointestinal diseases and were on a nonspecific omnivorous diet (metadata provided in Table S2.1).

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, UK) and an Oxoid anaerobic indicator (BR0055B, Oxoid, UK). Samples were transported to the laboratory anaerobically and processing occurred within 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 (985µm). This was conducted under strict anaerobic conditions as used for basal media preparation.

***In vitro* fermentation of fibre 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 fibre product concentration was maintained at 1% (w/v). A control sample was run in parallel with no

added fibre. 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 Fig. S2.1).

Culture vials were incubated anaerobically at 37°C with agitation (100 rpm). Cultures were left without agitation for 5-10 minutes, to allow the solids to settle, prior to collecting 2 mL aliquots from the top liquid fraction at 0, 24 and 48 hours. Harvested samples were stored at -80°C immediately prior to further analysis. The pH of the cultures at 48 hours 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 hours, insoluble fibre biomass (fibre fraction) was separated from each vial with fibre products by centrifugation at the 100 x g for 5-15 minutes. Separated fibre fraction samples were resuspended in Phosphate-buffered saline (PBS) prior to dissociation of tightly adherent microorganisms as described by Rosewarne *et al* 2010 [89]. According to this protocol the insoluble fibre 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 x g for 15 minutes. Harvested cells from the liquid and fibre 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 [90]. 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 seconds, 50°C for 60 seconds and 72°C for 90 seconds using 515 forward (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806 reverse (5′-GGACTACHVGGGT WTCTAAT-3′) primers with custom barcodes for Illumina MiSeq sequencing [91, 92]. Fibre 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) [93]. 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 [94].

Liquid fraction samples (n=216, 3 fibre products and a no added fibre control x 6 biological samples x 3 time-points x 3 technical replicates) resulted in a total of 21,052,381 reads (mean 97, 464 ± 25,865) prior to filtering out the OTUs with less than 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.

Fibre fraction samples (n=54, 3 fibre products x 6 biological samples x 3 technical replicates) were analysed with liquid fraction samples (n=54) at 48 hours 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 less than 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).

The 16S rRNA gene sequence data generated and analysed during the current study are available on the GenBank Sequence Read Archive (SRA) database under accession number SRP090829.

Rarefied and filtered OTUs were used for statistical analyses using PERMANOVA and pairwise tests were conducted using PERMANOVA+ [95] in the PRIMER-7 software package [96] 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 visualise 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 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 fibre product and no added fibre control at 48 hours were identified using LEfSe analyses (online Galaxy version 1.0) [97]. LEfSe analysis was conducted with treatment conditions as subject (no subclasses) and with all other default parameters. The significantly differentially abundant OTUs between the fibre 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 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) 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 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=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 described by Singleton *et al* [98]. Briefly 20 µL of sample was mixed with 1.58 mL of water and 100 µL of the Folin-Ciocalteu reagent. After 6 minutes of incubation, the solution was mixed with 300 µL of 7.5% (w/v) Na_2CO_3 and left to stand for 2 hours. 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 litre.

Ferric reducing antioxidant power (FRAP) was performed as described by Benzie *et al* [99]. 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 minutes. Ferrous sulfate standards ranged from 125 to 2500 µM. The absorbance was read at 593 nm and results reported in millimolar ferric ions converted to the ferrous form per litre.

Statistical analysis

Significant differences in the relative abundance of OTUs, Shannon diversity indices, pH measurements, concentration of SCFAs, TPC and FRAP measurements were determined through Tukey's multiple comparison tests between each fibre product and no added fibre control at 24 and 48 hours using GraphPad Prism (version 7) software (GraphPad Software, La Jolla California, USA). Biological samples were analysed 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.

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2. 8 Acknowledgements

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2.9 Figures and tables

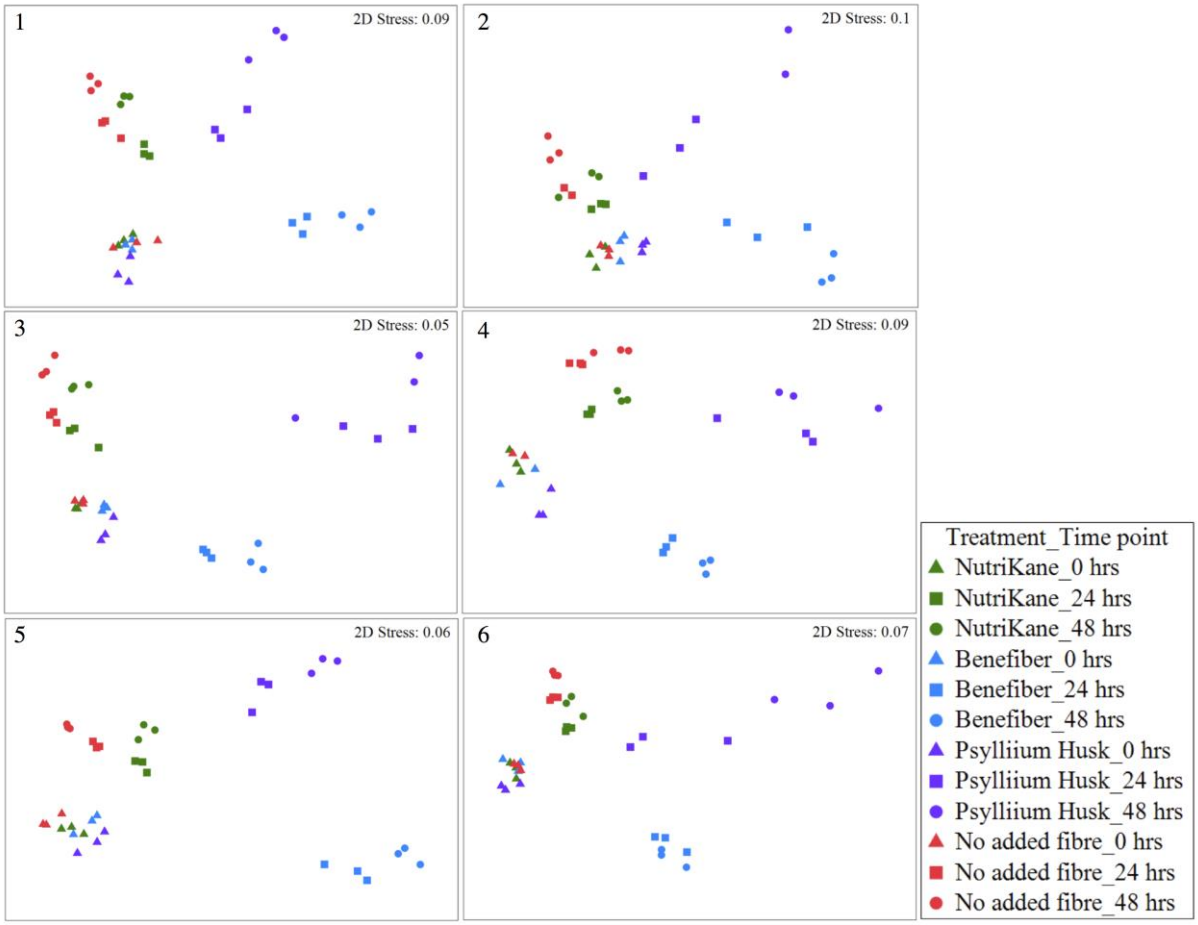

Figure 2.1 Ordination of the gut microbiota in each biological sample (sample 1-6) at 0, 24 and 48 hours. Data is shown as a Bray-Curtis similarity of Log (X+1) relative abundance based nMDS plots. At 0 hours (triangles) all samples group together. Fibre-dependent shifts were observed at 24 (squares) and 48 (circles) hours in all the treatments. NutriKane (green), Benefiber (blue) and Psyllium husk (purple) had different communities to the no added fibre control (red) while samples with Benefiber and Psyllium husk showed the most dramatic shifts compared to the no added fibre control and other treatments.

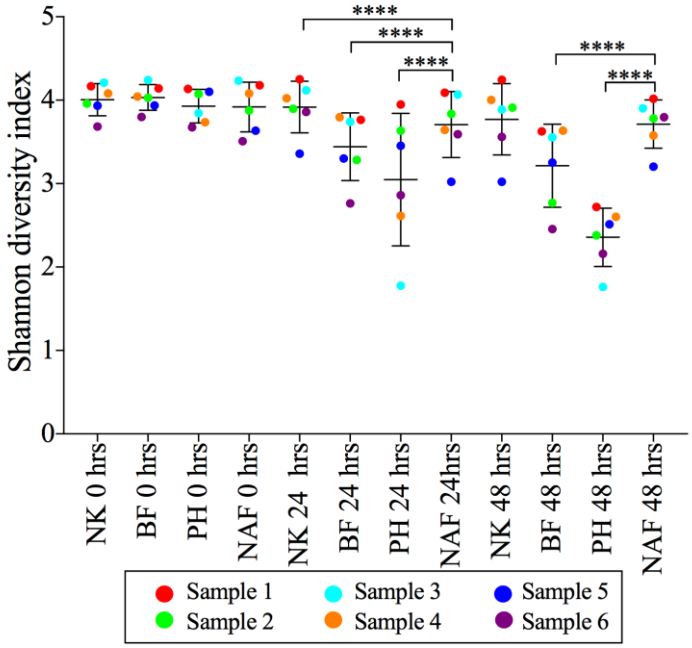

Figure 2.2 The Shannon diversity indices of microbial communities from each treatment at 0, 24 and 48 hours. The Shannon diversity index for each sample was determined using PRIMER-7 software. Data is shown as mean ± SD for samples with NutriKane (NK), Benefiber (BF), Psyllium husk (PH) and no added fibre control (NAF) at 0, 24 and 48 hours. Biological samples (sample 1-6) are indicted by colour-coded dots as shown in the key. Significance was determined using Tukey's multiple comparison tests (**** $P < 0.0001$).

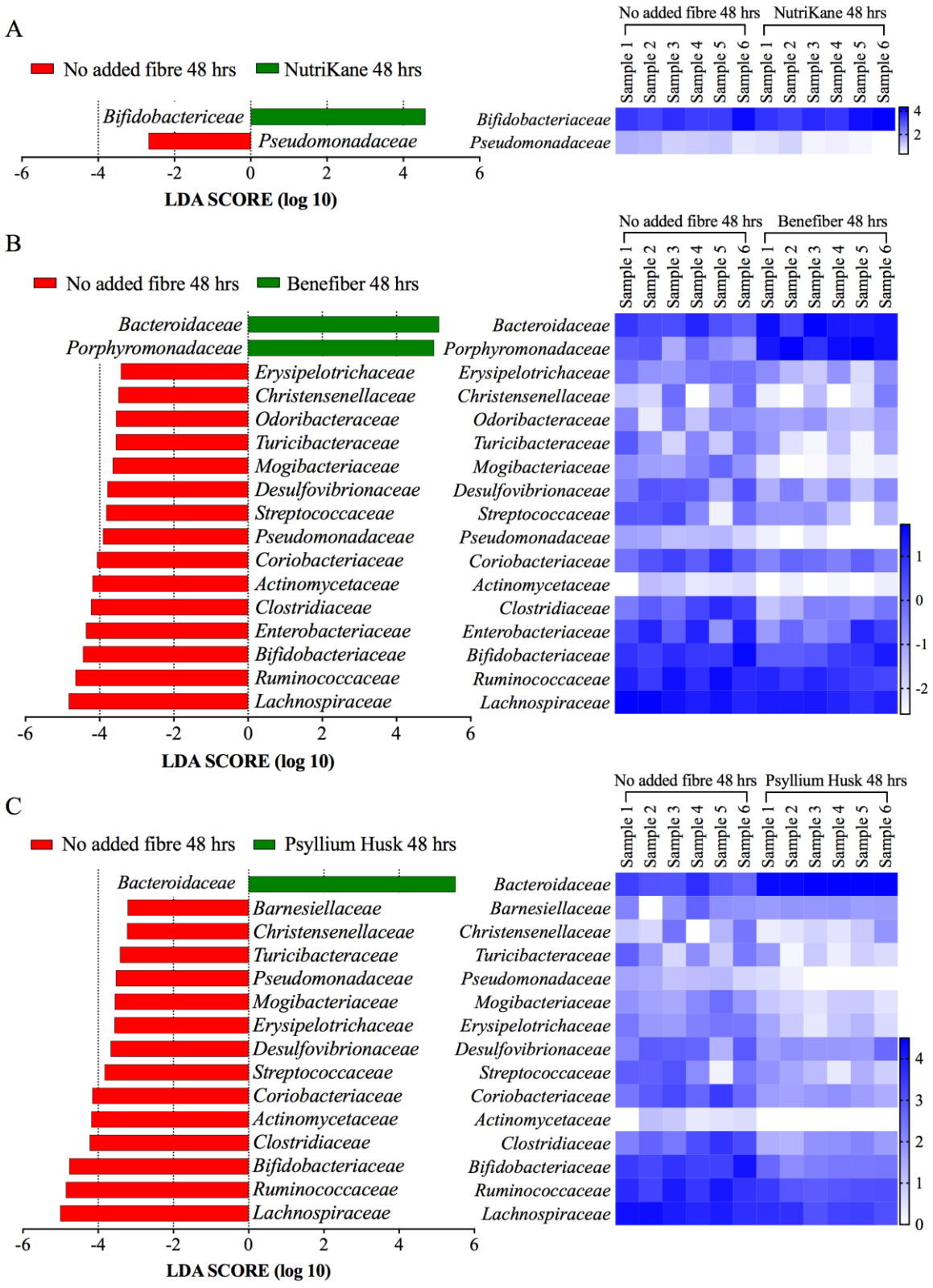

Figure 2.3 Key gut microbiota bacterial families that respond to fibre supplementation at 48 hours. Data was obtained using LEfSe analyses between **(A)** NutriKane vs no added fibre control, **(B)** Benefiber vs no added fibre control and **(C)** Psyllium husk vs no added fibre control. The left histogram 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.

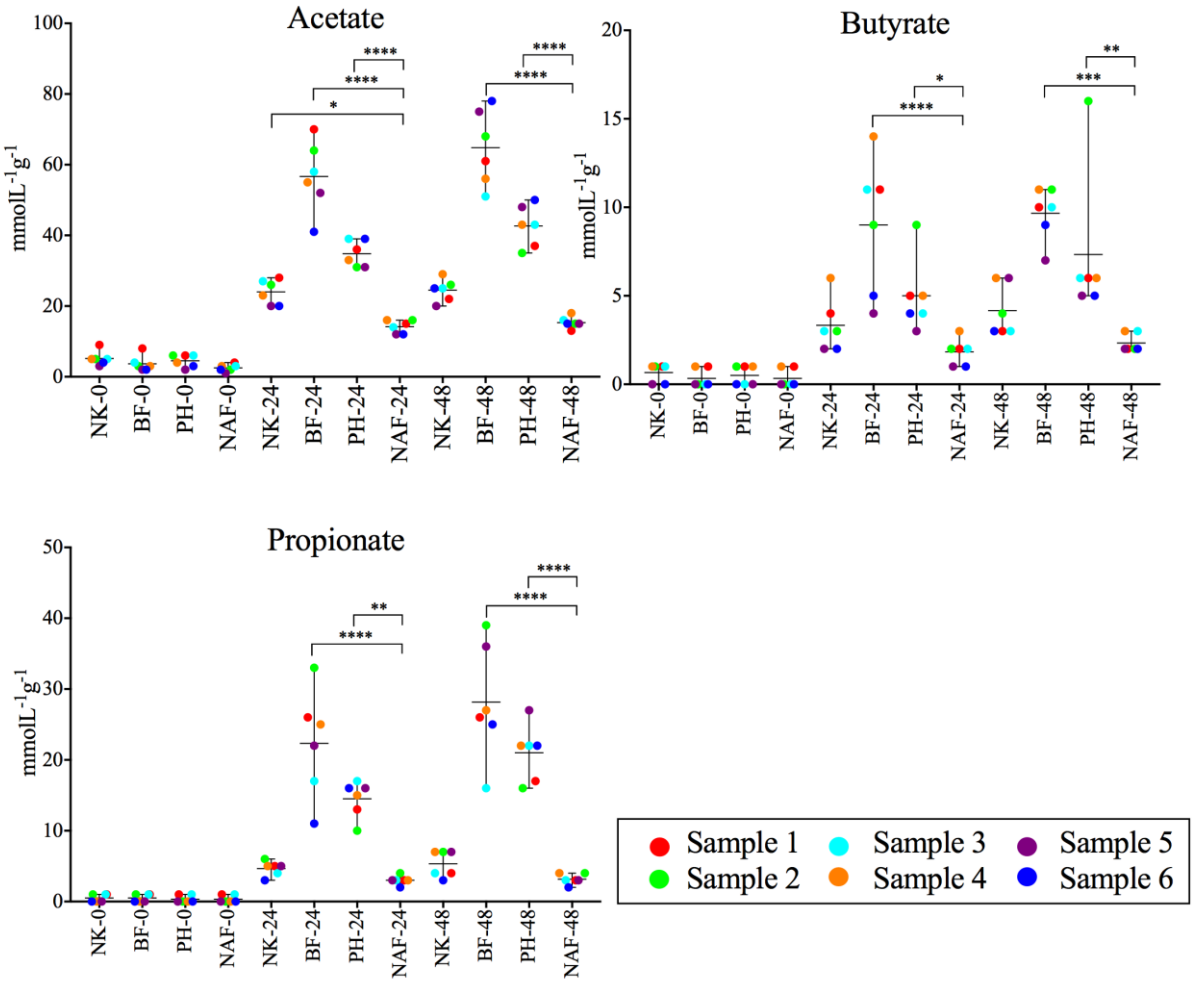

Figure 2.4 Average concentrations of acetate, butyrate and propionate in each sample at 0, 24 and 48 hours. Concentrations of all the SCFAs increased upon the fibre additions (NK- NutriKane, BF- Benefiber, PH- Psyllium husk) at 24 and 48 hours compared to the no added fibre control (NAF). The mean ± SD concentrations per treatment at 0, 24 and 48 hours are shown. The mean concentration for each biological sample (sample 1-6) is indicated by colour-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 Table S7.

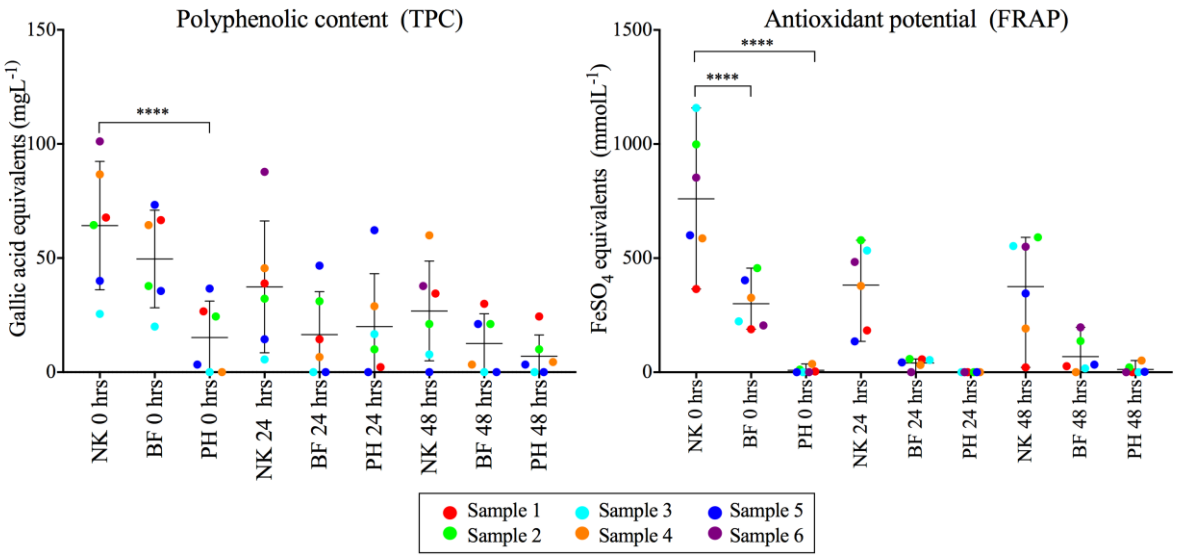

Figure 2.5 Comparison of the polyphenol content (mgL^{-1}) and antioxidant potential ($mmolL^{-1}$) of the fibre products (NK- NutriKane, BF- Benefiber, PH- Psyllium husk) in each sample at 0, 24 and 48 hours. Measurements for each fibre addition in each biological sample have been normalised against the no added fibre control at equivalent time points. Data is shown as mean ± SD for each treatment at 0, 24 and 48 hours. Biological samples are indicted by colour-coded dots as shown in the key. Significance was determined using Tukey's multiple comparison tests (**** $P < 0.0001$). The measured concentrations are provided in Table S8.

Table 2.1 The chemical composition and nutritional profile of fibre products. **(A)** The chemical composition of each fibre product determined as described in the Methods section. Values are expressed as g/100g 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.

A

Compound	NutriKane	Psyllium husk	Benefiber
Nitrogen	0.08 ± 0.01	0.24 ± 0.01	ND
Protein	0.54 ± 0.04	1.50 ± 0.09	ND
Fat	1.17 ± 0.09	0.46 ± 0.15	0.02 ± 0.04
Total dietary fibre	83.94 ± 0.60	77.24 ± 1.18	10.31 ± 1.19
Lignin	20.23 ± 1.08	4.69 ± 0.21	ND
Rhamnose	ND	2.39 ± 0.03	ND
Arabinose	5.83 ± 0.45	46.8 ± 0.49	1.27 ± 0.2
Xylose	31.8 ± 2.47	24.1 ± 0.25	0.19 ± 0.01
Mannose	1.54 ± 0.12	4.24 ± 0.04	21.3 ± 0.22
Glucose	17.3 ± 1.34	11.2 ± 0.12	74.5 ± 0.78
Galactose	0.74 ± 0.06	2.09 ± 0.16	ND

B

Fibre supplement	NutriKane	Benefiber	Psyllium husk
Ingredients	Sugarcane (sucrose removed) Pectin (from apple and citrus fruits)	100% wheat dextrin (derived from wheat)	100% organic psyllium husk
Dietary fibre content per 100 g	55.2 g	83 g (soluble fibre)	90 g
Nutritional information (average quantity per 100 g)	Energy 784 kJ Protein 0.8 g Fat total 0.1 g Saturated 0.1 g Carbohydrate 6.6 g Sugars 4.5 g Dietary fibre 55.2 g Sodium 15 mg Gluten ND Chromium 391 µg Potassium 5.7 g	Energy 913 kJ Protein Less than 1 g Fat total Less than 1 g Saturated Less than 1 g Carbohydrate 14.2 g Sugars Less than 1 g Dietary fibre (total) 83 g Sodium Less than 5 mg	Energy 759 kJ Protein 1.3 g Fat total Less than 1 g Saturated Less than 1 g Carbohydrate Less than 1 g Sugars Less than 1 g Dietary fibre 90.1 g Sodium 17 mg

2.10 Supplementary figures and tables

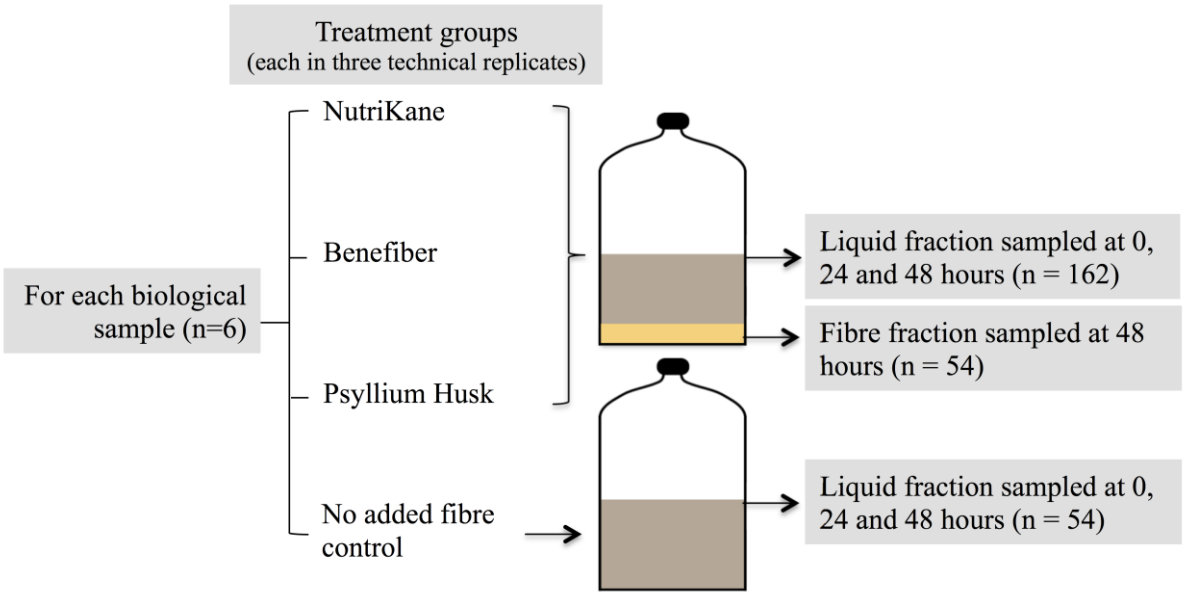

Figure S2.1 Experimental design. Fecal material obtained from six healthy adults as independent biological samples (n=6) were inoculated separately into the basal medium. For each biological sample, four treatments were applied, this included three fibre products (NutriKane, Benefiber and Psyllium husk) 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.

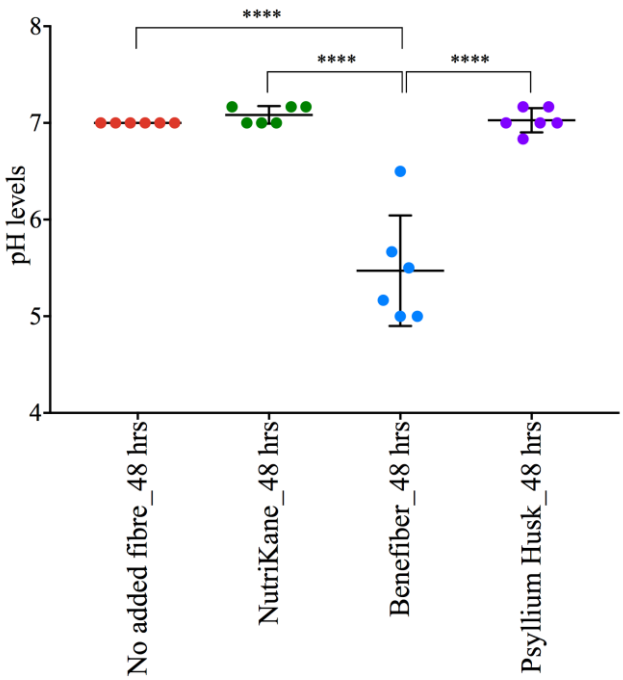

Figure S2.2 Measurements of pH for all cultures at 48 hours. Mean pH values for each of the three technical replicates in each of the six biological samples are indicated (dots). Bars represent the mean pH levels with ± SD for each treatment. Significance was determined using ANOVA with Tukey's multiple comparisons test (**** $P < 0.0001$).

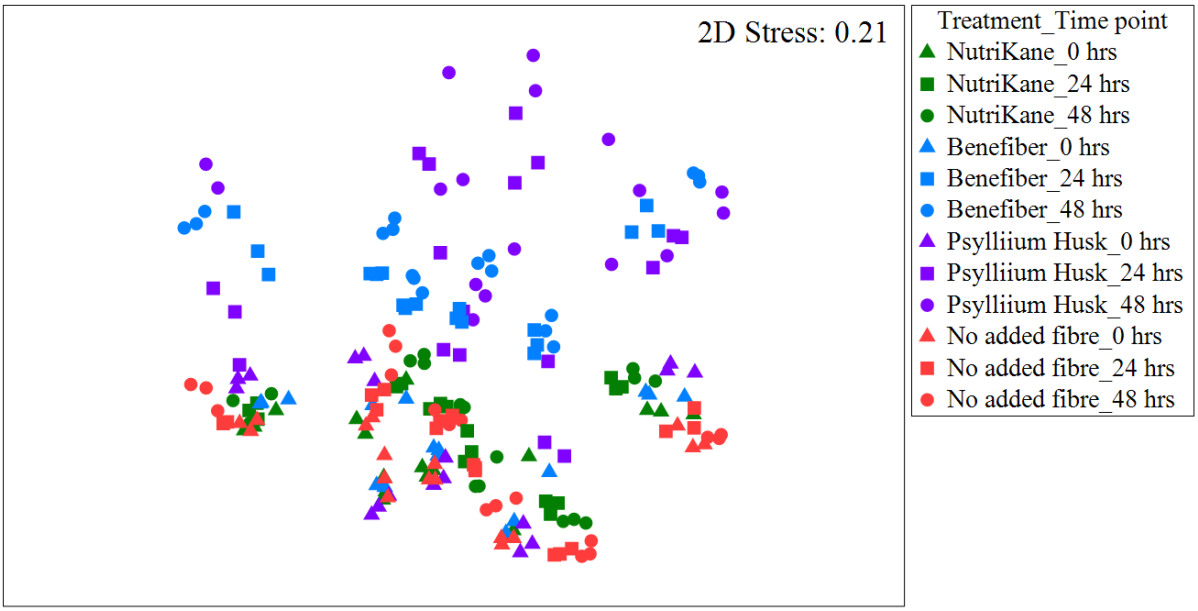

Figure S2.3 Bray-Curtis similarity based nMDS plot indicating the ordination of the gut microbiota at 0, 24 and 48 hours for all six individuals. All biological samples showed similar fibre-dependent shifts in the microbial community structure. Samples at 0 hours were grouped together independent of fibre addition. Fibre-dependent shifts were observed at 24 and 48 hours.

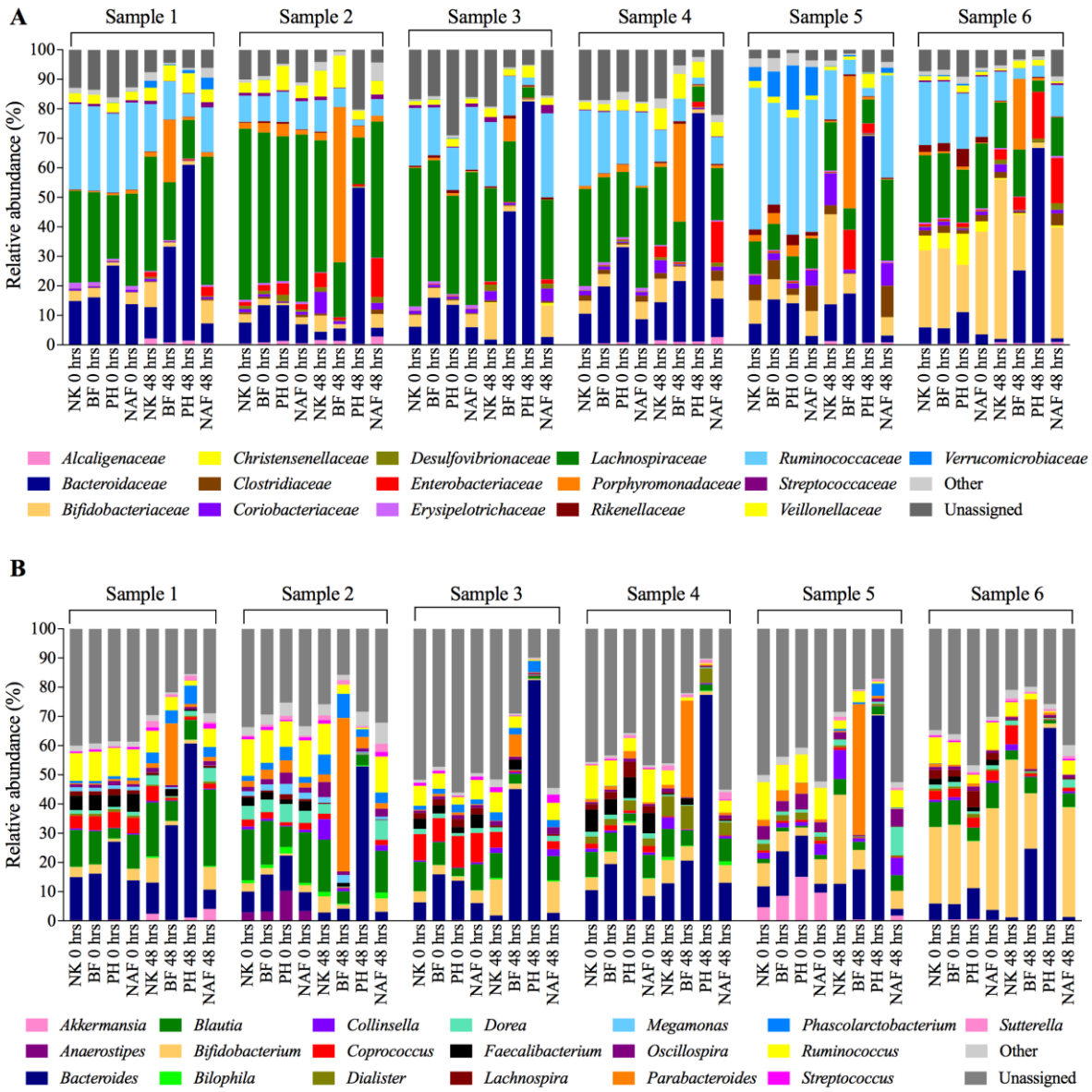

Figure S2.4 (A) Family and **(B)** genus level taxonomic composition of the microbial communities across treatments at 0 and 48 hours for each biological sample (Sample 1-6). The relative abundance of 16S rRNA gene amplicons in the families and genera were determined using QIIME and graphed using GraphPad 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.

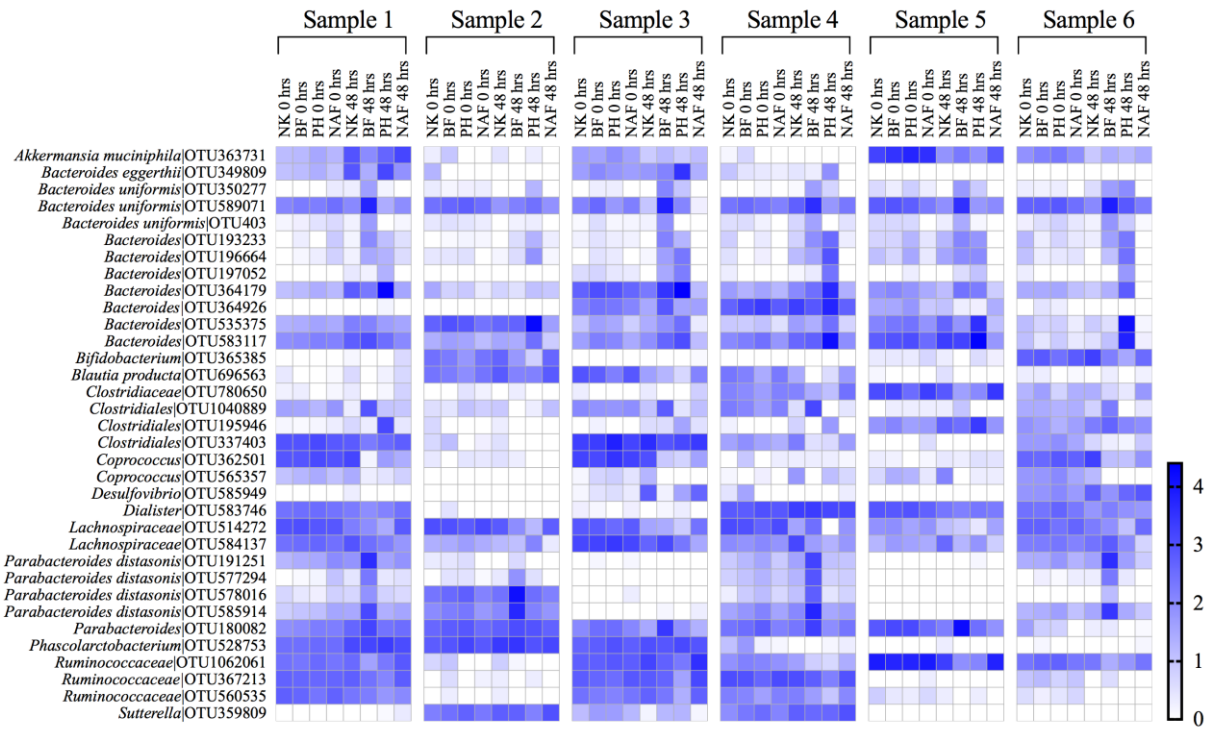

Figure S2.5 Fibre-dependent changes in the relative abundance of differentially abundant OTUs in each treatment at 0 and 48 hours. This subset of OTUs were shown to have different relative abundances in each fibre treatment at 48 hours compared to the no added fibre control at equivalent time points, 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 S2.2.

Figure S2.6 (provided on the CD) 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.

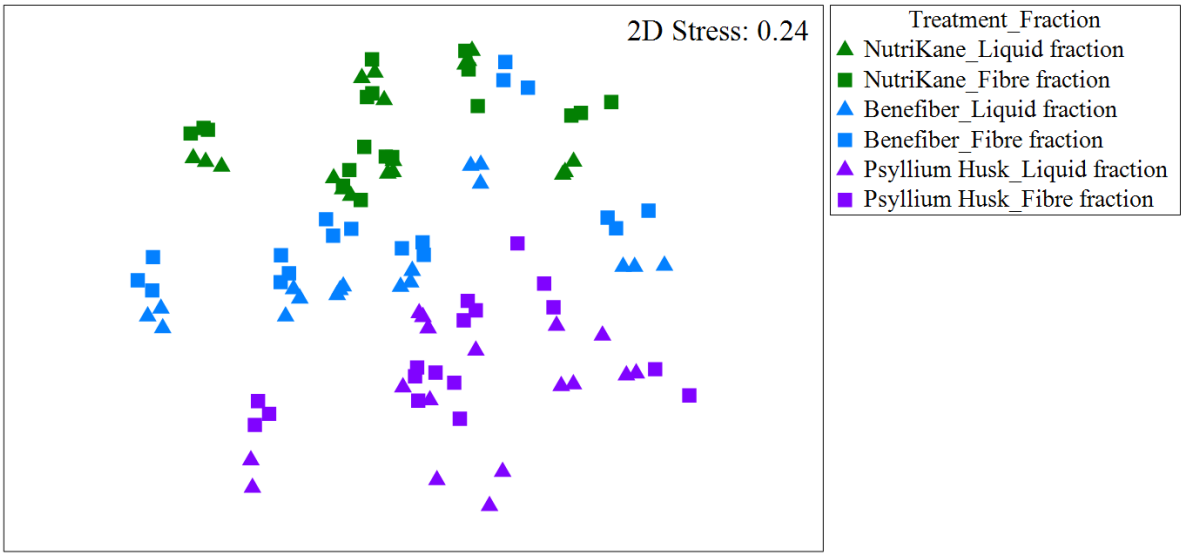

Figure S2.7 Bray-Curtis similarity based nMDS plot indicating the ordination of the fibre-adherent and liquid gut microbiota fractions. The community of bacteria adhered to the fibre material was compared to that of the liquid fraction. The community structure between the fibre and liquid fraction were similar in all fibre products.

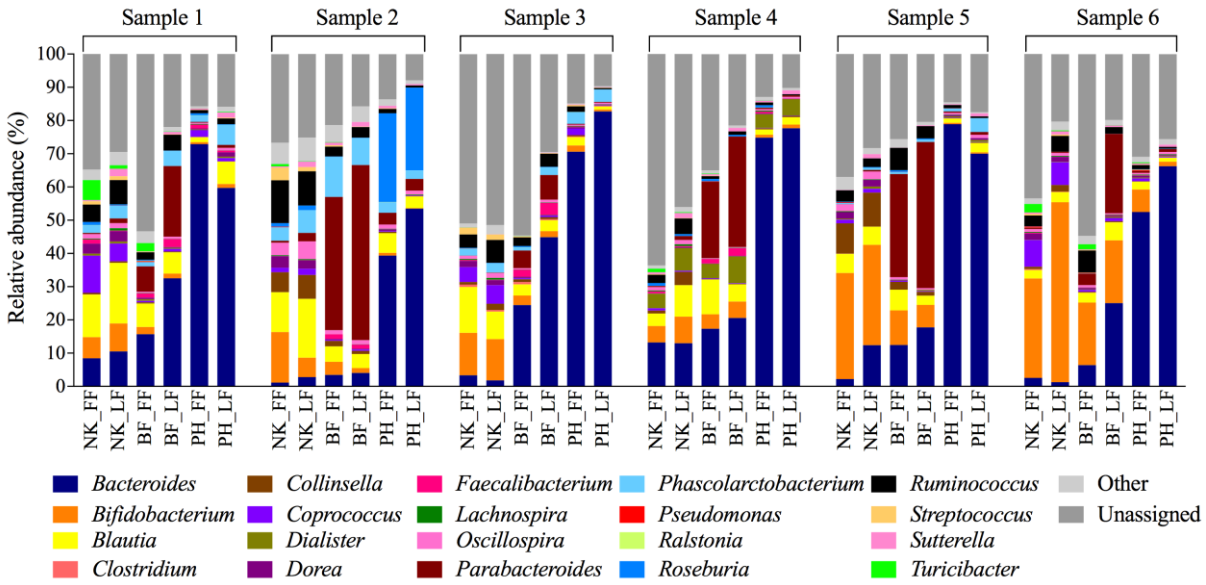

Figure S2.8 Differences in the bacterial relative abundance between the fibre fraction (FF) and liquid fraction (LF) microbiota at the genus level. Data is shown for each biological sample (sample 1-6) with each fibre product (NK- NutriKane, BF- Benefiber, PH- Psyllium husk) at 48 hours. Bacterial identifications 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".

Figure S2.9 (provided on the CD) 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 tables (provided on the CD)

Table S2.1 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.

Table S2.2 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.

Table S2.3 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.

Table S2.4 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).

Table S2.5 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.

Table S2.6 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).

Table S2.7 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.

Table S2.8 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.

CHAPTER 3

Effect of fibre supplementation on high fat diet-induced changes in the mouse gut microbiota

Title

Effect of fibre supplementation on high fat diet-induced changes in the mouse gut microbiota

Authors

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Running title - Fibre supplementation impacts mouse gut microbiota

Conflict of interest

MSB is an employee of Gratuk Technologies Pty Ltd, the producer of NutriKane™. All other authors have no conflict of interest.

3.1 Abstract

Dietary fibre supplementation is a possible strategy for therapeutic modulation of high fat diet-induced alterations in the gut microbiota. We fed mice a high fat diet for 17 weeks prior to supplementing with one of two commercially available dietary fibre products, NutriKane™ or Benefiber® for 15 weeks. We then investigated the effect of dietary supplementation on ameliorating high fat diet-induced changes in the gut microbiota, SCFA production and host physiological parameters. Analysis of the 16S rRNA gene amplicon sequencing data using a linear discriminant analysis effect size demonstrated significant fibre supplementation-mediated reductions in the abundance of the OTUs in *Clostridiaceae, Erysipelotrichaceae* and *Bacteroidaceae.* The relative abundance of the OTUs in the bacterial families *Lactobacillaceae, Lachnospiraceae, Porphyromonadaceae* and *Ruminococcaceae* increased in a fibre product-specific manner. Production of propionate increased significantly with only Benefiber addition, while, the production of acetate and butyrate was not significantly altered by fibre supplementation. However, neither fibre addition significantly impacted high fat diet-induced host physiological changes such as impaired glucose clearance, body weight gain or caecum mass.

Key words- Gut microbiota, high fat diet, dietary fibre supplementation, 16S rRNA gene and short chain fatty acids.

3.2 Introduction

The gut microbiota has profound effects on regulating the host physiology and inflammation. Accumulating evidence suggests a link between the dysbiosis of gut microbiota-host homeostasis and disease development [1]. Highly prevalent metabolic syndrome and concomitant health complications such as obesity, hyperlipidemia, hypertension, insulin resistance and diabetes mellitus (type 2) are associated with altered gut microbial composition and functions [2, 3].

Long- and short-term dietary habits heavily contribute to shaping the gut microbiota [4, 5] and play a key role in maintaining the host metabolism and immunity [6]. Complex polysaccharides that are non-digestible by human digestion are predominantly used as an energy source in the gut microbial ecosystem [7-9]. Microbial degradation of these dietary polysaccharides heavily impacts the composition, functions, spatial arrangement and diversity of the gut microbiota [10-12]. Degradation of complex polysaccharides/dietary fibre by the gut microbiota produces short chain fatty acids (SCFAs) such as acetate, propionate and butyrate. Apart from contributing up to 10% of the daily caloric requirement in the host [13], SCFAs regulate lipogenesis, gluconeogenesis and inflammation and modulate the secretion of hormones that impact the functions of the pancreas and release of insulin [6, 14]. Therefore, dietary fibre not only promotes the growth of microorganisms associated with complex carbohydrate digestion but also contributes to regulation of both host metabolism and the immune system.

Consumption of a high fat and low fibre diet has been linked to higher abundance of the phyla *Firmicutes* and *Proteobacteria* and lower abundance of *Bacteroidetes* in the gut microbiota [4, 15-17]. High fat diet-induced changes in the gut microbiota composition typically include an increase in the abundance of *Erysipelotrichaceae* and *Rikenellaceae,* and a decrease in *Lachnospiraceae* (formerly *Clostridium* cluster XIVa), *Lactobacillaceae, Ruminococcaceae* and *Prevotellaceae* [18-21]. Intake of high fat diets also generally lowers the gut microbial species diversity and gene richness [18, 22].

In addition to altering the composition, high fat feeding is related to functional changes in the gut microbiota. Feeding mice obesogenic diets has been associated with enrichment in phosphotransferase system (PTS) transporters, fructose and mannose catabolism and glycolysis/gluconeogenesis, whilst ABC transporters, bacterial chemotaxis and bacterial motility proteins were depleted [23, 24]. Corresponding changes have been observed in the production of bacterial metabolites, especially, lower concentrations of SCFAs with high fat

consumption [25, 26]. In addition, high fat intake typically increases the gut permeability and leads to inflammation, probably due to direct or indirect impact of the gut microbiota changes [27, 28].

The high prevalence of gut microbiota-associated diseases in the modern world can be linked to frequent perturbations to the gut microbiota and host symbiosis, especially through diets low in dietary fibre and high in fat [29, 30]. However, this relationship between the diet, gut microbiota and host may also indicate the potential for therapeutic modulation of high fat diet-induced gut microbiota dysbiosis [31]. Among the strategies that are currently in use for therapeutic modulation of the gut microbiota, dietary modulation is of high scientific and public interest [32, 33].

Many studies have investigated the potential for dietary modulation to alleviate high fat diet-induced complications. Addition of complex carbohydrates in the form of vegetables [34], fruits [35-37], dietary fibre [38-40], purified carbohydrates [41] and potential prebiotics [42] has been previously observed to modulate high fat diet-induced changes in the gut microbiota. For example, supplementation of high fat diet with a potential prebiotic, laminarin reduced the body weight gain, increased the abundance of carbohydrate active-enzymes, decreased the abundance of *Firmicutes* and increased the abundance of the genus *Bacteroides* in the gut microbiota of mice [42]. Kieffer *et al* reported an increase in relative abundance of the family *Rikenellaceae* and a decrease in *Lachnospiraceae* upon addition of high-amylose-maize resistant starch type 2 to a high fat diet [41].

Supplementation of a high fat diet with dietary fibre in purified or natural forms can increase the production of SCFAs [35, 43]. In addition, these supplementations can also alleviate certain high fat diet-induced complications in the host physiology and inflammation [36, 37]. Several dietary fibre supplementations have been linked to reduced body weight gain and lower plasma blood glucose levels [36-38]. However, several other studies have observed no

significant impact of dietary fibre supplementations on the body weight and host physiological parameters [34, 35, 42].

A popular and effective mode of dietary fibre supplementation frequently used by consumers is the intake of commercially available dietary fibre products [44]. Despite the availability of a variety of fibre supplements in the market, only limited work has been conducted to investigate the ability of these fibre products to mitigate high fat diet-induced effects on the gut microbiota and host. We investigated the impact of two commercially available fibre products, NutriKane™ and Benefiber® on alleviating high fat diet-induced changes in the gut microbiota, SCFAs and host physiology in mice.

3.3 Materials and methods

Animal trial and sample collection

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).

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^{o}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, produced by Speciality feeds, WA, Australia), mice were randomised into two groups based on the body weight at week 0. One group of mice (n=9) were fed the 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, produced by Speciality feeds, WA, Australia) 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. Group one (n=14) and two (n=13) were fed high fat diet with either NutriKane or Benefiber additions, respectively. The third group (n=14) was maintained on a high fat diet as a model control. Mice were maintained on these specific dietary groups for further 15 weeks (details of the experimental design are provided in Fig. S3.1).

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

Custom-made high fat diets with fibre additions contained 5% (w/w) of either NutriKane or Benefiber as a replacement of 5% (w/w) cellulose in the control high fat diet (produced by Speciality feeds, WA, Australia, compositions of the experimental diets and nutritional details of NutriKane and Benefiber are provided in Table 3.1). Normal chow, high fat diet, high fat diet supplemented with NutriKane and high fat diet supplemented with Benefiber will be referred to as NC, HF, HF-NK and HF-BF, respectively, here after.

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 group (week 0), before supplementation with the fibre products (week 17) and at two-time points after fibre supplementation (week 23 and 32). Fecal samples were stored at -80^{o}C prior to subsequent microbiota and metabolites analyses.

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.

All animals were anesthetised using 3% isofluorine (IsoFlo Isoflurane) with 0.5 L/min oxygen flow rate at week 32. Mice were euthanised by cervical dislocation. The liver, caecum, epididymal white adipose tissue and kidneys were excised and weighed with the content.

16S rRNA gene amplicons sequencing and bioinformatics analysis

Total community DNA was isolated from fecal 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 [45]. 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 [46, 47]. 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. [48]. Reads with high quality (-q 19) and full length were used to determine the Operational Taxonomic Units (OTUs) at 97% similarity using an open-reference protocol against the Greengenes (version 13_8) database [49]. 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.

Functional prediction using PICRUSt

The functional capacity of genes in each dietary group at week 0, 17, 23 and 32 were inferred from the 16S rRNA gene sequence data using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt, online galaxy version 1.1.0) [50]. All *de-novo* OTUs were removed from filtered and rarefied open-reference picked OTUs. The OTUs with Greengenes database (version 13_8) identifications were normalisation by the 16S rRNA gene copy number and used for analysis in PICRUSt. Functional groups were inferred using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology groups [51] at the third BRITE hierarchy level using PICRUSt.

Quantification of SCFAs

The concentration of SCFAs (acetate, propionate and butyrate) was quantified using fecal samples collected at week 17, 23 and 32. Approximately 20-50 mg of feces 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 fecal 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 fecal samples used for SCFA quantification.

Statistical analysis

Statistical analysis of the gut microbiota sequence data was conducted using PRIMER-7 software package [52, 53]. 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) and analysis of similarities (ANOSIM) were performed with 9999 permutations. PERMANOVA was conducted with pairwise tests and type III sums of squares. An ANOSIM R value closer to 1 indicates a higher separation between the groups, whilst R closer to 0 indicates a lower separation between the groups. The Shannon diversity index for each sample was also determined using PRIMER-7 software package.

Distinct phylotypes (bacterial phyla, families and OTUs) and the inferred functional pathways between dietary groups were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method (online Galaxy version 1.0) [54]. 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.

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.

The correlations between the relative abundance of the OTUs that responded to dietary changes and the Shannon diversity index, concentration of acetate, butyrate and propionate, the inferred relative abundance of functional pathways and physiological parameters (AUC of

IPGTT, caecum and epididymal white adipose tissue mass) were determined using Spearman's correlation analyses (two-tailed) on GraphPad Prism software (version 7). The significance levels of all statistical analyses are provided in the results section.

3.4 Results

To investigate the effect of dietary fibre supplementation on ameliorating high fat diet-induced gut microbiota changes, a group of mice (n=41) were initially fed a high fat diet (HF) for 17 weeks. Following HF feeding this group was randomised into HF (n=14), HF supplemented with NutriKane (HF-NK, n=14) and HF supplemented with Benefiber (HF-BF, n=13) dietary groups for a period of 15 weeks. A control group (n=9) fed a normal chow (NC) was maintained in parallel (Fig. S3.1). The effect of the fibre products on the gut microbiota, production of SCFAs and host physiological parameters were analysed using samples collected at week 0, 17, 23 and 32. The experimental design involved feeding mice a HF diet prior to fibre supplementation, resulting in development of high fat diet-induced changes in the gut microbiota and host physiology, before fibre-based dietary intervention. The goal of this was to determine if fibre supplementation could benefit individuals with pre-existing high fat diet-induced metabolic syndrome and alterations in the gut microbiota.

Overall structural changes of the gut microbiota

To examine the effect of different diets on the gut microbiota, the 16S rRNA gene amplicons were sequenced from fecal samples collected at week 0, 17, 23 and 32. A total of 18,527,820 reads were generated, after quality filtering and rarefaction 44,361 reads per each of the 200 samples were analysed.

Bray-Curtis similarity based nMDS plots were used to determine the effect of HF and dietary fibre additions on the gut microbiota structure (Fig. 3.1A). Observed variations were statistically confirmed using PERMANOVA and ANOSIM tests. NC fed mice showed significantly different ($P < 0.0001$, PERMANOVA and ANOSIM $R < 0.89$, $P < 0.0001$) gut

microbiota community structures at week 0, 17, 23 and 32 compared to mice fed any of the three high fat-based diets. Fibre supplementation using NutriKane or Benefiber did not significantly alter the high fat diet-induced state of the gut microbiota structure. Both HF-NK and HF-BF fed groups showed similar gut microbiota structures to the HF fed group and each other at week 23 and 32. Age-driven changes in the gut microbiota structure were insignificant, as all the samples from each dietary group clustered relatively close together, irrespective of the week of sampling.

The bacterial diversity of each sample was determined using a Shannon diversity index (Fig. 3.1B). The Shannon diversity indices of NC fed mice at week 0, 17, 23 and 32 were significantly higher ($P < 0.01$) compared to each of the three high fat based dietary groups. Fibre additions had no significant effect on high fat diet-induced reductions in the bacterial diversity at week 23 and 32.

Addition of fibre products altered the gut microbiota composition

The phyla *Bacteroidetes, Firmicutes, Actinobacteria*, *Verrucomicrobia* and *Proteobacteria* dominated the gut microbiota of all mice at week 0, 17, 23 and 32. However, the relative abundance of these phyla and *Firmicutes/Bacteroidetes* ratio significantly changed with HF feeding (Fig. S3.2). Most of the HF-induced changes in the gut microbial composition at the phylum level remained unchanged upon addition of the fibre products, except for the relative abundance of *Proteobacteria*, which significantly reduced with both HF-NK and HF-BF diets (Fig. S3.2 B and C).

Bacterial families that responded to high fat and fibre-based dietary interventions at week 32 were determined using LEfSe analyses between NC vs HF, HF vs HF-NK and HF vs HF-BF groups (Fig. 3.2, Table S3.1). The relative abundance of the families S24-7, *Lachnospiraceae, Paraprevotellaceae* and *Prevotellaceae* were significantly higher in the NC fed group, whilst the abundance of *Erysipelotrichaceae, Bacteroidaceae, Alcaligenaceae, Clostridiaceae* and

Verrucomicrobiaceae was higher in the HF fed group (Fig 3.2A). Both fibre additions significantly altered some of these HF-induced changes in the gut microbiota. The relative abundance of *Clostridiaceae* and *Alcaligenaceae* was lower in the HF-NK and HF-BF groups compared to the HF group. The relative abundance of *Bacteroidaceae* reduced, whilst the abundance of *Porphyromonadaceae* and *Rikenellaceae* further increased in the HF-BF group compared to the HF group (Fig. 3.2 B and C).

The OTUs that showed statistically significant changes in abundance between diets were identified through examination of the prokaryotic relative abundance using LEfSe. (Fig. 3.3, Table S3.2). These analyses were used to examine the impact of HF feeding (HF vs NC) and fibre supplementations (HF vs HF-NK/HF-BF). HF feeding altered the relative abundance of 80 OTUs, of which the abundance of 55 OTUs reduced and 25 OTUs increased compared to NC feeding.

Dietary fibre supplementation with NutriKane and Benefiber increased or reduced the abundance of 12 and 14 OTUs, respectively. The abundance of three OTUs (*Clostridiaceae*-OTU166, OTU591223 and *Erysipelotrichaceae*- OTU4379961) significantly decreased upon both fibre additions, these were significantly higher in the HF group compared to the NC group. HF-induced changes in the abundance of another three OTUs significantly changed only with HF-NK feeding. These included significant reductions in the relative abundance of *Bacteroidaceae*-OTU583117 and *Erysipelotrichaceae*-OTU1105860 and increase in the relative abundance of *Clostridiales*-OTU1106614 upon HF-NK feeding.

In comparison to the HF group, HF-BF feeding significantly increased the relative abundance of two OTUs in *Porphyromonadaceae* (OTU276149 and OTU578016), these OTUs had a significantly higher abundance in the HF fed group compared to the NC group.

The relative abundance of another six OTUs increased only in the HF-NK fed group, while the HF-BF diet resulted in an increase in the abundance of nine OTUs, which were all not

significantly altered by any other diet. The six OTUs that were more abundant only upon HF-NK feeding were in the order *Clostridiales* and families *Lactobacillaceae, Lachnospiraceae, Ruminococcaceae* and *Erysipelotrichaceae.* HF-BF feeding uniquely enhanced the abundance of nine OTUs within the families *Porphyromonadaceae, Rikenellaceae, Lactobacillaceae, Ruminococcaceae, Lachnospiraceae, Alcaliganaceae* and *Desulfovibrionaceae.*

Changes in the concentrations of SCFA in response to fibre additions

To investigate the effect of different diets on the production of SCFAs, concentrations of acetate, butyrate and propionate were measured from the fecal samples collected at week 17, 23 and 32 (Fig. 3.4, Table S3.3). HF feeding significantly reduced the concentrations of all three tested SCFAs, this was consistently observed at week 17, 23 and 32. None of the fibre additions significantly mitigated HF-induced reductions in the concentrations of SCFAs except for HF-BF, which significantly increased the production of propionate at week 23 and 32.

The correlation between the concentration of SCFAs and the relative abundance of the bacterial OTUs were determined using a Spearman's correlation (Fig. S3.3). The concentration of all three SCFAs showed positive correlations with the relative abundance of OTUs in the families S24-7, *Lachnospiraceae* and the order *Clostridiales* (Spearman's $r > 0.4$, $P < 0.0001$). Negative correlations were observed with the relative abundance of some OTUs in *Erysipelotrichaceae, Bacteroidaceae* and *Ruminococcaceae* (Spearman's $r < -0.4$, $P < 0.0001$). The concentration of acetate and butyrate correlated with the relative abundance of the OTU997439 (*Bifidobacteriaceae*), specific OTUs in the family S24-7 and the order *Clostridiales* (Spearman's $r > 0.4$, $P < 0.0001$). The concentration of propionate showed a positive correlation with the relative abundance of the OTU578016 (*Porphyromonadaceae*, Spearman's $r = 0.4$, $P < 0.0001$).

Predicted functional changes in response to fibre additions

To examine the effect of fibre additions on HF-induced changes in the gut microbiota functions, KEGG Orthology pathways in each dietary group at week 0, 17, 23 and 32 were inferred from the 16S rRNA gene abundances using PICRUSt. We then used LEfSe to determine the key inferred functional pathways that responded to different diets (Fig. 3.5). HF feeding altered the inferred relative abundance of nine functional pathways compared to the NC fed group. The inferred relative abundance of functional pathways for amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism and phosphotransferase system (PTS) transporters increased with HF feeding. The inferred abundance of functional pathways for bacterial chemotaxis, bacterial motility proteins, flagellar assembly, sporulation and methane metabolism was predicted to have decreased upon HF feeding.

NutriKane addition significantly reduced the inferred relative abundance of functional pathways for PTS transporters, which was highly abundant in the HF fed group. The inferred relative abundance for two-component systems also significantly increased with NutriKane addition. No significant changes in the relative abundance of functional pathways were observed in the HF-BF group compared to the HF fed group.

The correlations between the relative abundance of the OTUs and inferred relative abundance of KEGG Orthology functional pathways were determined using a Spearman's Correlation (Fig. S3.3). The relative abundance of the OTUs in *Erysipelotrichaceae* showed a strong negative correlation (Spearman's $r < -0.7$, $P < 0.0001$) with the inferred relative abundance of functional pathways for bacterial chemotaxis, bacterial motility proteins and flagellar assembly. Strong positive correlations were observed between the relative abundance of the OTUs in S24-7, *Clostridiales* and *Lachnospiraceae* and inferred abundance of methane metabolism (Spearman's $r > 0.7$, $P < 0.0001$). The inferred relative abundance of methane metabolism also showed positive correlations with the concentration of butyrate and acetate (Spearman's $r > 0.2$, $P < 0.0001$). Upon examination of the correlations between the inferred

relative abundance of the functional pathways, a strong positive correlation was observed between the PTS transporter systems and fructose and mannose metabolism (Spearman's r = 0.98, $P < 0.0001$).

Diet driven variations in feed intake and physiological responses in mice

Compared to NC fed mice, HF fed mice developed impaired glucose clearance, which was determined using an IPGTT at week 17 (Fig. 3.6A and B). Fibre supplementation did not significantly improve the impaired glucose tolerance in HF fed mice at week 23 (Fig. 3.6C and D). The AUC of IPGTT at week 17 and 23 positively correlated with the abundance of the OTUs in *Clostridiales* and *Erysipelotrichaceae* (Spearman's $r > 0.5$, $P < 0.0001$), whilst negatively correlated with the abundance of many OTUs in *Prevotellaceae,* S24-7, *Clostridiales* and *Lachnospiraceae* (Spearman's $r < -0.5$, $P < 0.0001$).

The feed intake of the three high fat-based diets was similar to each other but was significantly lower ($P < 0.0001$) compared to the NC group at all time points (Fig. S3.4A). However, weekly energy intake was similar between all dietary groups (Fig. S3.4B). In accordance with the similarities in energy intake, the average body weight was similar between HF and NC fed mice. Fibre supplementation driven alterations to the body weight were insignificant (Fig. S3.4C).

The caecum masses of NC fed mice were significantly higher compared to the HF and HF-NK groups at week 32 (Fig. S3.5). The mass of the epididymal white adipose tissues was higher in the HF, HF-NK and HF-BF fed groups compared to the NC fed group, while in each dietary treatment two significantly different groups were observed due to the higher degree of individual variation *(*$P < 0.05$). The liver and kidney masses were similar between the four groups.

3.5 Discussion

Therapeutic modulation of the gut microbiota through dietary fibre supplementation could hold potential in alleviating diet-induced health problems. While commercially available dietary fibre supplements are in popular customer demand for this purpose, only limited work has been conducted to investigate the effect of such products on alleviating high fat diet-induced changes in the gut microbiota. In this work, two commercially available fibre supplements were tested individually for their ability to alleviate high fat diet-induced changes in the gut microbiota and physiology of mice. The relative abundances of the OTUs in *Clostridiaceae, Erysipelotrichaceae* and *Bacteroidaceae* significantly changed upon supplementation with either NutriKane or Benefiber, in comparison to HF feeding. Both fibre products also altered the relative abundance of *Lactobacillaceae, Lachnospiraceae* and *Ruminococcaceae*, which are all associated with fibre digestion.

We observed significant HF feeding-induced alterations in the gut microbiota and host physiology of mice compared to the NC fed group. These included shifts in the overall gut microbiota structure, microbial composition, lower microbial diversity, lower concentration of SCFAs and changes in the host physiological parameters such as impaired glucose clearance, lower caecum mass and higher epididymal white adipose tissue mass. The most significant HF-induced gut microbiota alterations were observed in the abundance of the OTUs in the families S24-7, *Lachnospiraceae, Prevotellaceae* and order *Clostridiales*, which decreased with HF feeding and the abundance of the OTUs in *Erysipelotrichaceae, Bacteroidaceae, Verrucomicrobiaceae, Alcaligenaceae* and *Coriobacteriaceae*, which increased upon HF feeding. These HF-induced changes in the gut microbiota, host physiology and the correlation between the gut microbiota changes with physiological parameters are consistent with several previous studies [17-19, 21, 39, 55-61].

Both NutriKane and Benefiber additions significantly reduced the relative abundance of the phylum *Proteobacteria*. This phylum has been identified as a potential diagnostic signature of gut microbiota dysbiosis [58]. Therefore, the decrease in the abundance of *Proteobacteria* may indicate the potential of the two fibre products to alleviate high fat diet-induced changes in the gut microbiota. In addition, we also observed fibre addition-mediated significant differences in the relative abundance of 6 OTUs, which are in the families *Clostridiaceae*, *Erysipelotrichaceae* and *Bacteroidaceae.* These bacterial families are typically abundant in HF fed mice [55-57], and a decrease in the abundance of these groups may lead to improved gut microbiota health.

A large proportion of the OTUs that were significantly differentially abundant upon NutriKane or Benefiber additions were fibre product-specific. The relative abundance of potential fibre-digesting bacterial OTUs in the families *Lactobacillaceae* (genus *Lactobacillus*), *Lachnospiraceae* (genus *Coprococcus*), *Ruminococcaceae* (genus *Oscillospira*) and some members in the order *Clostridiales* increased with HF-NK feeding. Whereas the relative abundance of a different set OTUs in the families *Porphyromonadaceae* (genus *Parabacteroides*), *Lactobacillaceae* (genus *Lactobacillus*), *Ruminococcaceae* and *Lachnospiraceae* (genus *Coprococcus*) was significantly higher upon HF-BF feeding. Members of these families have been associated with fibre digestion [7, 9, 62-64], therefore, may flourish with the addition of dietary fibre rich NutriKane and Benefiber. NutriKane supplementation appears to promote the growth of fibre-digesting OTUs in the phylum *Firmicutes*, whereas Benefiber mostly enhanced the growth of fibre-digesting members of *Bacteroidetes.* This could be linked to the higher proportion of insoluble fibre in NutriKane compared to Benefiber. These observations are largely in agreement with the preference of *Firmicutes* and *Bacteroidetes* to digest insoluble and soluble fibre, respectively [65-67].

We observed fibre product-specific changes in the abundance of potential fibre-digesting bacteria, which could be due to the distinct chemical properties of the two fibre products and

availability of bioactive components such as polyphenols [68]. NutriKane, which is derived from dried whole sugarcane stem and pectin is rich in cellulose, hemicellulose and lignin [69-71], and therefore, may contain a range of β-1, 4 and α-1, 4 linkages between glucose, xyloglucans, xylans, glucomannan, arabinoxylan, glucuronoxylan and D-galacturonic acid [72-75]. In comparison, Benefiber is derived from wheat dextrin and 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 [76, 77]. The changes in the gut microbiota composition observed in this study are consistent with published data on the ability of members of the family *Porphyromonadaceae* to digest chemically modified starch [78], and members of the families *Ruminococcaceae* and *Lachnospiraceae* to digest starch as well as cellulose, hemicellulose and pectin [7, 79].

The production of all three SCFAs was significantly affected by HF-feeding. While most of the HF-induced changes in the SCFA production remained unchanged with fibre supplementation, HF-BF addition significantly increased the production of propionate in comparison to HF and HF-NK feeding. The correlation between the concentration of propionate and the relative abundance of OTUs in *Porphyromonadaceae* is consistent with the ability of the members of this family to produce propionate [80]. Propionate has been shown to impact gluconeogenesis and production of gut hormones that reduce the appetite [81, 82]. However, while HF-BF feeding promoted the production of propionate no significant attenuations to HF-induced impaired glucose clearance or feed intake were observed.

The inferred relative abundance of the functional pathways for fructose and mannose metabolism and PTS transporters increased with HF feeding. The strong positive correlation between these two functional pathways is in agreement with the preference of the PTS transporter to uptake a variety of simple sugars over complex carbohydrates [83]. Bacterial PTS transporters are essential for carbohydrate scavenging during limited availability [84],

which may explain the higher abundance of this functional pathway upon HF feeding in comparison to NC, as NC has a higher carbohydrate content. The gut microbiota of HF fed mice also showed a higher inferred abundance for amino sugar and nucleotide sugar metabolism, which has been previously linked with type 2 diabetes and metabolic syndrome [85]. Previous metagenomic studies have also reported on HF-induced higher abundance of these functional pathways [5, 23, 86, 87]. Although most of these inferred changes in the gut microbiome remained unchanged upon supplementation with the fibre products, HF-NK significantly reduced the HF-induced increase in the abundance of the PTS. This could be explained by the higher availability of complex carbohydrates in NutriKane compared to cellulose in the HF diet or dextrin in Benefiber.

The inferred abundance of functional pathways for bacterial chemotaxis, bacterial motility proteins and flagellar assembly reduced upon HF feeding. The abundance of these three functional pathways also showed strong negative correlations with the relative abundance of *Erysipelotrichaceae,* which could be attributed to the lack of motility in most of the members of this family [23, 88]. The inferred abundance of methane metabolism also decreased with HF-feeding and showed strong positive correlations with the concentration of butyrate and acetate. This is consistent with the gut microbial conversion of H_2, a by-product of bacterial SCFA production to methane [89]. These high fat diet-induced alterations have been previously observed in multiple metagenomics studies [23, 90, 91].

Inferred functional pathways for two-component systems were highly abundant in HF-NK group compared to HF group. Two-component systems allow prokaryotic cells to adopt to new environmental conditions under selective pressure [92]. Lower abundance of the two-component systems has been previously observed in mice fed a high fat and high sugar diet [23]. While further metagenomic studies are essential, current observations of an increase in the abundance of these systems with NutriKane addition may be indicative of a beneficial alteration in the microbial community function. While both HF-NK and HF-BF changed the

abundance of several OTUs, there were no significant changes in PICRUSt predicted functional pathways in HF-BF compared to HF fed mice.

Despite having significant effects on the abundance of specific gut bacteria and SCFA production, the fibre additions did not significantly alter HF-mediated changes in the gut microbiota species diversity, host glucose tolerance and other tested host physiological parameters. Previous studies have also observed limited effect of dietary supplementation on alleviating high fat diet-induced changes in the host physiology [35, 41, 42, 93]. Dietary supplementation with wheat dextrin has been previously reported to lower caloric intake [94] but have no significant effect on blood glucose levels in humans [76, 95]. Supplementation of a high fat diet with sugarcane fibre lowered the total body fat mass in mice and improved insulin sensitivity [38]. However, none of these studies examined the effect of wheat dextrin or sugarcane supplementation on the host gut microbiota.

The high fat diet regimen tested here resulted in many host physiological alterations that were not significantly ameliorated by the subsequent inclusion of NutriKane and Benefiber as part of the fibre component of the diet. Alterations in experimental design could be used to further investigate the capability of these fibre products to mitigate high fat diet-induced changes in the gut microbiota with corresponding host physiological changes. For example, different periods of exposure, altered fat content in the HF diets, increased fibre content from the supplementation or changing other experimental parameters may impact observed host outcomes.

3.6 Conclusion

This study showed that NutriKane and Benefiber supplementation of a high fat diet resulted in specific changes to the mouse gut microbial community composition. High fat diet-induced increase in the relative abundance of the OTUs within *Erysipelotrichaceae, Clostridiales* and *Bacteroidaceae* was significantly reduced with NutriKane and Benefiber supplementation.

Addition of both fibre products also promoted the growth of the beneficial *Lactobacillaceae* and other fibre-digesting bacteria. The increase in the abundance of specific potential fibre-digesting bacteria was product-dependent, potentially due to distinct variations in the chemical structure of the fibre within NutriKane and Benefiber. Corresponding changes in the production of SCFAs were observed only with HF-BF feeding. Given the large number of fibre supplement products coming to market further investigation into the ability of these products to alter gut microbiota composition and improve host health remains important.

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3.8 Acknowledgments

This work was supported under the Australian Research Council's Industrial Transformation Training Centre funding scheme, through funding the centre for Molecular technology in the food industry (IC130100009). We thank Mamdouh Nasseim, Ella Glover, Wilson Wong, Dr Mugdha Joglekar and Dr Andrzej Januszewski at the NHMRC Clinical Trials Centre, Sydney Medical School, The University of Sydney, Australia for providing technical support. We also acknowledge the industry partner of this project, Gratuk Technologies Pty Ltd, Sydney, Australia.

3.9 Figures and tables

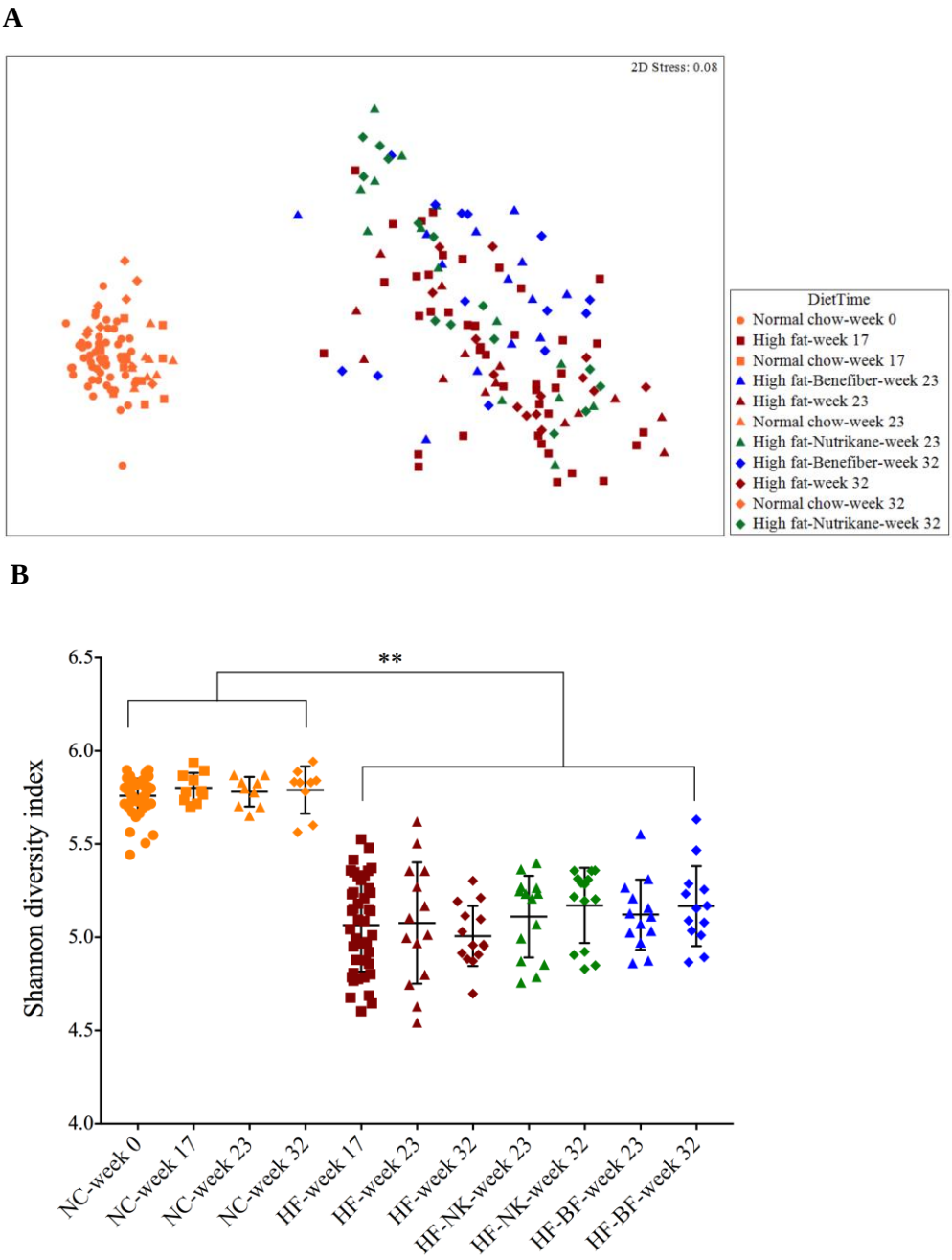

Figure 3.1 Ordination and microbial diversity of the gut microbiota in mice fed each of the four diets at weeks 0, 17, 23 and 32. **(A)** Ordination of the gut microbiota shown as a Bray-Curtis similarity of the relative abundance based nMDS plot. **(B)** Gut microbial diversity shown as Shannon diversity indices. Significance was determined using Mann-Whitney test. ** ($P < 0.01$) denotes significantly different Shannon diversity indices in the three high fat based groups compared to the normal chow group at week 0, 17, 23 and 32. Mean values with ± SD are shown.

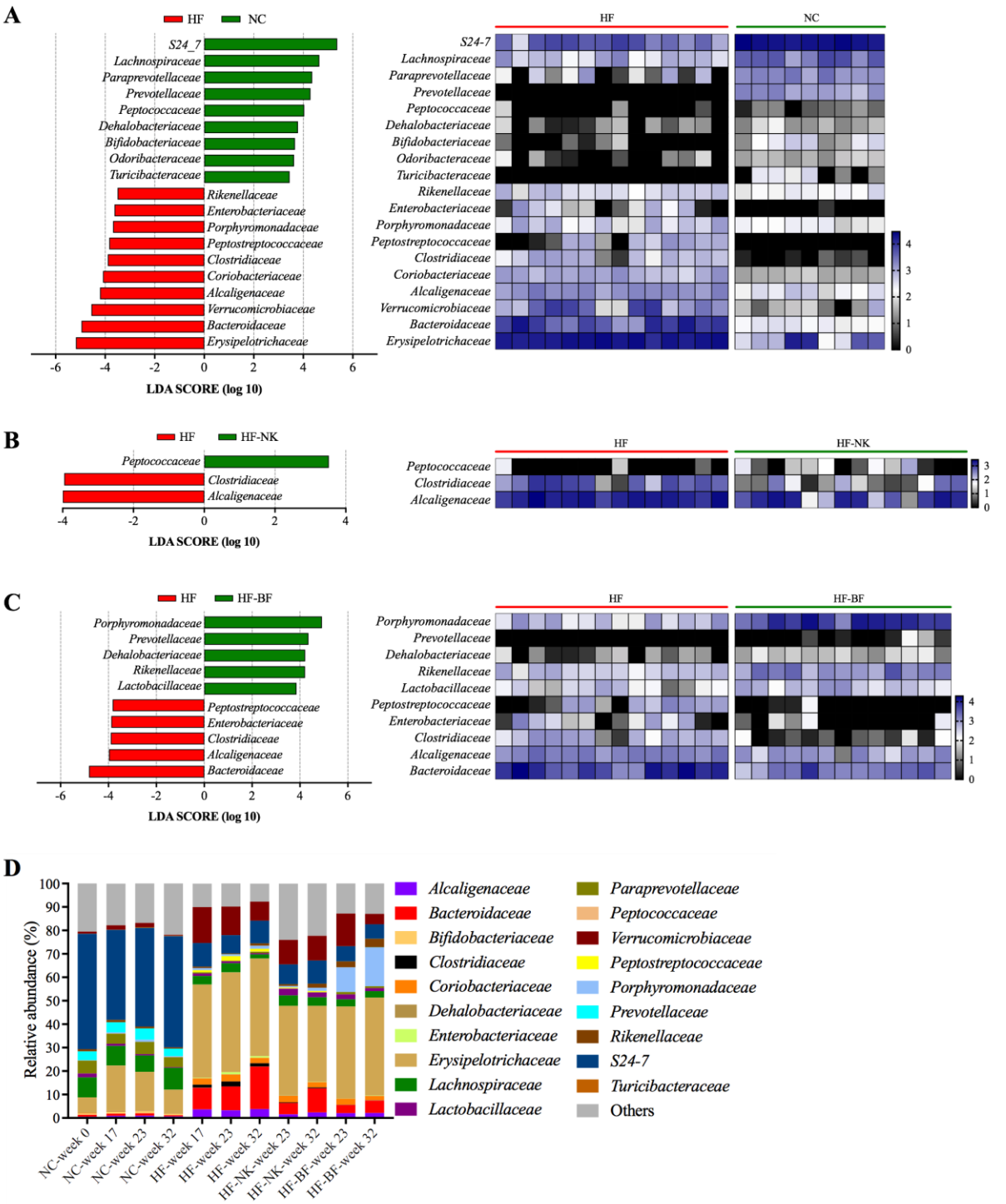

Figure 3.2 Key bacterial families of the gut microbiota responding to dietary changes at week 32. Data was obtained using LEfSe analyses between **(A)** HF vs NC, **(B)** HF vs HF-NK and **(C)** HF vs HF-BF groups. The left histogram shows the LDA scores computed for each bacterial family and the right 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. The relative abundance of these families are provided in Table S3.1.

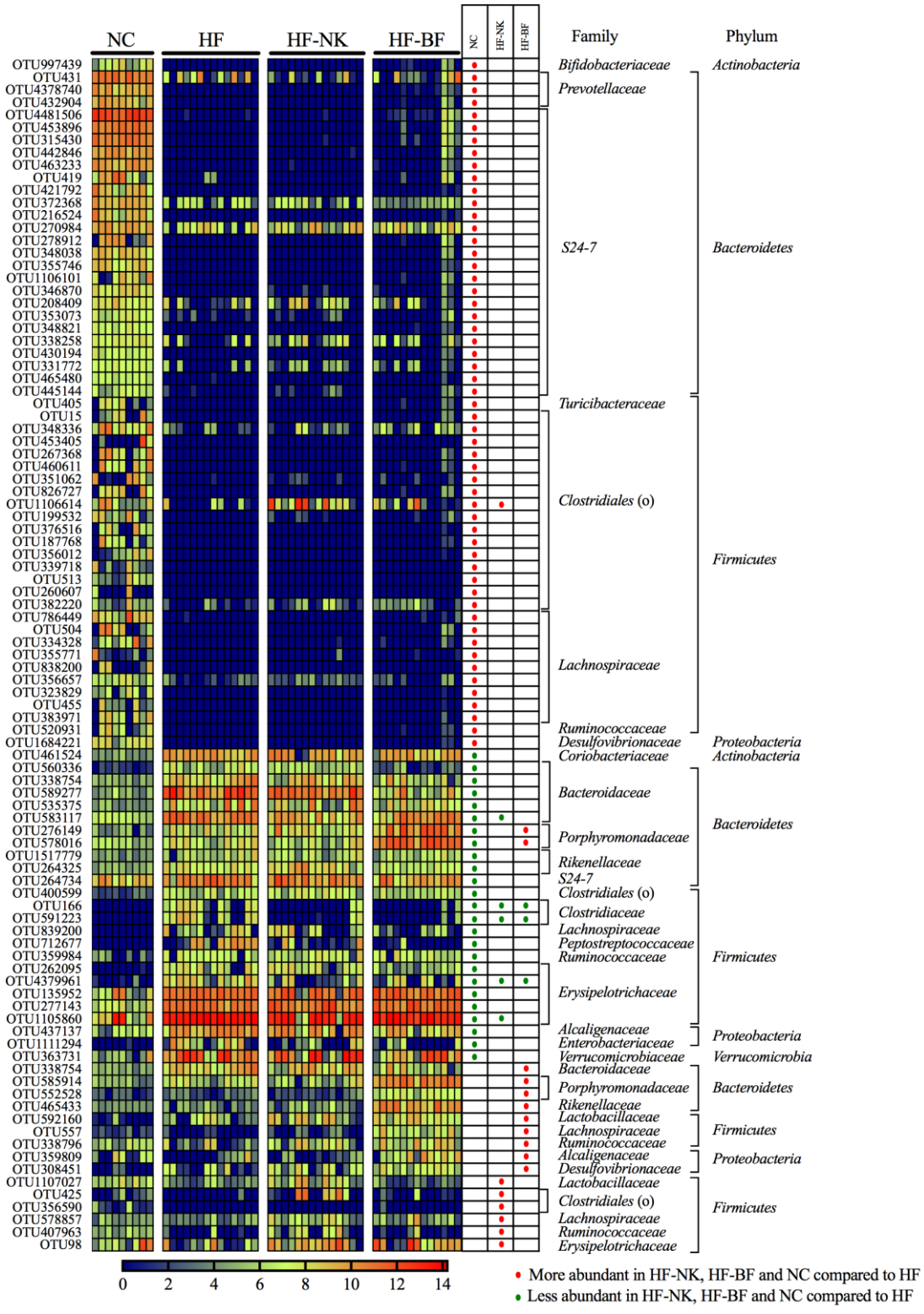

Figure 3.3 The relative abundance (Log_{10} transformation) of the OTUs that were significantly different between dietary groups, shown per mouse. Data was obtained through LEfSe analyses between HF vs NC, HF vs HF-NK and HF vs HF-BF groups. Rows in the heat map correspond to the abundance of the OTUs 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.

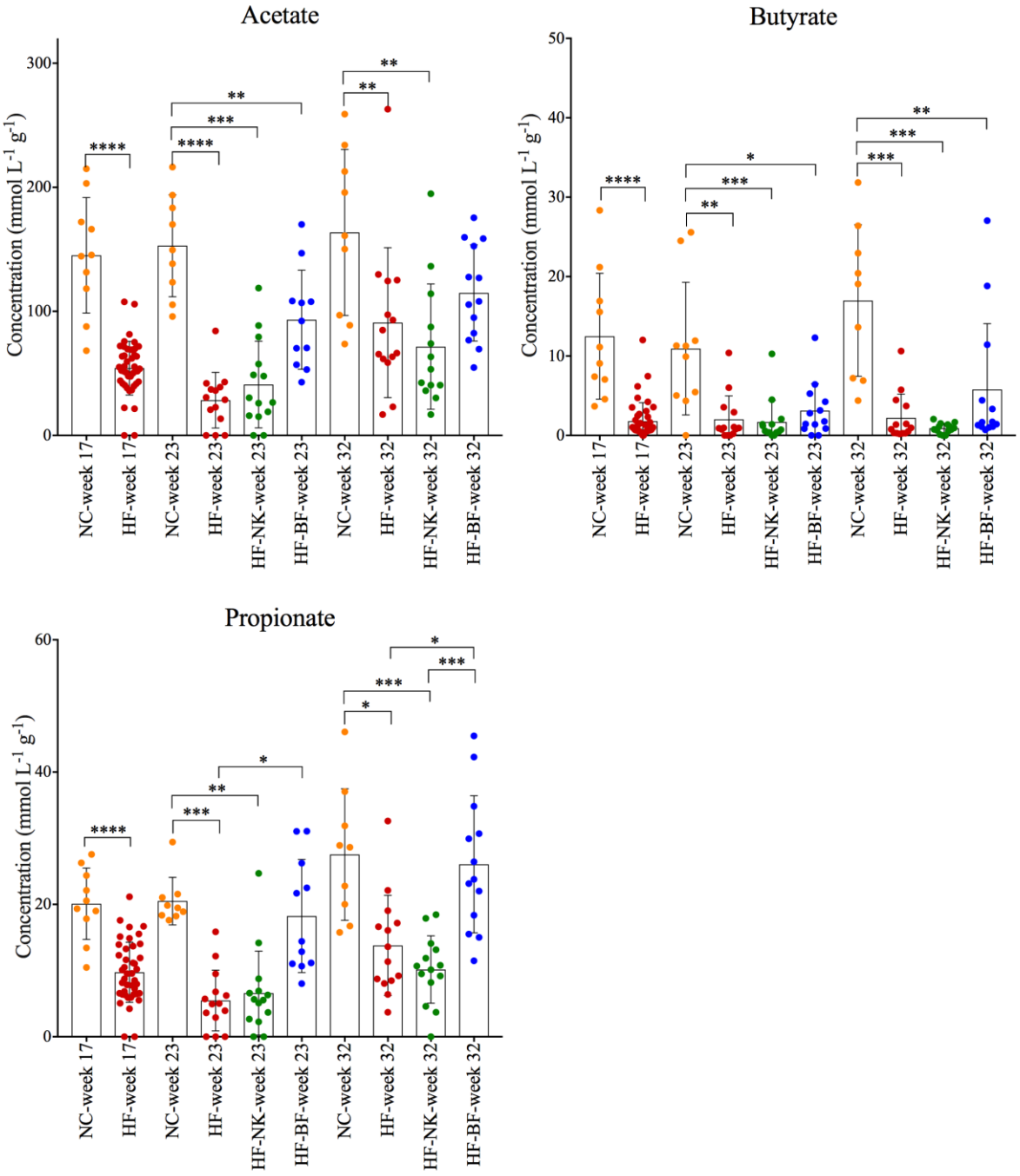

Figure 3.4 Concentration (mmol$L^{-1}g^{-1}$) of acetate, butyrate and propionate per mouse in each dietary group at week 17, 23 and 32. Mean values with ± SD are shown. Significance (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$) was determined using Mann-Whitney test. The concentrations are provided in Table S3.3.

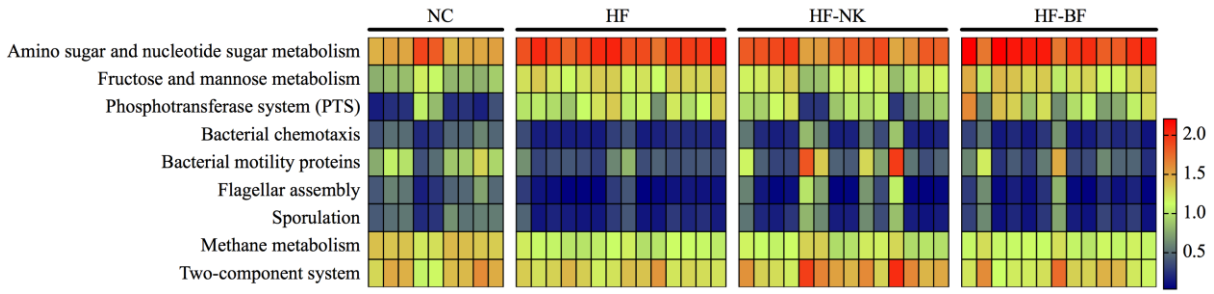

Figure 3.5 The inferred relative abundance of KEGG Orthology functional pathways in the gut microbiota of each mouse. Data is shown only for the key functional pathways determined through independent LEfSe analyses between NC vs HF, HF vs HF-NK and HF vs HF-BF groups. Heat map shows the relative abundance of the KEGG Orthology pathways (rows) in each mouse in each dietary group (columns) at week 32. Red and blue represent the highest and lowest relative abundance respectively. Intensity of the colour denotes the level of the relative abundance (as shown in the legend).

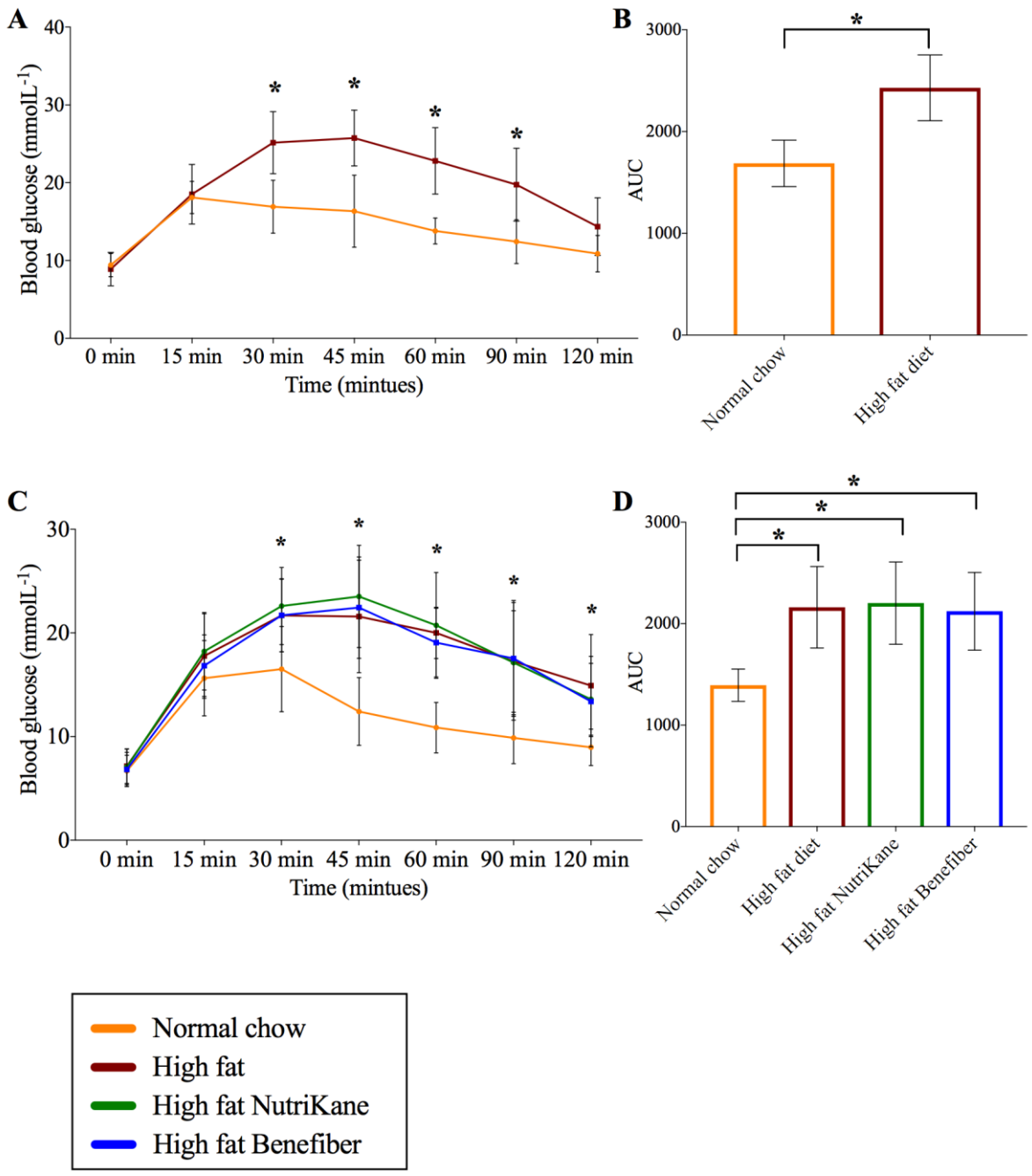

Figure 3.6 The glucose tolerance of mice determined using IPGTT. Blood glucose levels during the IPGTT and area under curve (AUC) of IPGTT shown for: **(A)** and **(B)** at week 17, before fibre based dietary intervention. **(C)** and **(D)** at week 23, after fibre based dietary intervention. Significance was determined based on Bonferroni's multiple comparisons test (for A and C) and Mann Whitney test (for B and D). * $P < 0.05$ compared to the normal chow group. Mean values per group with ± SD are shown.

Table 3.1 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.

A

Nutritional parameters	High fat (Speciality feeds SF02-006)	Normal chow (Speciality feeds meat free rat and mouse cubes)
Protein	19.40%	20.00%
Total fat	60.00%	4.80%
Crude fibre	4.70%	4.80%
Acid detergent fibre	4.75	7.60%
Neutral detergent fibre	NP	16.40%
Total carbohydrate	NP	59.40%
Digestible energy	24 MJ/Kg	14.0 MJ/Kg
% Total calculated energy from protein	81.00%	23.00%
% Total calculated energy from lipids	13.00%	12.00%
Ingredients	Casein, sucrose, canola oil, coca butter, clarified butter fat, cellulose (5% w/w), DL methionine, Calcium carbonate, Sodium carbonate, AIN93 trace minerals, Potassium citrate, Potassium dihydrogen phosphate, Potassium sulphate, Choline chloride, AIN93 vitamins	Wheat, barley, lupins, soya meal, fish meal, mixed vegetable oils, canola oil, salt, Calcium carbonate, Dicalcium phosphate, Magnesium oxide and a vitamin and trace metal premix.

B

Fibre product	NutriKane™	Benefiber®
Ingredients	Sugarcane (sucrose removed) Pectin (from apple and citrus fruits)	100% wheat dextrin (derived from wheat)
Dietary fibre content per 100 g	55.2 g	83 g (soluble fibre)
Nutritional information (Average quantity per 100 g)	Energy 784 kJ Protein 0.8 g Fat total 0.1 g -Saturated 0.1 g Carbohydrate 6.6 g -Sugars 4.5 g Dietary fibre 55.2 g Sodium 15 mg Gluten ND Chromium 391 µg Potassium 5.7 g	Energy 913 kJ Protein Less than 1 g Fat total Less than 1 g -Saturated Less than 1 g Carbohydrate 14.2 g -Sugars Less than 1 g Dietary fibre (total) 83 g Sodium Less than 5 mg

3.10 Supplementary figures and tables

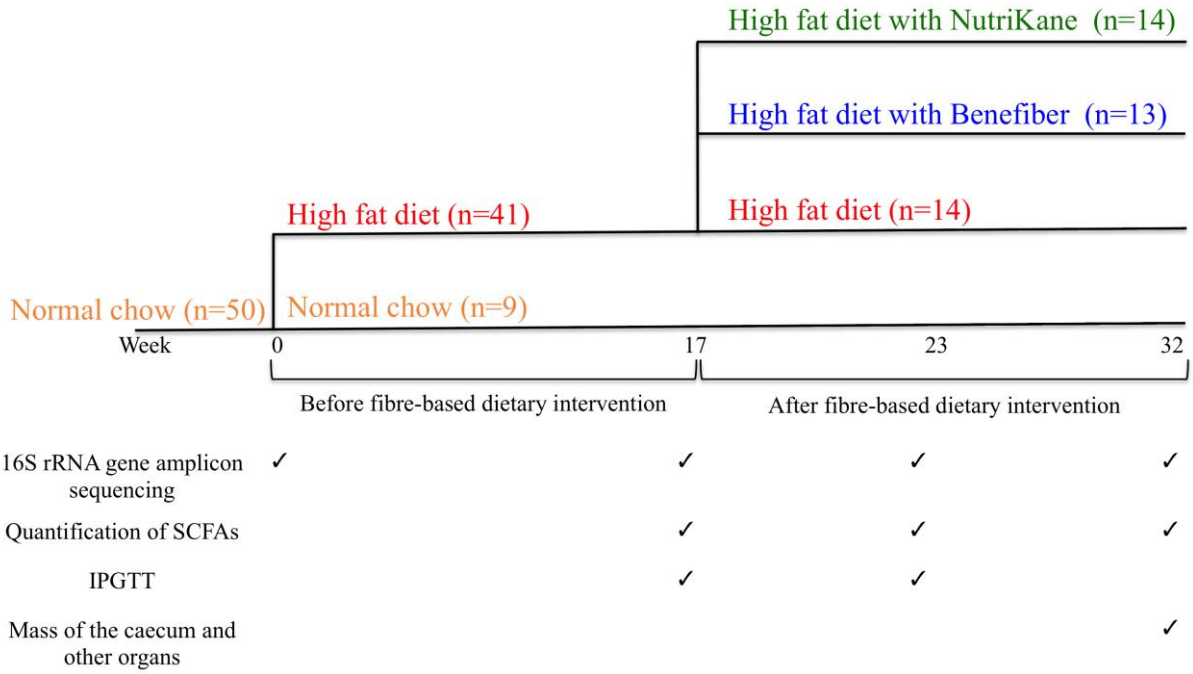

Figure S3.1 Experimental design. After a two-week acclimatisation, at week 0, mice were randomised based on the body weight into two groups (normal chow and high fat diet). After 17 weeks, the high fat group was randomly divided based on the body weight and IPGTT results into three groups, high fat diet, high fat diet supplemented with NutriKane and high fat diet supplemented with Benefiber. These diets were continued until week 32. Fecal samples were collected at weeks 0, 17, 23 and 32 for gut microbiota analysis and quantification of SCFAs. IPGTT was performed at week 17 and 23. Following euthanisation, body organs were excised and weighed. Weekly body weight per mouse and feed consumption per cage were measured.

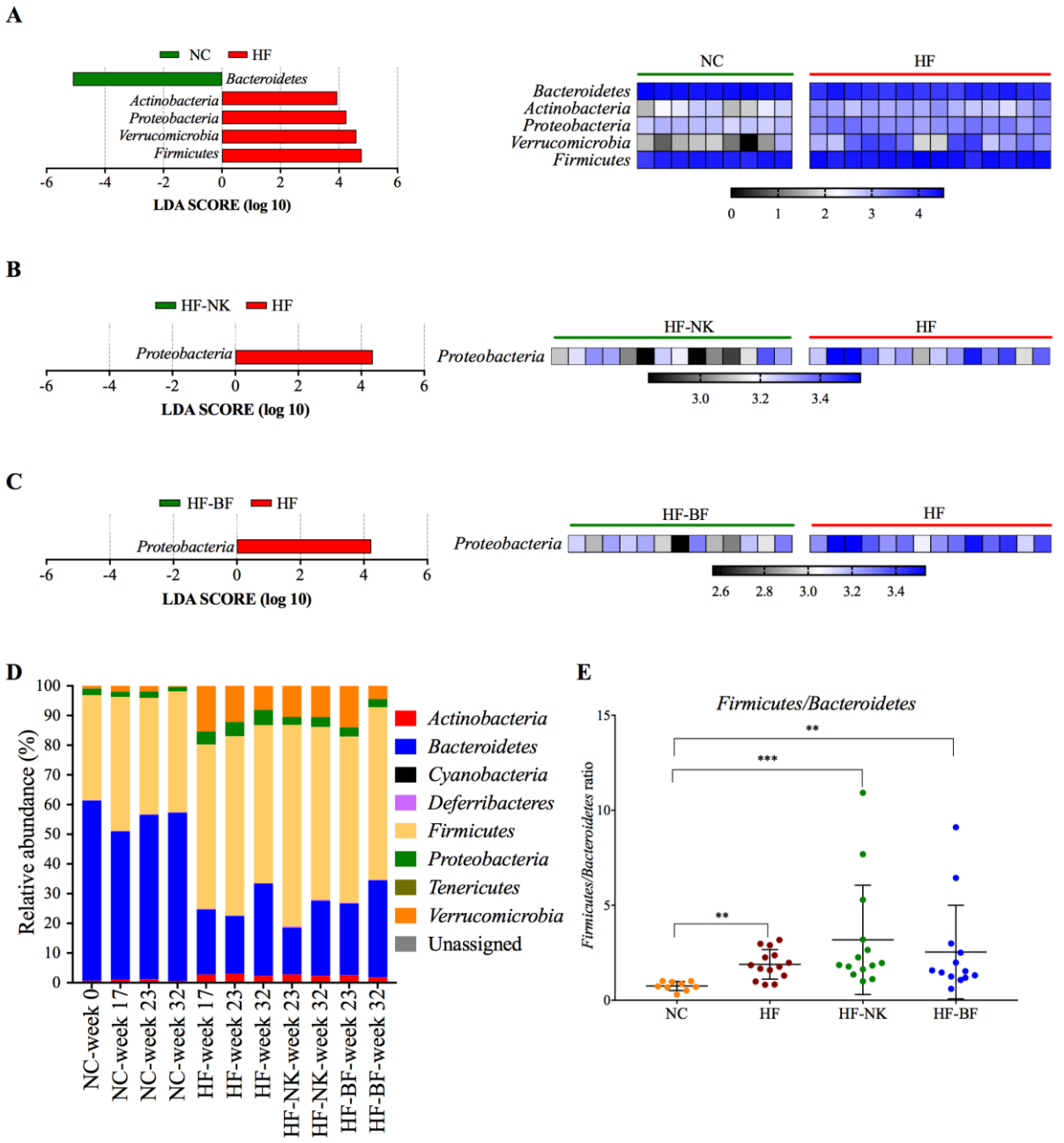

Figure S3.2 Key bacterial phyla of the gut microbiota responding to dietary changes. Data was obtained using LEfSe analysis between **(A)** HF vs NC, **(B)** HF vs HF-NK and **(C)** HF vs HF-BF groups at week 32. The left histogram shows the LDA scores computed for each bacterial phylum and the right heat map shows the relative abundance (Log_{10} transformation) per mouse. In the heat map, rows correspond to bacterial phyla and columns correspond to individuals. Blue and black denote the highest and lowest relative abundance as shown in the legends. **(D)** Relative abundance (%) of all phyla at week 0, 17, 23 and 32. Bacterial identifications that are not assigned to a phylum are labelled as "Unassigned". **(E)** The ratio between the relative abundance of *Firmicutes/Bacteroidetes* per mouse in the dietary groups at week 32. Significance (** $P < 0.01$, *** $P < 0.001$) was determined using a Mann Whitney test.

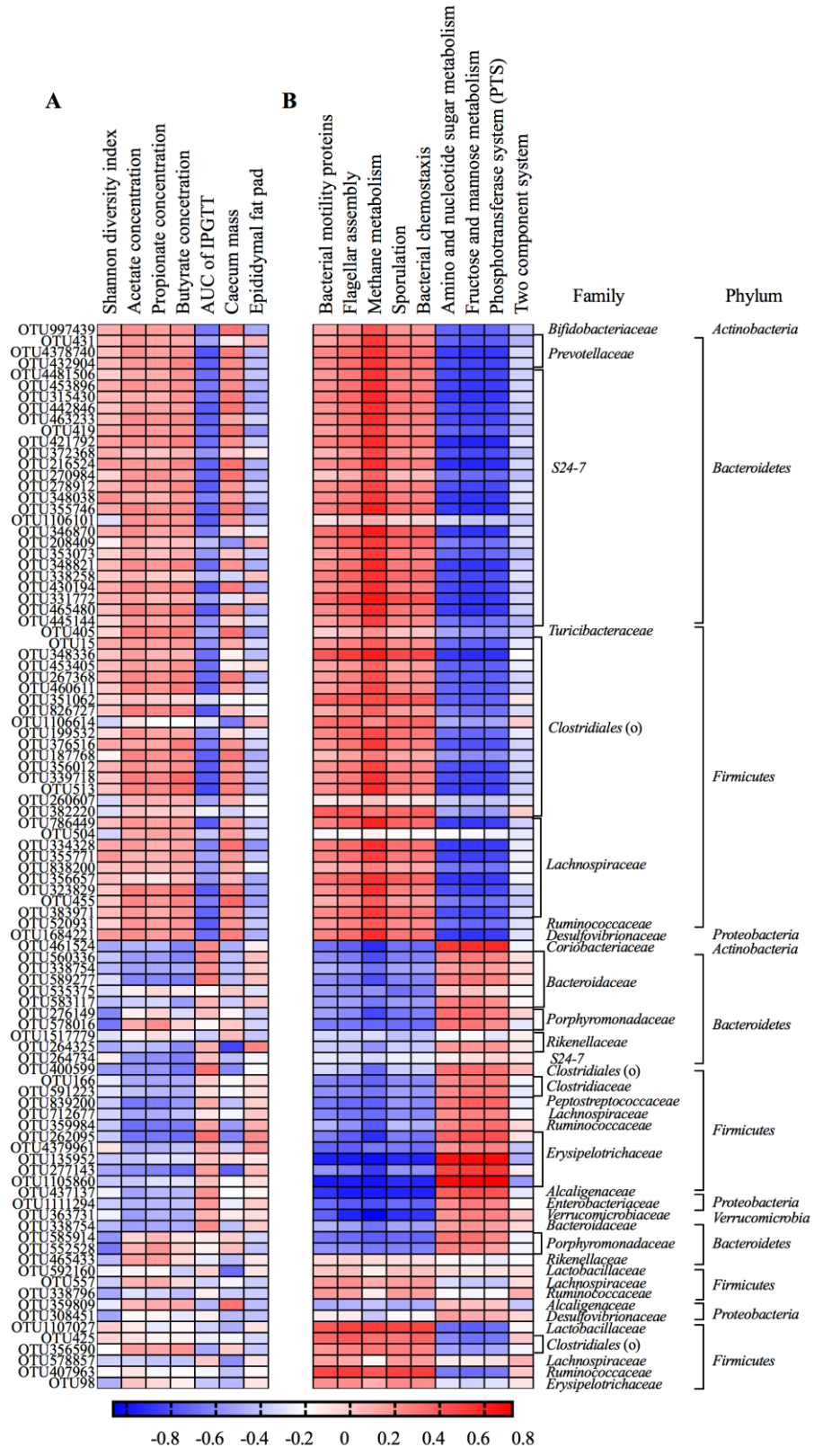

Figure S3.3 The Spearman's correlation (*r*) between gut microbial and host physiological parameters. Data shows the correlation between the relative abundance of key OTUs and **(A)** The Shannon diversity index, concentration of SCFAs (acetate, propionate and butyrate), AUC of IPGTT, caecum and epididymal adipose tissue mass, **(B)** The inferred relative abundance of key KEGG Orthology functional pathways. Red and blue represent the highest and lowest correlation respectively. Intensity of the colour denotes the level of correlation. The taxonomies of the OTUs (family and phylum) are shown on the right.

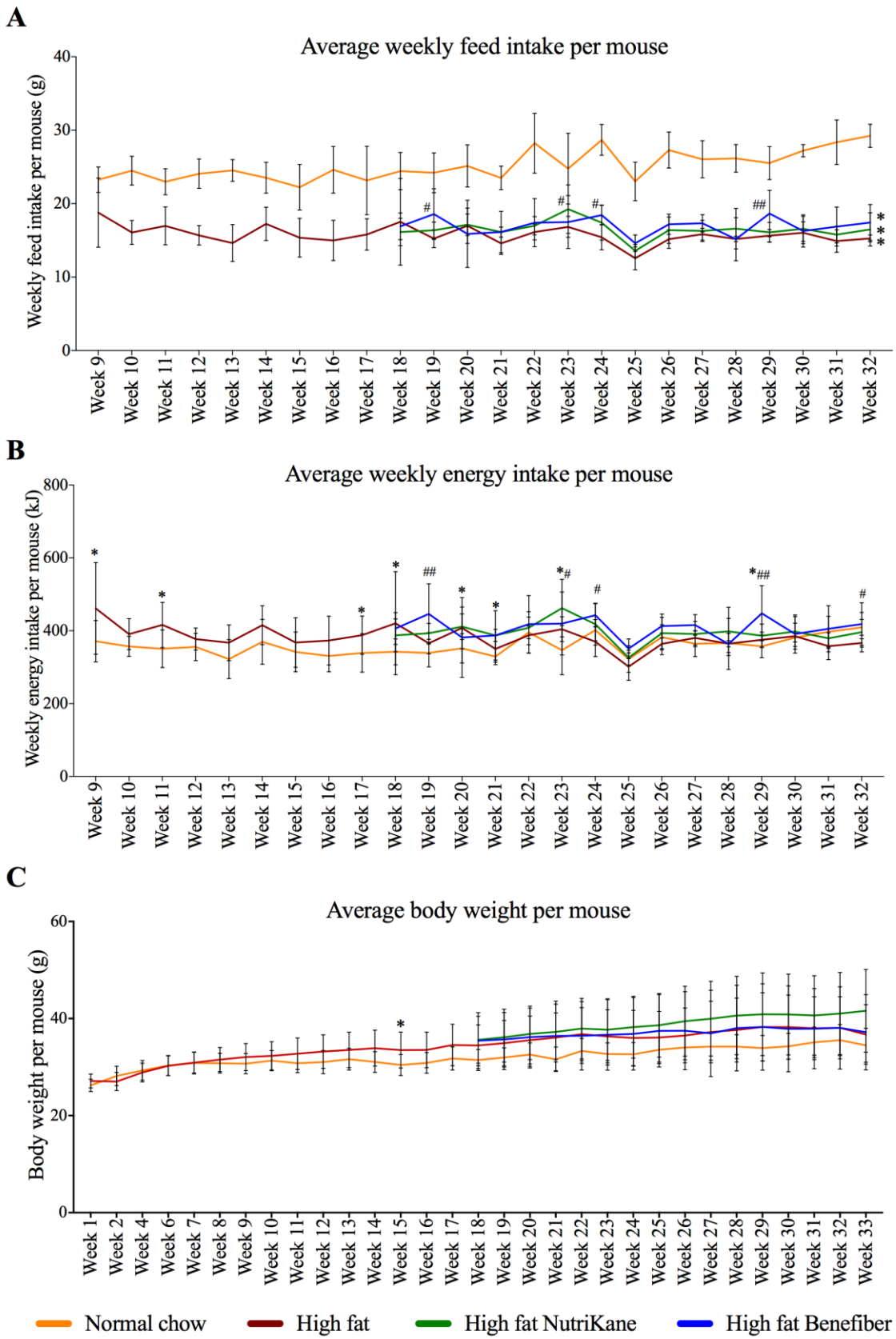

Figure S3.4 Weekly **(A)** feed intake, **(B)** energy intake and **(C)** body weight per mouse. Mean values per dietary group with ± SD are shown. Significance was determined based on Bonferroni's multiple comparisons tests. Significance ($P < 0.05$) is shown as, * compared to normal chow, # compared to high fat and ## compared to both high fat and high fat NutriKane. An asterisk (*) at the end of a data set denotes significant differences compared to the normal chow at all weeks.

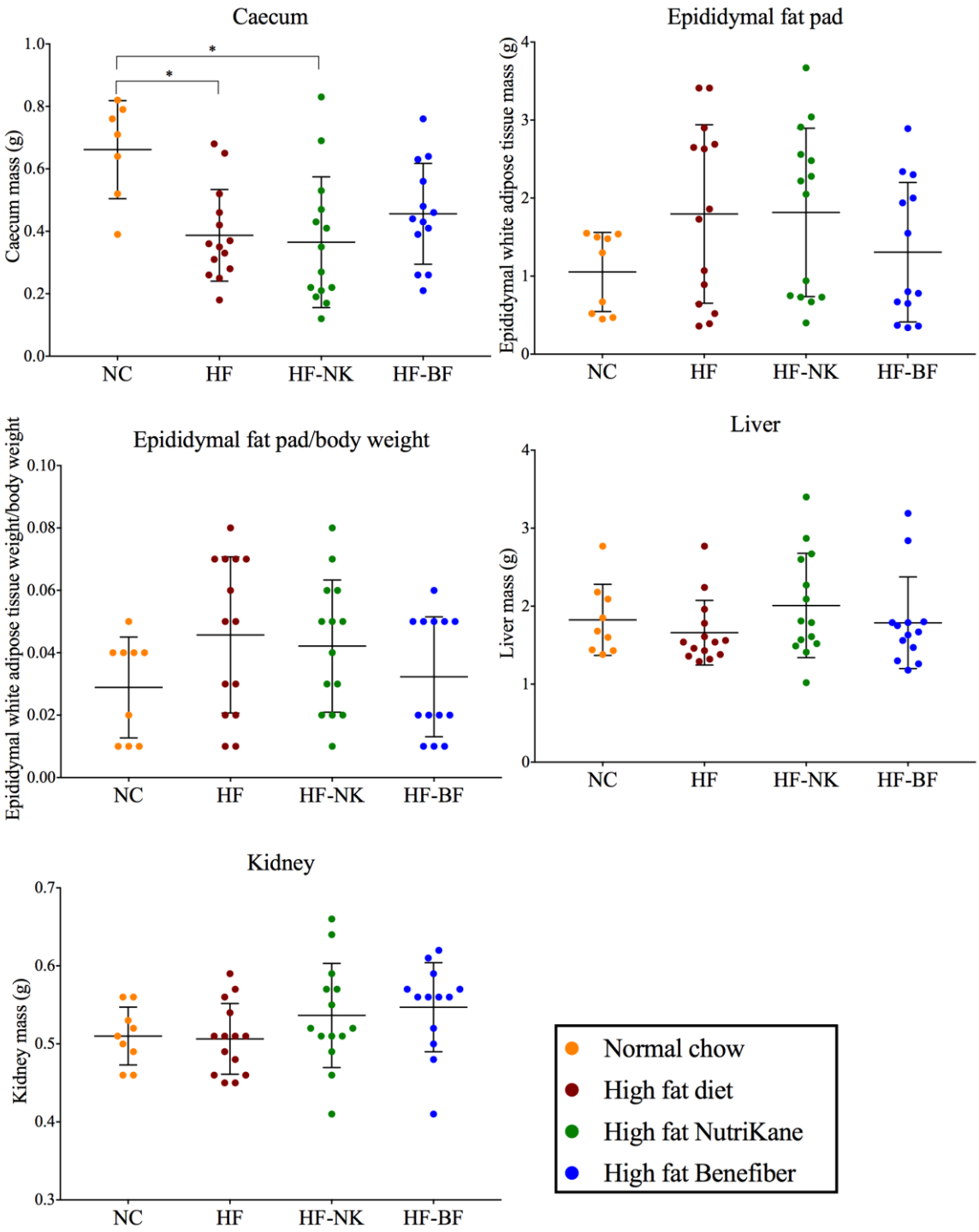

Figure S3.5 Mass of the organs excised from mice at week 32. Data is shown for the mass of the caecum, epididymal white adipose tissue, liver, kidneys and ratio between epididymal white adipose tissue mass and body weight per mouse. Mean values with ± SD are shown. Significance (* $P < 0.05$) was determined using a Mann-Whitney.

Supplementary tables (provided on the CD)

Table S3.1 The relative abundance of bacterial families 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 S3.2 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 S3.3 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 S3.4 The predicted relative abundance of KEGG Orthology pathways of each mouse gut microbiota inferred using PICRUSt. Data is shown for individual mice at week 0, 17, 23 and 32.

Table S3.5 The Spearman's correlation (r) and the significance of the correlations between the relative abundance of significantly differentially abundant OTUs across dietary groups at week 32 and gut microbial, host physiological parameters. Data is shown for the correlations between the Shannon diversity index, concentration of SCFAs (acetate, propionate and butyrate), AUC of IPGTT, caecum and epididymal adipose tissue mass, inferred relative abundance of the key KEGG Orthology pathways.

Table S3.6 The abundance of the OTUs identified in the gut microbial communities of each mouse at week 0, 17, 23 and 32.

CHAPTER 4

Effect of weekly overnight fasting and fibre supplementation on the gut microbiota of mice fed a high fat diet

4.1 Preamble

This chapter describes the results from an experiment conducted prior to the experimental work described in Chapter 3. In this earlier experiment we fed mice one of four diets, normal chow, high fat diet and high fat diet supplemented with either NutriKane™ or Benefiber® (as in Chapter 3), however we measured blood glucose levels after overnight fasting (14-18 hours) weekly. Since mice primarily consume feed at night, this effectively may have been a fasting of ~36 hours, hence subjecting the mice to very regular, lengthy fasting periods. In this study after 30-week high fat diet consumption (81% of total energy from fat), the glucose clearance of the high fat diet fed mice showed no significant difference compared to mice on normal chow (12% of total energy from fat). Dietary fibre supplementation of high fat diet with either NutriKane or Benefiber did not change the glucose clearance.

Based on the findings of this initial experiment a subsequent study (described in Chapter 3) was conducted with no weekly overnight fasting but otherwise was very similar in design. As described in Chapter 3, with no weekly overnight fasting we observed significant differences in the glucose clearance between high fat diet and normal chow fed groups. Comparing the outcome of the two studies led to the hypothesis that subjecting mice to a regular fasting period may have prevented the development of high fat diet-induced impaired glucose clearance. To look at this in more detail we sought to compare the gut microbiota of mice from the two experiments. This comparison is described in detail in Chapter 4. However, as the two studies were not designed to look specifically at this question, there are some features of each, which prevent firm conclusions from being drawn directly from the experiments described here, as discussed within the chapter.

Title

Effect of weekly overnight fasting and fibre supplementation on the gut microbiota of mice fed a high fat diet

Authors

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4.2 Abstract

Dietary fibre supplementation and dietary restriction through fasting have been individually studied as potential strategies for therapeutic modulation of the gut microbiota. However, to our best knowledge, the combined effect of the two strategies on the gut microbiota has not been studied before. We examined the effect of weekly overnight (14-18 hours) fasting on the gut microbiota and host physiology of mice fed either a normal chow, high fat diet or high fat diet supplemented with one of two commercially available fibre products, NutriKane™ and

Benefiber®. The 16S rRNA gene amplicon sequencing results demonstrated significant fasting-mediated shifts in the overall gut microbiota community structure and composition. Linear discriminant analysis effect size analyses showed several OTUs in the genera *Allobacullum, Bacteroides, Lactococcus* and *Parabacteroides,* which were differentially abundant between the groups with fasting and *ad libitum* access to feed. Fasting also resulted in significant alterations in the response of the gut microbiota to high fat and fibre supplementation based dietary interventions. High fat feeding-induced changes in the abundance of the OTUs in the genera *Bacteroides, Clostridium* and *Allobacullum* significantly reduced with fibre supplementation. The mice with *ad libitum* access to high fat diet, high fat diet supplemented with NutriKane or Benefiber showed significantly lower glucose tolerance compared to the normal chow group with *ad libitum* access to feed. In contrast, the glucose tolerance between the same dietary groups with fasting showed no significant difference.

4.3 Introduction

The co-evolutionary relationship between the gut microbiota and host heavily contributes in nutrient metabolism and regulation of inflammation in the host [1-5]. The human digestive system lacks many essential enzymes for the digestion of complex polysaccharides, and therefore depends largely on the gut microbiota to digest this otherwise non-digestible material [6, 7]. A variety of dietary compounds that are not digested nor absorbed in the upper gastrointestinal tract reach the colon and become an energy source for the gut microbiota [8]. Microbial metabolism of these dietary materials produces a range of metabolites that are used in the host as an energy source or signalling molecules to regulate host physiology and immunity [2, 9, 10]. Hence changes in the gut microbial nutrient and energy sources through long- and short-term dietary habits largely contribute to shaping the gut microbiota [11, 12].

A high fat and low fibre diet induces clear changes in the human and mouse gut microbiota, generally including a decrease in the abundance of the bacterial families *Prevotellaceae, Ruminococcaceae, Lachnospiraceae* (formerly *Clostridium* cluster XIVa), *Bifidobacteriaceae* and *Lactobacillaceae* as well as promotion of the growth of *Bacteroidaceae* and *Erysipelotrichaceae* [11, 13-16]. In addition, consumption of a high fat diet typically leads to a lower species diversity and gene richness in the gut microbiota [17]. High fat and low fibre diet-induced changes in the gut microbiota can also trigger the production of endotoxins and increase gut permeability, which results in higher levels of endotoxins in the plasma, development of inflammation and metabolic diseases [18-20]. Therefore, intake of a high fat and low fibre diet heavily contributes in jeopardising the gut microbiota-host relationship and promoting the onset of metabolic and inflammatory diseases.

There is also a growing recognition of the effect of factors such as circadian rhythm and dietary behaviour on the gut microbiota- and metabolism-associated diseases [21-23]. This is most likely due to the complex crosstalk between natural cycles of fasting and feeding that occurs depending on daily light/dark cycle, gut microbiota and host [22, 24-26]. For example, mice fed a high fat diet during the day gained more weight and adipose compared to mice that consumed the same diet and amount of energy at night (active phase) [27]. Therefore, modern dietary behaviour with disruptions to natural fasting and feeding cycles also risks the gut microbiota-host symbiosis.

The current epidemic of gut microbiota-associated diseases such as obesity, diabetes mellitus (type 1 and 2) and inflammatory bowel disease indicates the importance of therapeutic modulation of the gut microbiota for prevention of such diseases or enhancement of the treatments [10, 28]. Dietary restriction is a promising strategy for the therapeutic modulation of the gut microbiota and has been studied in association with extending the lifespan of mice and alleviating metabolic syndrome in both human and animal models [29, 30]. This is generally performed through the restriction of specific macronutrients or time-restricted

feeding with little or no decrease in overall caloric intake [31]. Time-restricted feeding without caloric restrictions has been associated with improved nutrient utilisation and energy expenditure, and therefore, treats or reduces the risk of developing obesity, diabetes mellitus (type 2) and cardiovascular disease in Rhesus monkeys [32] and mice fed obesogenic diets [33, 34]. Similar metabolic and physiological benefits of dietary restriction, including lower risk factors related to cardiovascular disease and cancer have been observed in humans and several other animal models [20, 30, 35-38].

A lifelong study of high fat fed mice with dietary restrictions showed life expectancies, glucose metabolism and lipid profiles similar to that of the low-fat fed group without dietary restrictions [39]. A continuation of the same study examined the effect of low fat or high fat diets with or without dietary restrictions on the gut microbiota. Comparison of the gut microbiota of low fat fed groups with and without dietary restrictions indicated that dietary restrictions increased the relative abundance of *Lactobacillus* spp. and lowered the abundance of *Streptococcus* and TM7, changes which are all associated with reducing inflammation [40]. The relative abundance of *Porphyromonadaceae* was higher in mice fed a high fat diet with dietary restrictions than mice under the same diet without dietary restrictions [40].

Another potential strategy for therapeutic modulation of the gut microbiota is increased intake of microbiota-accessible carbohydrates through dietary fibre or prebiotic supplementation [41, 42]. In addition to positive outcomes such as stool bulking and regulation of appetite, metabolism of prebiotics selectively stimulates the growth and/or activity of the gut microbiota and increases the production of short chain fatty acids (SCFAs). SCFAs provide a range of physiological benefits such as regulation of glucose and lipid metabolism, modulation of immune function, improvement of the gut barrier integrity and protection against pathogens [43]. Some prebiotics or candidate prebiotics are known to promote the growth of *Bifidobacterium, Lactobacillus, Porphyromonadaceae* and groups of *Clostridiales* [44-46]. Due to the direct or indirect effect of the gut microbiota, some prebiotics have been

shown to ease the symptoms of obesity, diabetes mellitus and inflammatory bowel disease [47-49].

Therapeutic modulation of the gut microbiota could have potential in alleviating high fat diet-induced gut microbiota dysbiosis and host health issues. Fasting and intake of dietary fibre have been individually studied as potential strategies for modulating the gut microbiota. However, to our knowledge the effect of the combination of two therapies has not been studied before. In this work we investigated the effect of weekly overnight fasting and different dietary regimes including supplementation with two dietary fibre products on the gut microbiota and host physiology of high fat fed mice. A total of 95 mice were fed a normal chow, high fat diet or high fat diet supplemented with one of two dietary fibre products, namely NutriKane™ (dried whole sugarcane stem and pectin) or Benefiber® (wheat dextrin). Each dietary group was maintained with weekly overnight fasting or *ad libitum* access to feed prior to examining the effect of fasting and dietary fibre supplementation on the gut microbiota.

4.4 Materials and methods

Animal trial

The experimental protocols and procedures used in this study 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). Each experiment comprised four dietary groups, normal chow (containing 14.0 kJg^{-1}, 12% of total energy from fat, produced by Speciality feeds, WA, Australia), high fat diet (containing 24.0 kJg^{-1}, 81% of total energy from fat, serial number SF02-006, produced by Speciality feeds, WA, Australia) and two custom-made high fat diets replacing 5% (w/w) cellulose in SF02-006 with either NutriKane™ or Benefiber® (these diets were also produced by Speciality feeds, WA, Australia). NutriKane™ was provided by Gratuk technologies Pty Ltd,

Australia and Benefiber® was purchased from a local Australian supermarket. Composition and nutritional information of the experimental diets, NutriKane and Benefiber are provided in Table 4.1. Normal chow, high fat diet, high fat diet supplemented with NutriKane and high fat diet supplemented with Benefiber will be referred as NC, HF, HF-NK and HF-BF, respectively, here after.

Eleven-week-old male C57BL/6J mice (Animal Resource Centre (ARC), Canning Vale, WA, Australia) were cohoused (2-3 mice per cage) in a temperature (20-26 ^{o}C), humidity (40-60%) and light (12-hour light/dark cycles) monitored facility. Mice were acclimatised with *ad libitum* access to normal chow and water for two weeks prior to commencing the experiments.

Two independent experiments were conducted with and without weekly overnight fasting (14-18 hours). The group with fasting (n=45) was randomised based on the body weight into one of the two treatment groups. These two groups were fed either NC (n=6) or HF (n=39). After two weeks, the HF group was further randomised into three dietary groups based on the body weight. These three groups were maintained on HF (n=12), HF-NK (n=12) or HF-BF (n=15) diets for 12 weeks.

The group with *ad libitum* access to feed (n=50) was randomised in to one of the two groups fed NC (n=9) or HF (n=41) based on the body weight. After 17 weeks, the HF group was randomised into three groups based on the body weight and these groups were fed HF (n=14), HF-NK (n=14) or HF-BF (n=13) for a period of 15 weeks. Further details of the experimental designs are provided in Fig. S4.1.

Sample collection

Individual body weight and feed consumption per cage were measured weekly. Energy intake per mouse was calculated based on feed consumption and total energy of each feed. Fecal samples were aseptically collected from the groups with and without fasting after 12 and 15 weeks, respectively, on specific diets and stored at -80°C prior to microbiota analysis.

Intraperitoneal glucose tolerance tests

Intraperitoneal glucose tolerance tests (IPGTT) were performed at week 12 and 23 for the groups with and without fasting, respectively. The group with fasting were feed deprived overnight (14-18 hours) and the group with *ad libitum* access to feed was fasted for six hours during the light cycle. Glucose (Sigma-Aldrich, Australia) was injected at 2.0 gkg^{-1} intraperitoneally. Blood glucose concentrations were measured from the tail vein using a Freestyle blood glucose monitoring system (Abbott Pty Ltd, Australia). Initial blood glucose measurements were obtained before (0 mins) glucose injection. Subsequent measurements were obtained after glucose injection at 30, 60, 90 and 120 mins and 15, 30, 45, 60, 90 and 120 mins in each of the groups with and without fasting, respectively.

Sequencing of the 16S rRNA gene amplicons and bioinformatics analysis

Total community DNA was isolated from each of the 95 fecal samples using a FastDNA spin kit (MP Biomedicals) according to the manufacturer's instructions. The lysing matrix in the kit was replaced with Lysing matrix E (MP Biomedicals, Australia) according to previously optimised protocols [50]. The V4 region of 16S rRNA gene was amplified using 515 forward (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806 reverse (5′-GGACTACHVGGGTWTCT AAT-3′) primers with custom barcodes for Illumina MiSeq sequencing [51, 52]. Each 25 µL PCR reaction contained 0.2 µM of the reverse and forward primers, 1 X reaction buffer (Five prime hot master mix, VWR, Australia) containing 45 mM KCl, 2.5 mM Mg^{2+}, 200 µM of each of the dNTPs and 1 unit Taq polymerase and 5-10 ng of template DNA. The PCR comprised a denaturation step at 94°C for 3 minutes, followed by 30 cycles of 94°C for 45 seconds, 50°C for 60 seconds and 72°C for 90 seconds. Final extension step was maintained at 72°C for 10 minutes.

All amplicons were quantified using a Quant-iT™ PicoGreen® (Invitrogen, Australia) assay kit according to the manufacturer's instructions. Amplicons from each sample was pooled and

gel purified using a Wizard® SV gel and PCR clean up system (Promega, Australia). Purified barcoded amplicons were sequenced using an Illumina Miseq platform (250 bp paired-end run) at the Ramaciotti Centre for Genomics, Australia.

Demultiplexed, paired-end raw sequence data were processed using Quantitative Insights Into Microbial Ecology (QIIME) software (version 1.9.1) [53]. High quality and full-length reads (-q 19 with other default parameters) were searched against the Greengenes (V 13_8) database [54] using default QIIME parameters to determine the Operational Taxonomic Units (OTUs) at 97% similarity using an open reference protocol. This resulted in a total of 11,256,544 reads (mean 118,489 ± 29,219 reads). Following filtering out OTUs with less than 0.005%, reads for each sample was rarefied at 60,937, this resulted in 5,789,015 reads in a total of 95 samples.

Functional prediction using PICRUSt

Functional genes in each dietary group were inferred from the 16S rRNA gene amplicon sequence data of both groups with and without fasting using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt, online galaxy version 1.1.0) [55]. OTUs with Greengenes database (version 13_8) identification were used for PICRUSt. Following normalisation based on the 16S rRNA gene copy number, functional genes were identified using Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology groups at the third BRITE hierarchy level [56].

Statistical analysis

All statistical analyses were performed using filtered and rarefied OTUs. Bray-Curtis similarity of Log (x+1) transformed values of the abundance of the OTUs were used to construct non-metric multidimensional (nMDS) plots using PRIMER-7 software package [57, 58]. This allowed the visualisation of the effect of different dietary groups and fasting on the gut microbiota communities. Permutational Multivariate Analysis of Variance

(PERMANOVA) was performed on Bray-Curtis similarity matrices to determine the statistical significance of the community differences. PERMANOVA *P*-values were determined using a pairwise tests and type III sums of squares with 9999 permutations using PRIMER-7 software package. The bacterial diversity of each sample was determined using a Shannon diversity index for each sample using PRIMER-7 software package.

Key OTUs and inferred functional pathways that responded to fasting and dietary interventions were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method (online, Galaxy version 1.0) [59]. Feeding pattern or dietary groups were used as the classes of the subjects (with no subclasses). LEfSe was conducted using alpha value for the Kruskal-Wallis test among classes ($P < 0.05$), pairwise Wilcoxon test between classes < 0.01, the threshold on the logarithmetic LDA score for discriminative features > 3.0 and with other default parameters.

Statistical significance of the Shannon diversity indices and physiological parameters was examined using a Mann-Whitney test, Kruskal-Wallis with Dunn's multiple comparisons test or Bonferroni's multiple comparisons test where appropriate using GraphPad Prism (version 7) software (GraphPad software, USA). The correlations between the relative abundance of the OTUs and AUC of IPGTT, and the inferred relative abundance of functional pathways were determined using Spearman's correlation analyses (two-tailed) using GraphPad Prism software. The significance levels of each statistical analysis are provided in the results section.

4.5 Results

To investigate the effect of fasting and dietary fibre supplementation on the gut microbiota, we fed mice a normal chow (NC), high fat diet (HF), high fat diet supplemented with NutriKane (HF-NK) or high fat diet supplemented with Benefiber (HF-BF). Mice in each of the four dietary regimes were maintained with weekly overnight fasting (14-18 hours) or with

ad libitum access to feed (Fig. S4.1). Fasting during the active phase might have exposed the mice to ~36 hours feed deprivation as mice typically consume feed at night. Fecal samples for the gut microbiota analysis were collected from groups with and without fasting after 12 and 15 weeks, respectively, on specific diets. To examine the effect of different diets and fasting on the gut microbiota, the 16S rRNA gene amplicons were sequenced. After quality filtering, each sample was rarefied to 60,937 reads prior to further analysis.

Fasting-mediated changes in the overall gut microbiota structure

The effect of the different diets and fasting on the gut microbiota community structure was examined using Bray-Curtis similarity matrix-based nMDS plots and PERMANOVA tests. First, each of the four dietary groups was examined independently to determine the effect of fasting on overall gut microbiota community structure (Fig. 4.1). This showed significant fasting-mediated shifts in the microbiota community structures in each dietary group ($P < 0.05$, PERMANOVA). The effect of fasting was most evident ($P < 0.001$, PERMANOVA) in mice fed NC compared to HF, HF-NK and HF-BF fed mice.

Secondly, the effect of each treatment on the gut microbiota structure was analysed through examining the microbiota communities of mice fed NC, HF, HF-NK or HF-BF with or without fasting (Fig. 4.2). Mice on NC showed significantly different ($P = 0.0001$, PERMANOVA) microbiota structures compared to HF, HF-NK and HF-BF fed groups, irrespective of fasting. The gut microbiota structures of mice fed HF, HF-NK or HF-BF were similar, irrespective of fasting or supplementation with fibre products.

The microbial diversity of each sample was examined using a Shannon's diversity index (Fig. 4.3). The diversity of the gut microbiota in the NC fed group was significantly higher ($P < 0.01$) compared to HF and HF-BF groups, irrespective of fasting, except for the HF-NK group. The gut bacterial diversity of mice fed HF-fasted and HF-NK-fasted diets were

significantly higher ($P < 0.05$) compared to the same dietary groups with *ad libitum* access to feed.

Fasting-mediated effects on the gut microbiota composition

The gut microbiota of all mice was dominated by the phyla *Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria* and *Verrucomicrobia,* irrespective of the feeding pattern or diet (Fig. S4.2). However, the relative abundance of each phylum significantly varied between dietary groups and feeding patterns.

The OTUs in each dietary group that responded to the feeding patterns were determined using LEfSe analyses (Fig 4.4). The effect of the feeding pattern was substantially higher in the gut microbiota of mice fed NC compared to mice fed HF, as a total of 46 and 28 OTUs, respectively, were identified as key OTUs that responded to fasting. In NC-fasted group compared to the NC-*ad libitum* group, the relative abundance of 20 OTUs was higher, whist 26 OTUs were lower (Fig. 4.4A). The relative abundance of the OTUs in the genera *Akkermansia* (OTU363731), *Lactobacillus* (OTU592160), and *Bifidobacterium* (OTU997439) and the family *Coriobacteriaceae* (OTU293 and OTU461524) were higher in the NC-fasted group compared to the NC-*ad libitum* group.

Most of the OTUs responding to fasting in the NC group were not found in the HF group, except for five OTUs in the genera *Allobacullum* (OTU237 and OTU277143), *Lactobacillus* (OTU592160), family *Coriobacteriaceae* (OTU461524) and order *Bacteroidales* (OTU3013444), which were increased by fasting in both NC and HF groups. In HF fed mice, the relative abundance of the OTUs in the families *Parabacteroidaceae* (OTU585914 and OTU578016) and *Rikenellaceae* (OTU264325 and OTU151779) were higher in the group fed *ad libitum* (Fig. 4.4B).

A total of 12 OTUs responded to fasting in the HF-NK group (Fig. 4.4C), nine OTUs of these similarly responded to fasting in the HF fed group. In the HF-BF group, the relative

abundance of 25 OTUs altered with fasting (Fig. 4.4D), 11 of these OTUs similarly responded to fasting in the HF fed group. In the HF-BF-*ad libitum* group, four OTUs in the genus *Parabacteroides* were significantly lower in abundance compared to the HF-BF-fasted group.

Most of the OTUs that responded to fasting were not similar between HF, HF-NK and HF-BF groups, except for the relative abundance of the OTUs in *Allobacullum* (OTU277143), *Coriobacteriaceae* (OTU330) and *Lactococcus* (OTU716006), which increased, and the relative abundance of *Bacteroidales* (OTU535375 and OTU589277), which decreased by fasting in mice fed HF, HF-NK or HF-BF.

Fasting-mediated effects on the gut microbiota response to fibre additions

To determine the effect of fasting on the diet-responsive OTUs, the gut microbiota in mice with and without fasting were independently analysed using LEfSe analyses between the NC vs HF, HF vs HF-NK and HF vs HF-BF groups (Fig. 4.5). In mice fed *ad libitum,* 83 OTUs were differentially abundant between the HF and NC groups, whereas, between the same diets with fasting only 59 OTUs were significantly differentially abundant. Thirty-five of these OTUs responded similarly to HF feeding irrespective of the feeding pattern, whilst 72 OTUs that responded to HF feeding were feeding pattern-specific. A number of these OTUs that responded differentially to HF feeding due to fasting were in S24-7, *Lachnospiraceae, Clostridiales* and *Bacteroides.*

A larger number of OTUs were significantly differentially abundant between the NC vs HF diets compared to HF vs HF-NK or HF-BF diets (Fig. 4.5). In groups with and without fasting, HF-NK feeding significantly altered the abundance of 7 and 14 OTUs, respectively, compared to the HF groups. Whereas mice fed HF-BF with and without fasting had 16 and 17 significantly differentially abundant OTUs, respectively, compared to the HF groups.

Supplementing HF with NutriKane or Benefiber significantly altered the abundance of specific gut bacteria irrespective of fasting, whereas, some OTUs that responded to fibre

addition varied depending on the feeding pattern. The relative abundance of two and four OTUs significantly decreased in the HF-NK groups with and without fasting, respectively. Of these OTUs, the abundance of an OTU in *Allobacullum* (OTU4379961) significantly decreased with HF-NK feeding irrespective of the feeding pattern. The relative abundance of eight OTUs each significantly changed in HF-BF groups with and without fasting compared to the HF fed groups. Of these OTUs, the relative abundance of two OTUs (*Bacteroides* OTU589277 and *Allobaculum* OTU4379961) significantly reduced by HF-BF feeding irrespective of the feeding pattern.

Addition of NutriKane and Benefiber induced alterations in the abundance of fibre product-dependent sets of OTUs. In groups without fasting, HF-NK and HF-BF feeding changed the abundance of seven and nine OTUs, respectively, while in the groups with fasting HF-NK and HF-BF feeding altered the abundance of three and six OTUs, respectively, that were product-dependent.

Fasting-induced changes in the inferred functional pathways

The gut microbiota functional pathways were inferred from the 16S rRNA gene sequences using PICRUSt. The key inferred functional pathways that responded to fasting and dietary interventions were determined using LEfSe analyses (Fig. S4.3). The inferred abundance of the functional pathways for ABC transporters, phosphotransferase system (PTS) transporters and general transporters were higher in *ad libitum* fed groups of NC, HF and HF-BF compared to the groups fed the same diets with fasting. In comparison to NC-*ad libitum* group, the inferred functional pathways for the PTS transporters and fructose and mannose metabolism were less abundant in NC-fasted group, whilst the inferred relative abundance of bacterial motility proteins and flagellar assembly were higher. HF feeding *ad libitum* incurred an increase in the inferred relative abundance of the PTS transporters and fructose and mannose metabolism compared to the group fed NC *ad libitum.* We also observed a decrease

in the inferred relative abundance of functional pathways for bacterial motility proteins and flagellar assembly in mice fed HF *ad libitum* compared to the group fed NC *ad libitum.*

Effect of fasting on host physiology, feed and energy intake

Glucose tolerance was determined using an IPGTT (Fig. 4.6). In the groups fed *ad libitum*, NC fed mice showed significantly lower ($P < 0.05$) IPGTT blood glucose measurements and IPGTT-AUC values compared to the HF group. Fibre supplementations did not attenuate HF-induced impaired glucose clearance. However, in the group with fasting, NC-fasted, HF-fasted and HF-BF-fasted mice showed no significant differences in the glucose clearance, expect for HF-NK-fasted, which had significantly higher IPGTT-AUC values compared to NC-fasted.

The correlation of the gut microbiota with IPGTT-AUC results was determined using Spearman's correlation. The AUC of IPGTT negatively correlated with the relative abundance of the OTUs in S24-7 (OTU355746, OTU1106101, OTU465480, OTU169), *Clostridiales* (OTU459276, OTU346764, OTU290338, OTU339718, OTU415491) and *Lachnospiraceae* (OTU356657, OTU323829) (Spearman's $r < -0.4$, $P < 0.001$).

Compared to the NC-*ad libitum* group, HF, HF-NK and HF-BF fed *ad libitum* groups consumed significantly lower ($P < 0.05$) amounts of feed at all time points (Fig. S4.4). However, in the group with fasting, the feed intake was similar between all four dietary groups. Effect of different diets or fasting was not significant in the energy intake and body weight as all four dietary groups had similar energy intake and bogy weight values in each group with and without fasting.

4.6 Discussion

Modulation of the gut microbiota for alleviation of metabolic syndrome is of increasing scientific and public interest. Dietary restriction through fasting has been studied for its ability

to alter the gut microbiota and improve host health and lifespan. Another potential strategy for therapeutic modulation of the gut microbiota is the increased intake of microbiota-accessible carbohydrates through consumption of commercially available fibre supplements. In the current study, we investigated the effect two commercially available fibre products, NutriKane™ and Benefiber® in combination with fasting on the gut microbiota and physiology of high fat fed mice.

Dietary restriction in the present study was carried out through weekly overnight fasting (14-18 hours) with *ad libitum* access to water. Mice primarily consume feed at night, therefore, overnight fasting can significantly reduce the feed intake, and this has been previously shown to provoke a catabolic state in mice [60, 61]. Overnight fasting has been also linked with significant loss of total body, lean body, total body fat mass and hepatic glycogen in lean mice [61]. In this work we demonstrate that overnight fasting can also induce shifts in the gut microbiota composition, demonstrating that the gut microbiota can be substantially modulated only through weekly fasting during the active phase.

The overall gut microbiota community structure and the specific OTUs that responded to fasting were significantly different across NC, HF, HF-NK and HF-BF dietary groups. The most dramatic response to fasting was observed in the gut microbiota of mice fed NC, as the overall gut microbiota structure showed a clearer separation and the relative abundance of a higher number of OTUs changed in response to fasting compared to HF, HF-NK or HF-BF groups. This is consistent with previous observations on diet-dependent response of the gut microbiota towards the feeding pattern [40].

The relative abundance of many OTUs in *Allobaculum* (in family *Erysipelotrichaceae)* were significantly higher in all four dietary groups upon fasting, of these only one OTU (OTU277143) responded similarly to fasting across all dietary groups. Although members of the family *Erysipelotrichaceae* have been previously shown to positively correlate with the dietary fat intake and inflammation in mice [18, 62, 63], some studies have also observed an

increase in the abundance of *Allobaculum* upon caloric restriction and exercise [64, 65]. These observations potentially reflect the functional dynamics of different members of this family.

Specific OTUs in the genus *Lactococcus* were more abundant in the groups with fasting compared to the groups with *ad libitum* access to feed. Previous studies have reported on potential anti-inflammatory properties in the members of the genus *Lactococcus* [66, 67]. Our observation of fasting-mediated reduction in the abundance of *Bacteroides* is also in agreement with previous reports that suggested a positive link between the relative abundance of this genus with the intake of dietary fat and impaired glucose clearance [11, 12, 68, 69]. However, a study on the gut microbiota of rats limited to 1-hour feeding periods for 6 days demonstrated an increase in the abundance of *Bacteroides* [70]. The phylum *Bacteroidetes* responds to significant reductions in the energy load [71], which may be the reason for contradictory results of this study compared to our work, as mice in the present study were only feed deprived weekly with no significant loss in the energy intake.

Compared to NC feeding, HF feeding resulted in significant shifts in the overall gut microbiota community structure and microbial composition and lowered the microbial diversity irrespective of the feeding pattern. These HF-induced changes are largely in agreement with previous studies [16, 62, 72, 73]. While fibre supplementation with NutriKane and Benefiber did not attenuate HF-induced changes in the overall gut microbiota structure and microbial diversity, we observed significant alterations in the abundance of some OTUs. However, the specific OTUs that responded to each fibre addition and the effect on the microbial diversity varied between the two feeding patterns.

The microbial diversity of HF-fasted and HF-NK-fasted groups was significantly higher compared to the groups with *ad libitum* access to HF and HF-NK. Loss of microbiota diversity has been identified as the most constant finding of the gut microbiota dysbiosis in association with metabolic and inflammatory diseases [74]. The higher diversity of the gut

microbiota observed in fasted mice in this study may indicate the potential of fasting to control HF-induced loss of the gut microbial diversity.

Supplementation with either NutriKane or Benefiber significantly changed the relative abundance of specific OTUs in the genera *Bacteroides, Clostridium* and *Allobacullum* compared to HF feeding irrespective of the feeding pattern. A higher relative abundance of these bacterial genera are typically linked to high fat diet-induced gut microbiota dysbiosis [62, 71, 75], hence fibre supplementation-mediated reduction in the abundance of these genera might indicate an improvement in the gut microbiota irrespective of the feeding pattern.

NutriKane and Benefiber addition also promoted the growth of fibre product-specific OTUs, most of which have been previously associated with fibre digestion [7, 76-78]. These product-specific changes in the gut microbiota could be due to variations in the chemical structure of the dietary fibre within NutriKane and Benefiber [79]. Specific OTUs that changed with fibre supplementation varied between the two feeding patterns.

Fasting also induced changes in the inferred functions of the gut microbiota. The inferred relative abundance of one or more functional pathways associated with transporter systems (PTS transporter systems, ABC transporters or general transporters) decreased with fasting in groups fed NC, HF or HF-BF. An increase in the abundance of these pathways has been previously linked to gut microbiota dysbiosis and metabolic diseases [80-82]. While further metagenomic studies are essential to draw firm conclusions, current inferred results may indicate the potential of overnight fasting in improving the gut microbiota health irrespective of the type of diet.

The inferred functional pathways that were differentially abundant between NC-fasted and NC-*ad libitum* groups were also differentially abundant between the NC-*ad libitum* and HF-*ad libitum* groups. Previous metagenomics studies have reported HF-induced higher

abundance in the functional pathways for PTS transporter systems, fructose and mannose metabolism, and have observed lower abundance in the pathways for bacterial motility proteins and flagellar assembly in relation to metabolic diseases [68, 80] Therefore, it is tempting to speculate that fasting during the active phase has the potential to further improve the gut microbiota functions in NC fed mice.

HF-feeding significantly affected the glucose clearance in mice fed *ad libitum*, whilst in mice fasted, HF-feeding did not mediate significant differences in the glucose clearance compared to that of NC-fasted group. This suggests the possibility that fasting could be used to control HF-induced impaired glucose clearance [39]. The negative correlation of the AUC of IPGTT with specific OTUs in *Clostridiales*, S24-7 and *Lachnospiraceae* agrees with previous studies [83, 84]. However, other studies have observed a fasting-mediated improvement in the glucose clearance in the NC fed groups [39], whereas we did not.

The experimental design of the current study has several limitations such as the differences in age and duration of exposure to dietary treatments at the time of fecal sample collection and IPGTT between the groups with and without fasting. Variation of these factors in addition to the feeding pattern between the treatment groups limit us from concluding fasting to be the only factor that resulted in observed changes in the gut microbiota and glucose tolerance. However, previous studies have reported the adult mouse gut microbiota to be more stable over time [40, 85] and is able to respond to dietary changes in less than 48 hours [12]. Although these evidences might indicate a minimal impact of the above-mentioned limitations on the observed differences in the present study, we are unable to confirm this statistically. Hence, future experiments directly aimed to examine the effect of weekly overnight fasting on diet-mediated gut microbiota alterations are essential extensions to this work.

4.7 Conclusion

In the present work we demonstrated a strong effect of weekly fasting during the active phase on the gut microbiota composition, overall structure and microbial diversity of mice fed NC, HF, HF-NK or HF-BF. Fasting-mediated changes in the gut microbiota included higher abundance of the OTUs in *Lactococcus, Allobaculum, Bacteroides* and *Parabacteroides.* Our results also demonstrate the ability of fasting to control the degree of HF-mediated gut microbiota alterations. The abundance of the OTUs in *Bacteroides*, *Clostridium* and *Allobacullum* changed upon fibre supplementation irrespective of the feeding pattern, however, the specific bacterial OTUs that responded to NutriKane and Benefiber additions varied between the two feeding patterns.

Despite the limitations in the experimental design of the present study, our observations show that weekly overnight fasting have the potential to modulate the gut microbiota as well as the impact of dietary interventions on the gut microbiota of mice. Future experiments with designs directly aimed to investigate the effect of fasting on the gut microbiota and microbial metabolites would be useful in gaining further insight.

4.8 References

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4.9 Acknowledgements

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4.10 Figures and tables

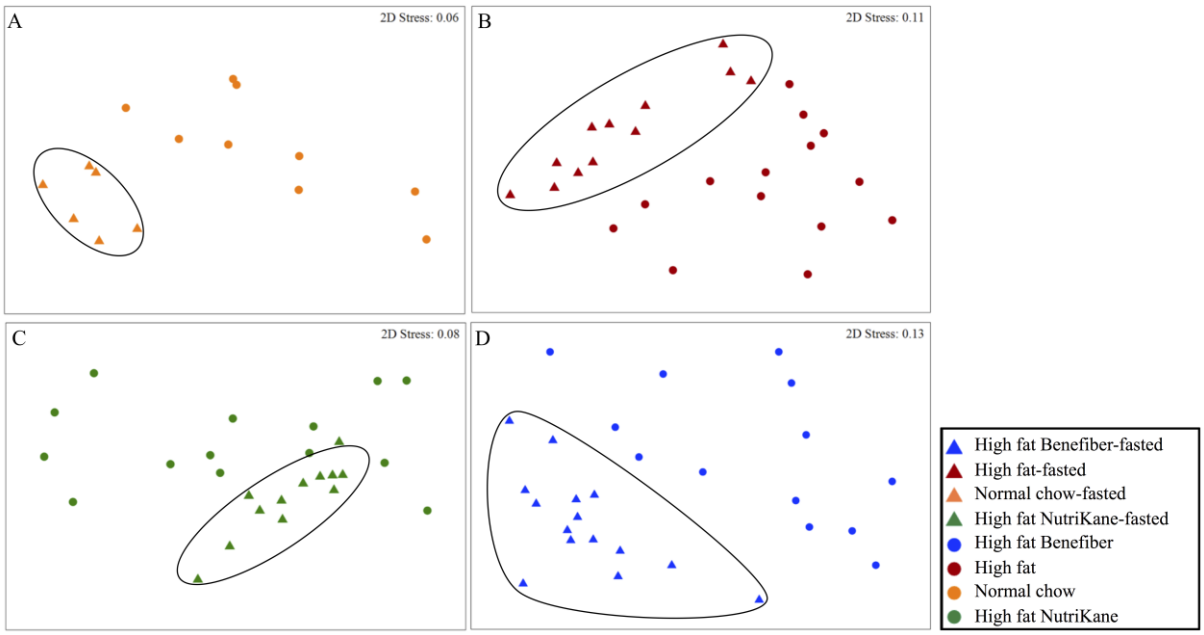


Figure 4.1 Ordination of the gut microbiota in mice fed each of the four diets with or without fasting. Data is shown as Bray-Curtis similarity of Log (X+1) relative abundance based nMDS plots. Mice fed **(A)** Normal chow, **(B)** High fat diet, **(C)** High fat diet supplemented with NutriKane and **(D)** High fat diet supplemented with Benefiber are shown. A triangle or a dot corresponds to a mouse with or without fasting respectively. Mice that were fed one the four diets with weekly fasting are circled with a solid line.

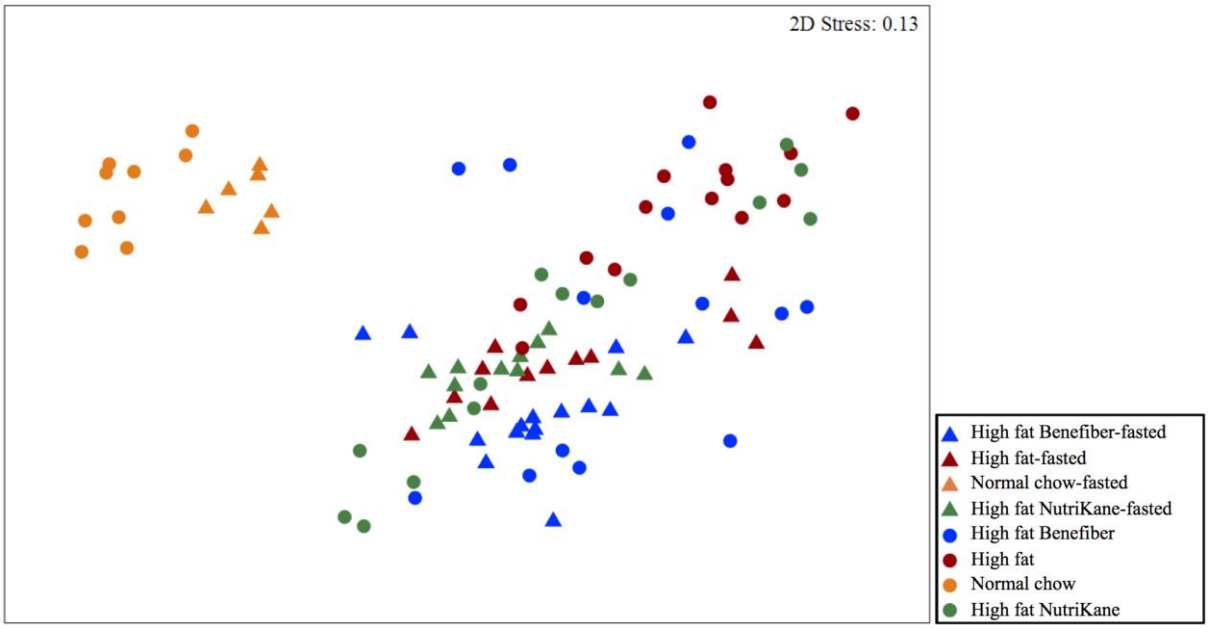


Figure 4.2 Ordination of the gut microbiota in mice with (triangles) and without (circles) weekly overnight fasting shown as Bray-Curtis similarity of Log (X+1) relative abundance based nMDS plots. Normal chow fed mice (orange) demonstrated a different gut microbiota composition compared to high fat diet (red), high fat diet supplemented with NutriKane (green) and high fat diet supplemented with Benefiber (blue) fed groups irrespective of the feeding pattern.

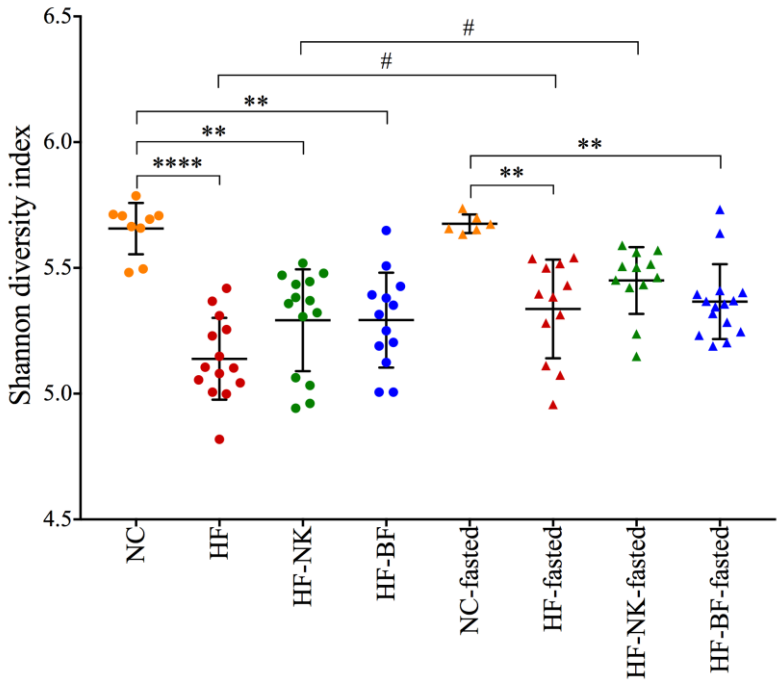
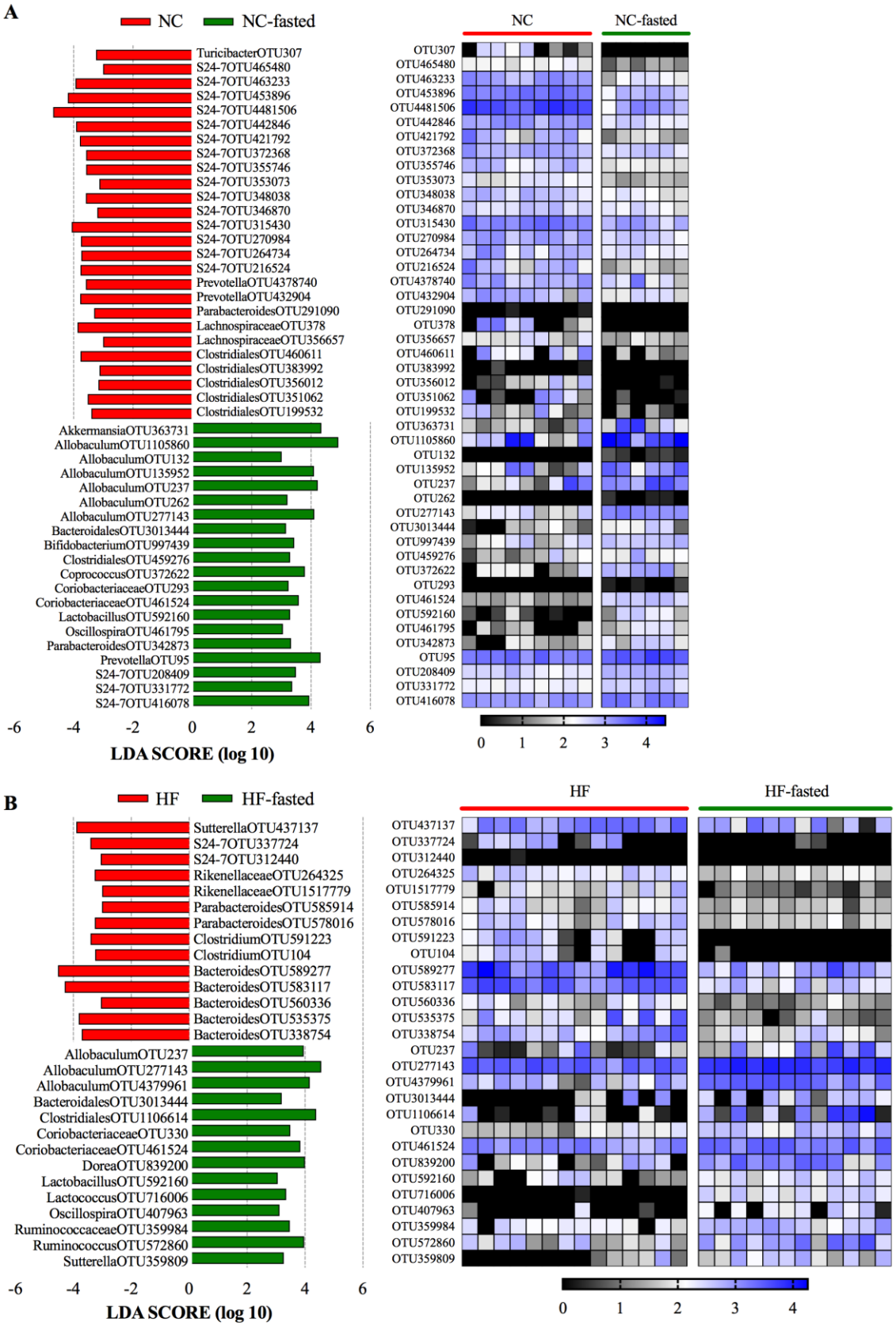


Figure 4.3 The gut microbial diversity shown as a Shannon diversity index per mouse in each dietary group with or without fasting. Data is shown for mice fed normal chow (NC), high fat diet (HF), high fat diet supplemented with NutriKane (HF-NK) or high fat diet supplemented with Benefiber (HF-BF). Mean values with ± SD are indicated. Significance was determined based on a Kruskal-Wallis with Dunn's multiple comparisons test (** $P<0.01$, **** $P<0.0001$) or Mann-Whitney test (# $P<0.05$).


A
NC
NC-fasted
TuricibacterOTU307
S24-7OTU465480
S24-7OTU463233
S24-7OTU453896
S24-7OTU4481506
S24-7OTU442846
S24-7OTU421792
S24-7OTU372368
S24-7OTU355746
S24-7OTU353073
S24-7OTU348038
S24-7OTU346870
S24-7OTU315430
S24-7OTU270984
S24-7OTU264734
S24-7OTU216524
PrevotellaOTU4378740
PrevotellaOTU432904
ParabacteroidesOTU291090
LachnospiraceaeOTU378
LachnospiraceaeOTU356657
ClostridialesOTU460611
ClostridialesOTU383992
ClostridialesOTU356012
ClostridialesOTU351062
ClostridialesOTU199532
AkkermansiaOTU363731
AllobaculumOTU1105860
AllobaculumOTU132
AllobaculumOTU135952
AllobaculumOTU237
AllobaculumOTU262
AllobaculumOTU277143
BacteroidalesOTU3013444
BifidobacteriumOTU997439
ClostridialesOTU459276
CoprococcusOTU372622
CoriobacteriaceaeOTU293
CoriobacteriaceaeOTU461524
LactobacillusOTU592160
OscillospiraOTU461795
ParabacteroidesOTU342873
PrevotellaOTU95
S24-7OTU208409
S24-7OTU331772
S24-7OTU416078
-6
-4
-2
0
2
4
6
LDA SCORE (log 10)
NC
NC-fasted
OTU307
OTU465480
OTU463233
OTU453896
OTU4481506
OTU442846
OTU421792
OTU372368
OTU355746
OTU353073
OTU348038
OTU346870
OTU315430
OTU270984
OTU264734
OTU216524
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OTU461524
OTU592160
OTU461795
OTU342873
OTU95
OTU208409
OTU331772
OTU416078
0
1
2
3
4
B
HF
HF-fasted
SutterellaOTU437137
S24-7OTU337724
S24-7OTU312440
RikenellaceaeOTU264325
RikenellaceaeOTU1517779
ParabacteroidesOTU585914
ParabacteroidesOTU578016
ClostridiumOTU591223
ClostridiumOTU104
BacteroidesOTU589277
BacteroidesOTU583117
BacteroidesOTU560336
BacteroidesOTU535375
BacteroidesOTU338754
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AllobaculumOTU277143
AllobaculumOTU4379961
BacteroidalesOTU3013444
ClostridialesOTU1106614
CoriobacteriaceaeOTU330
CoriobacteriaceaeOTU461524
DoreaOTU839200
LactobacillusOTU592160
LactococcusOTU716006
OscillospiraOTU407963
RuminococcaceaeOTU359984
RuminococcusOTU572860
SutterellaOTU359809
-6
-4
-2
0
2
4
6
LDA SCORE (log 10)
HF
HF-fasted
OTU437137
OTU337724
OTU312440
OTU264325
OTU1517779
OTU585914
OTU578016
OTU591223
OTU104
OTU589277
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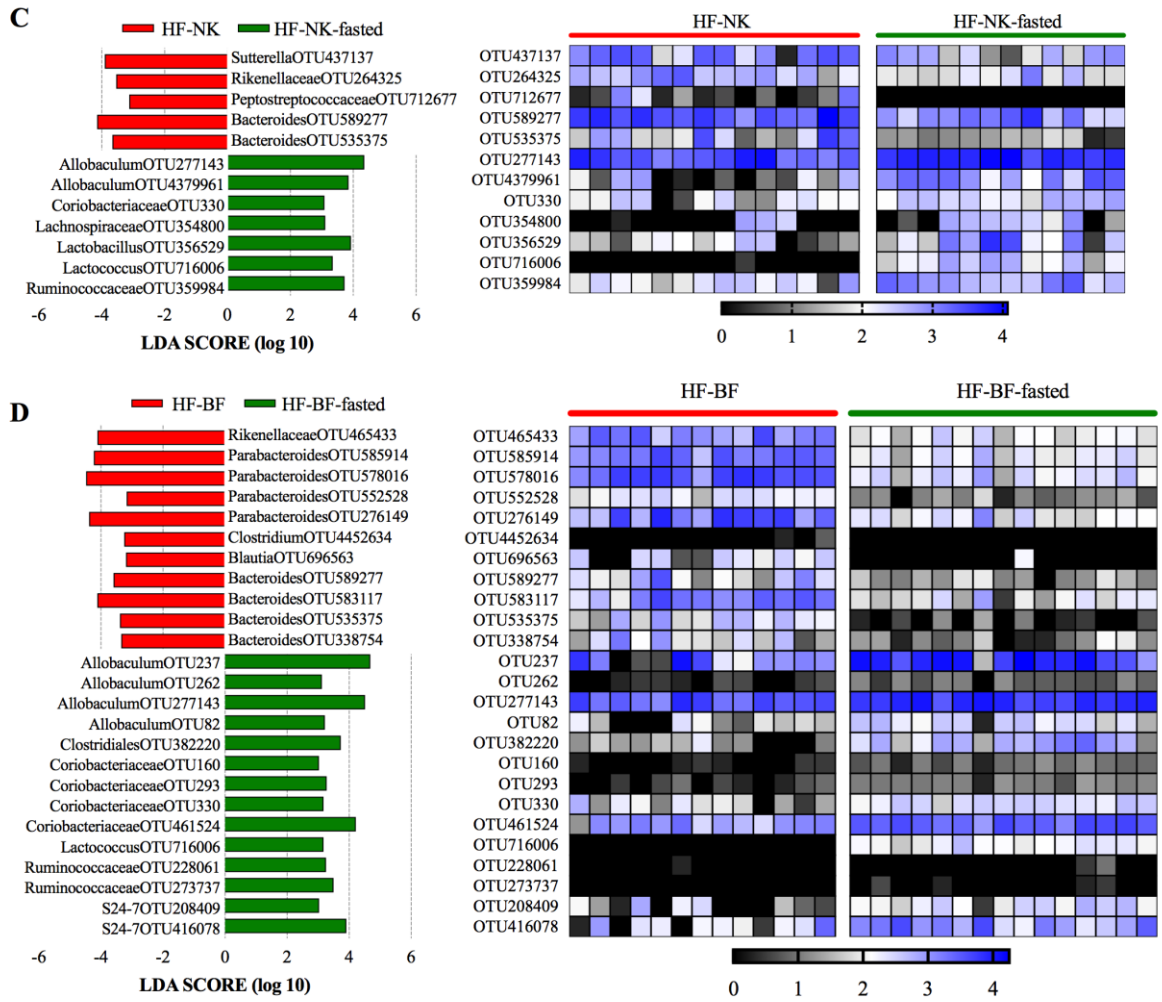

Figure 4.4 Key OTUs responding to fasting in mice fed **(A)** normal chow (NC), **(B)** high fat diet (HF), **(C)** high fat diet supplemented with NutriKane (HF-NK) and **(D)** high fat diet supplemented with Benefiber (HF-BF). Data was obtained based on LEfSe analyses between mice with and without fasting in each dietary group. The left histogram shows the LDA scores for each differentially abundant OTU between the feeding patterns. The right heat map shows the relative abundance (Log10 transformation) of the OTUs (rows) per mouse in each dietary group (column). Black and blue correspond to the lowest and highest relative abundance respectively.

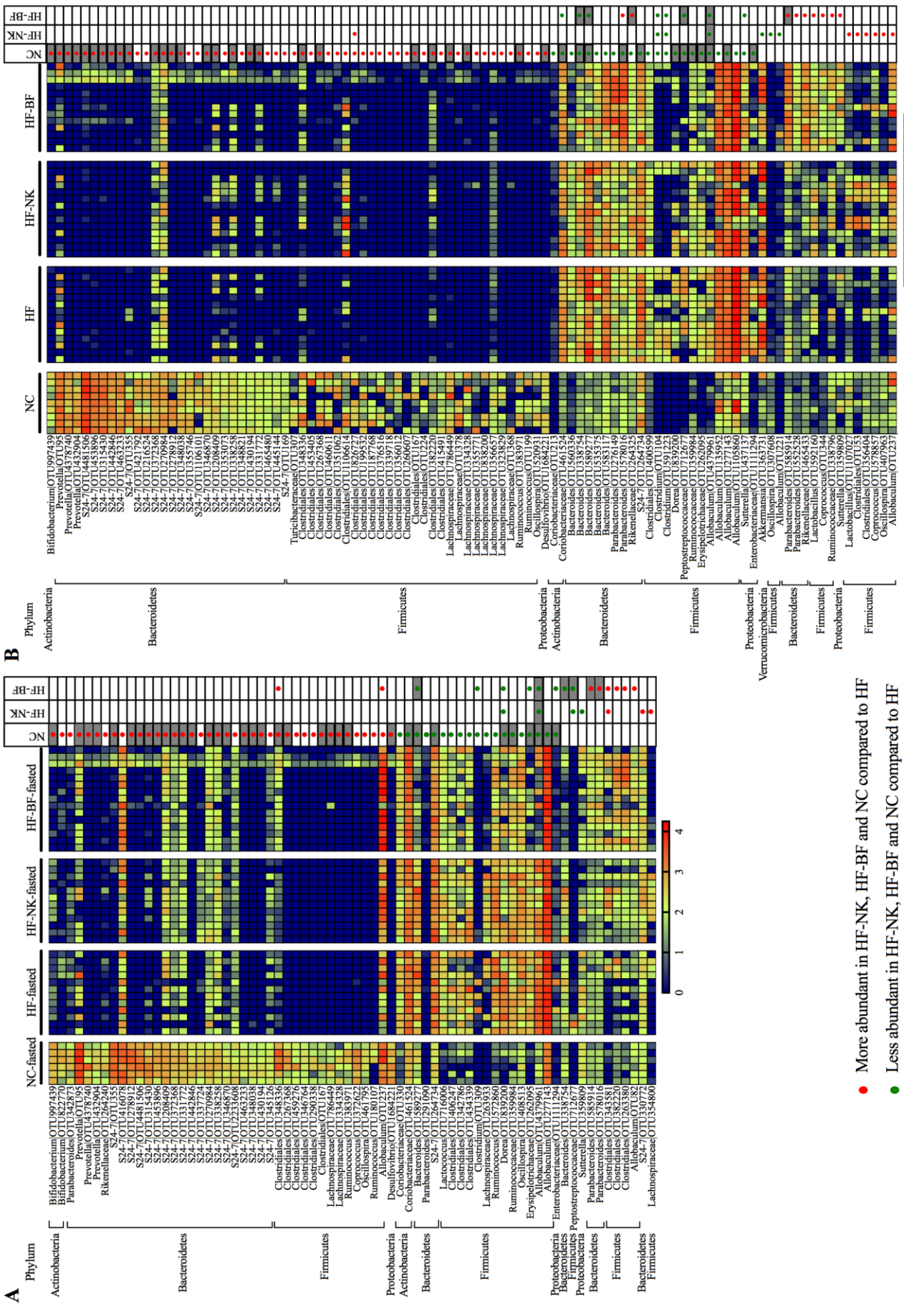
A
Phylum
NC-fasted
HF-fasted
HF-NK-fasted
HF-BF-fasted
NC
HF-NK
HF-BF
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Bacteroidetes
Firmicutes
Bacteroidetes
Firmicutes
B
Phylum
NC
HF
HF-NK
HF-BF
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Verrucomicrobia
Firmicutes
Bacteroidetes
Firmicutes
Proteobacteria
Firmicutes
More abundant in HF-NK, HF-BF and NC compared to HF
Less abundant in HF-NK, HF-BF and NC compared to HF

Figure 4.5 The relative abundance (Log_{10} transformation) of significantly differentially abundant OTUs between dietary groups. Data was obtained using LEfSe analyses between HF vs NC, HF vs HF-NK and HF vs HF-BF groups for mice **(A)** with fasting and **(B)** without fasting. Rows in the heat maps correspond to 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 taxonomy of the OTUs (best taxonomic identification and phylum) is shown on the left. 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. OTUs that changed similarly irrespective of fasting are highlighted in grey.

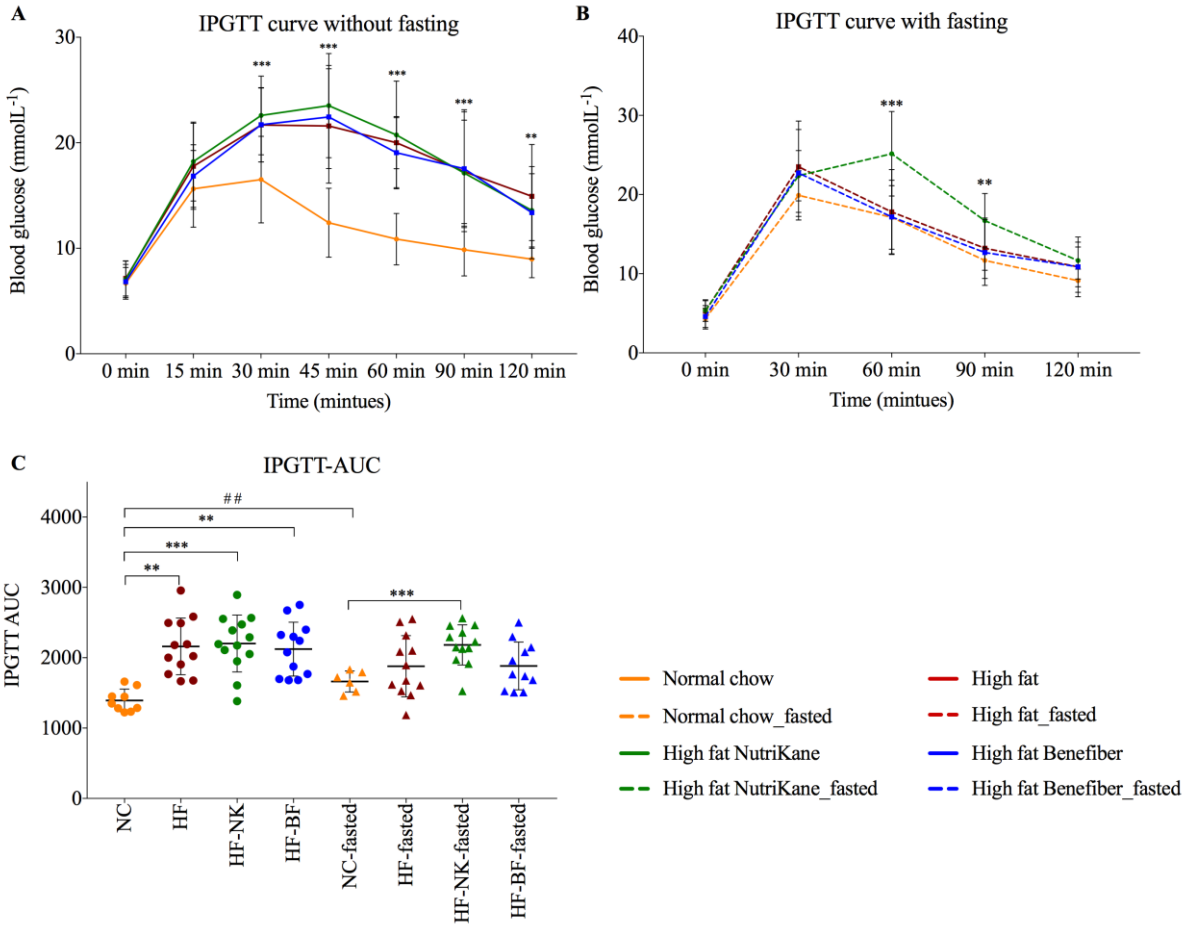

Figure 4.6 The effect of fasting on the glucose tolerance of mice determined using an IPGTT. Blood glucose measurements obtained from intraperitoneal glucose tolerance test (IPGTT), **(A)** without and **(B)** with weekly fasting. **(C)** Area under curve (AUC) of IPGTT. Significance in (A) and (B) was determined using Bonferroni's multiple comparisons test (* $P < 0.05$ compared to normal chow). In (C), significance was determined using a Kruskal-Wallis with Dunn's multiple comparisons test (*** $P < 0.001$ and ** $P < 0.01$) or a Mann-Whitney test (# # $P < 0.01$). Mean values with ± SD are indicated.

Table 4.1 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 in the high fat diet with 5% (w/w) NutriKane and Benefiber respectively). **(B)** Ingredients and nutritional information of NutriKane and Benefiber.

A

Nutritional parameters	High fat (Speciality feeds SF02-006)	Normal chow (Speciality feeds meat free rat and mouse cubes)
Protein	19.40%	20.00%
Total fat	60.00%	4.80%
Crude fibre	4.70%	4.80%
Acid detergent fibre	4.75	7.60%
Neutral detergent fibre	NP	16.40%
Total carbohydrate	NP	59.40%
Digestible energy	24 MJ/Kg	14.0 MJ/Kg
% Total calculated energy from protein	81.00%	23.00%
% Total calculated energy from lipids	13.00%	12.00%
Ingredients	Casein, sucrose, canola oil, coca butter, clarified butter fat, cellulose (5% w/w), DL methionine, Calcium carbonate, Sodium carbonate, AIN93 trace minerals, Potassium citrate, Potassium dihydrogen phosphate, Potassium sulphate, Choline chloride, AIN93 vitamins	Wheat, barley, lupins, soya meal, fish meal, mixed vegetable oils, canola oil, salt, Calcium carbonate, Dicalcium phosphate, Magnesium oxide and a vitamin and trace metal premix.

B

Fibre product	NutriKane™	Benefiber®
Ingredients	Sugarcane (sucrose removed) Pectin (from apple and citrus fruits)	100% wheat dextrin (derived from wheat)
Dietary fibre content per 100 g	55.2 g	83 g (soluble fibre)
Nutritional information (Average quantity per 100 g)	Energy 784 kJ Protein 0.8 g Fat total 0.1 g -Saturated 0.1 g Carbohydrate 6.6 g -Sugars 4.5 g Dietary fibre 55.2 g Sodium 15 mg Gluten ND Chromium 391 µg Potassium 5.7 g	Energy 913 kJ Protein Less than 1 g Fat total Less than 1 g -Saturated Less than 1 g Carbohydrate 14.2 g -Sugars Less than 1 g Dietary fibre (total) 83 g Sodium Less than 5 mg

4.11 Supplementary figures and tables

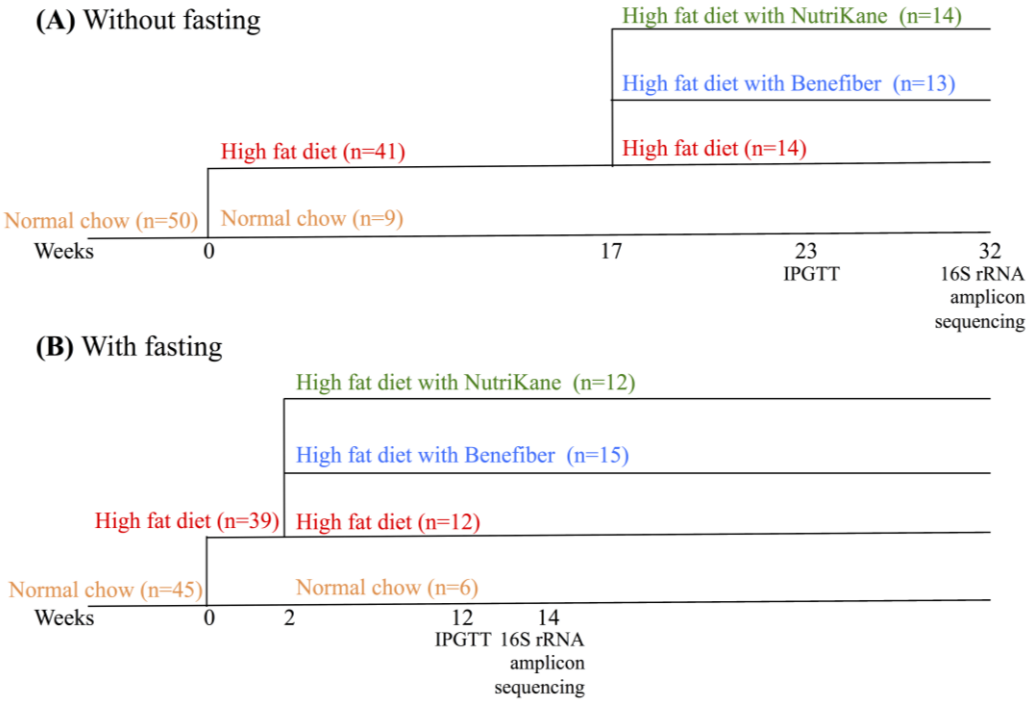

Figure S4.1 Experimental designs. Mice **(A)** without and **(B)** with weekly fasting. After two weeks acclimatisation, mice from each group were randomised based on the body weight into two dietary groups (normal chow and high fat diet). High fat fed mice with and without fasting were further randomised in to three groups (high fat diet, high fat diet supplemented with NutriKane or high fat diet supplemented with Benefiber) based on the weight after 2 and 17 weeks on the high fat diet respectively. Gut microbiota analysis was conducted using the fecal samples collected from mice with and without fasting after 12 and 15 weeks on the four different diets.

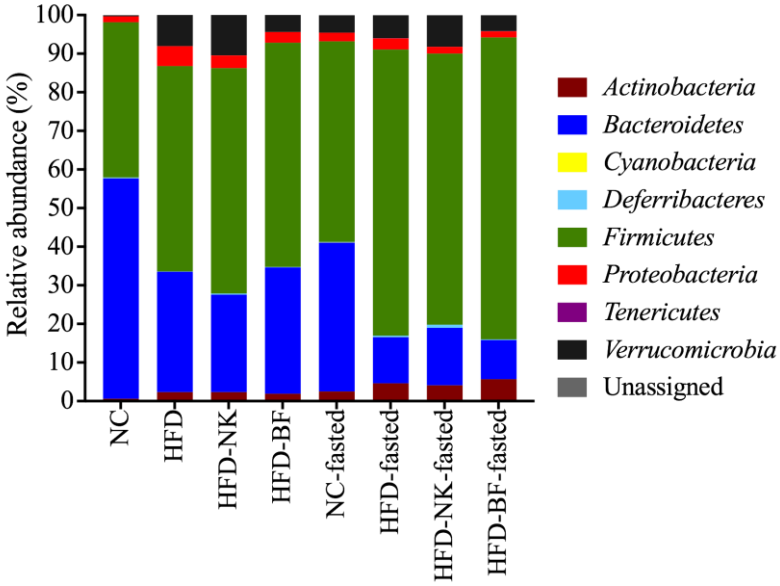

Figure S4.2 Phylum level taxonomic composition of the gut microbiota in mice under different dietary groups and feeding patterns. The average relative abundance of the phyla in each feeding pattern and dietary group was determined using QIIME and GraphPad Prism (V7). Bacterial identifications that were not assigned to a phylum are categorised as "Unassigned".

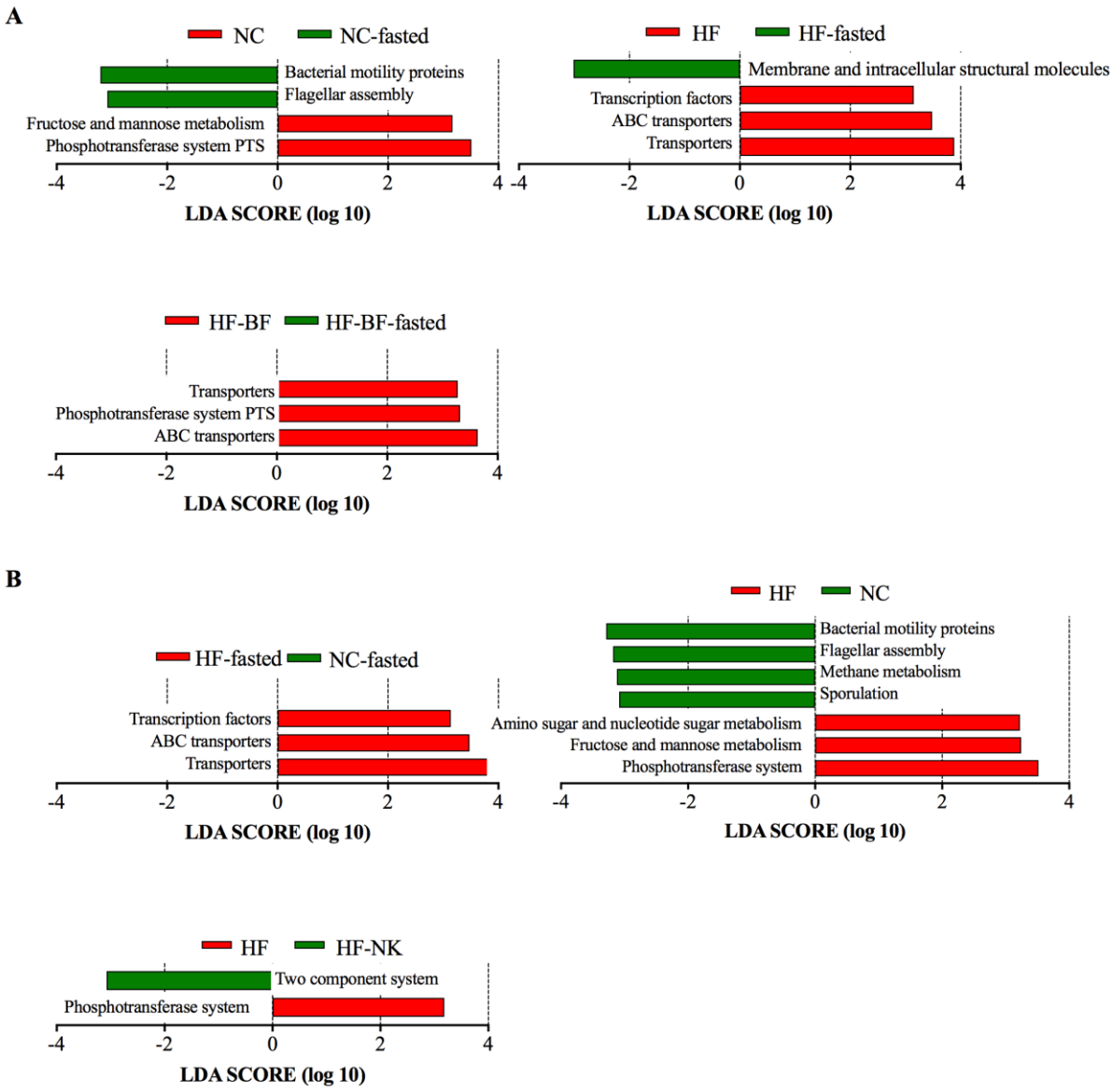

Figure S4.3 The inferred relative abundance of KEGG Orthology functional pathways in the mouse gut microbiota, which were found to be significantly different between the dietary groups. Data was obtained using LEfSe analyses between **(A)** NC vs NC-fasted, HF vs HF-fasted and HF vs HF-fasted, **(B)** HF-fasted vs NC-fasted, HF vs NC and HF vs HF-NK groups. None of the inferred functional pathways were found to be significantly differentially abundant between NK vs NK-fasted, HF vs HF-BF, HF-fasted vs HF-BF-fasted, HF-fasted vs HF-NK-fasted groups.

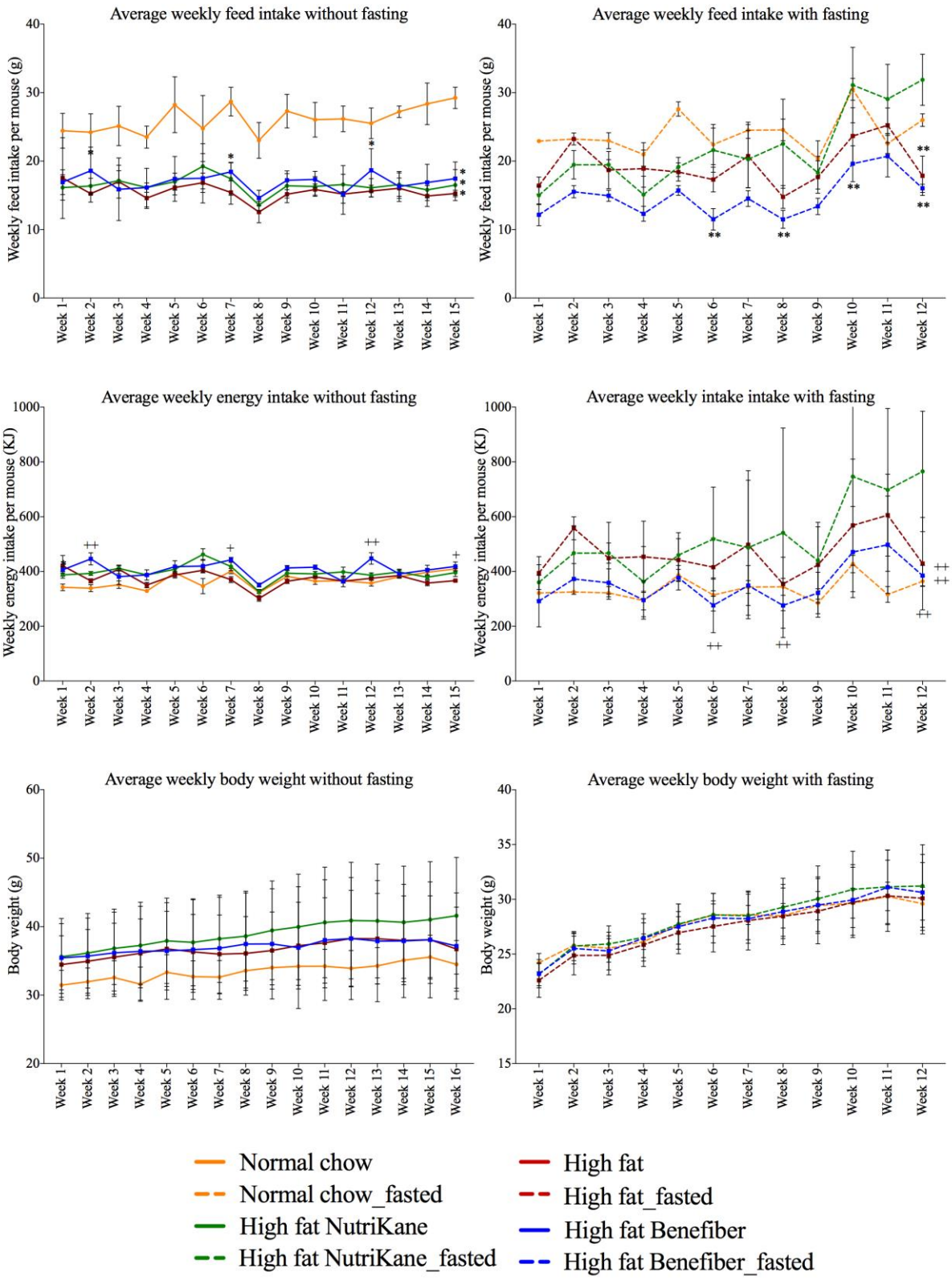

Figure S4.4 Average weekly intake of the feed, energy and body weight per mouse in groups with and without fasting. Mean values ± SD are shown. Significance was determined based on a Bonferroni's multiple comparisons test. * $P < 0.05$ compared to normal chow group and ++ $P < 0.05$ compared to the high fat diet supplemented with NutriKane group.

Supplementary tables (provided on the CD)

Table S4.1 The relative abundance of the OTUs that were found to be significantly responding to fasting in each of the four dietary groups. Data was obtained through LEfSe analyses between NC vs NC-fasted, HF vs HF-fasted, HF-NK vs HF-NK-fasted and HF-BF vs HF-BF-fasted. 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 S4.2 The relative abundance of the OTUs, which were significantly differentially abundant between dietary groups based on LEfSe analyses. Data was obtained through analysing groups with and without fasting independently using LEfSe for NC vs HF, HF vs HF-NK and HF vs HF-BF. 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 S4.3 The inferred relative abundance of the gut microbiota functional pathways predicted using PICRUSt. Data is shown for each mouse fed each of the four diets with or without fasting.

Table S4.4 The abundance of the OTUs identified in the gut microbial communities of each mouse fed one of the four diets with or without fasting.

CHAPTER 5

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

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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 $\times$ 3 time points $\times$ 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

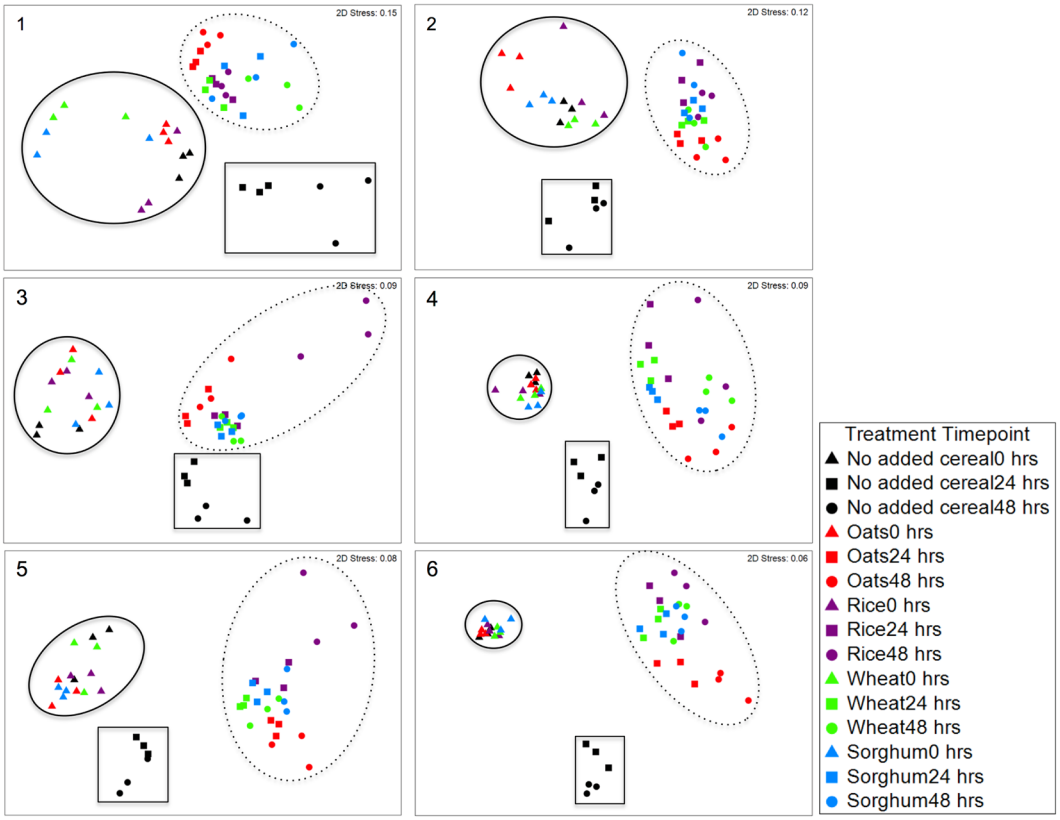

Figure 1. Ordination of the gut microbiota in each biological sample (1–6) at 0, 24 and 48 hours. Data is shown as Bray-Curtis similarity of Log (X + 1) transformed relative abundance based nMDS plots. Treatments and time points are colour coded as shown in the legend. All cereal additions shifted the 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 ($P < 0.0001$).

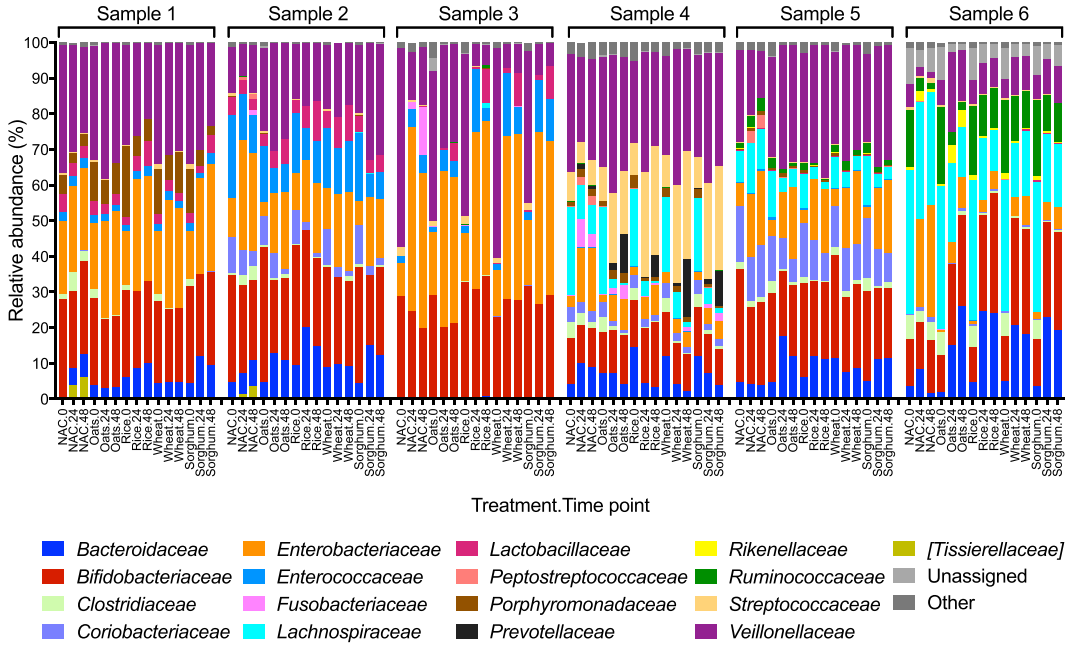

Figure 2. Family level taxonomic compositions of the microbial communities for each biological replicate. The relative abundances of the families were determined using QIIME and GraphPad Prism (V7). Each bar is labelled first by treatment, followed by time point (0, 24 and 48 hours). No added cereal 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].

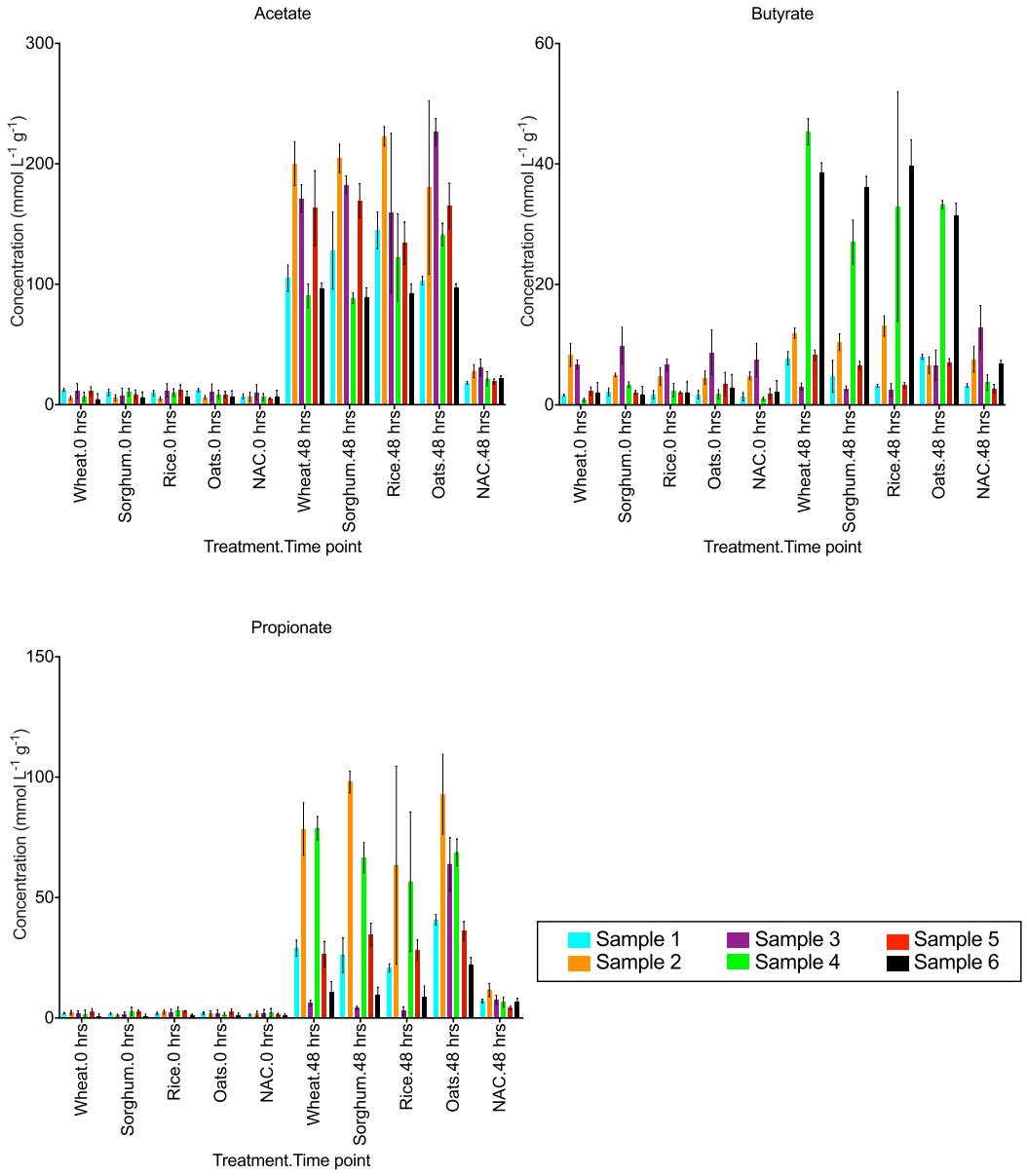

Figure 3. Concentration ($mmolL^{-1}g^{-1}$) of acetate, butyrate and propionate in each treatment at 0 and 48 hours. Concentration measurements at 24 and 48 hours for all three SCFAs were similar, therefore only 48 hours are shown. Mean ± SD concentration for all treatments with each biological sample (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].

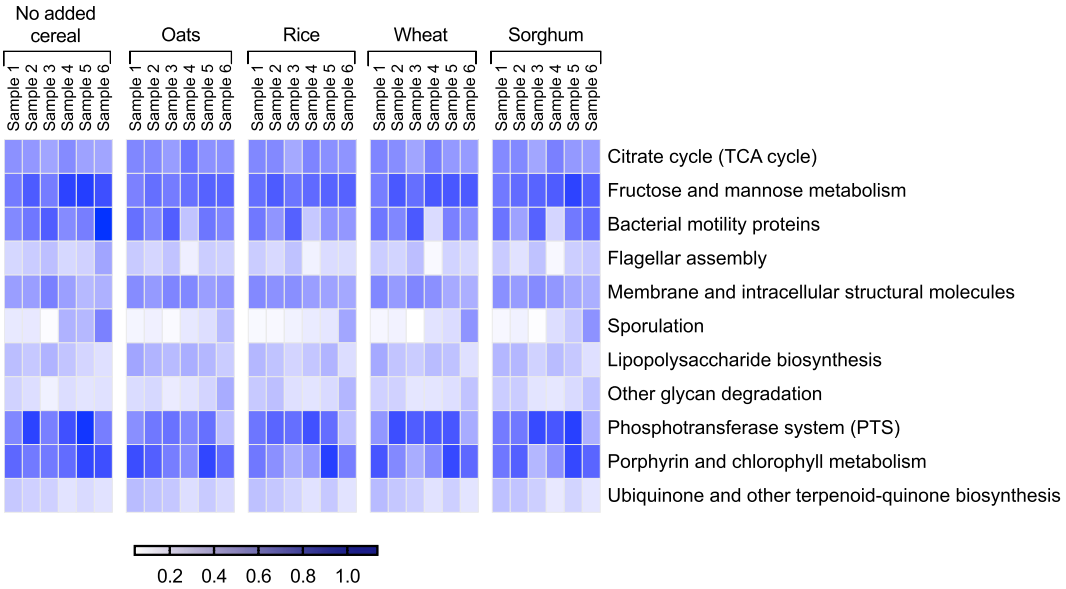

Figure 4. The predicted relative abundance of KEGG Orthology pathways for each sample with different cereal additions inferred using PICRUSt. The heat map shows the relative abundance of KEGG Orthology pathways (rows) with significant differences between treatments at 48 hours (columns) in at least five 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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Supplementary figures and tables

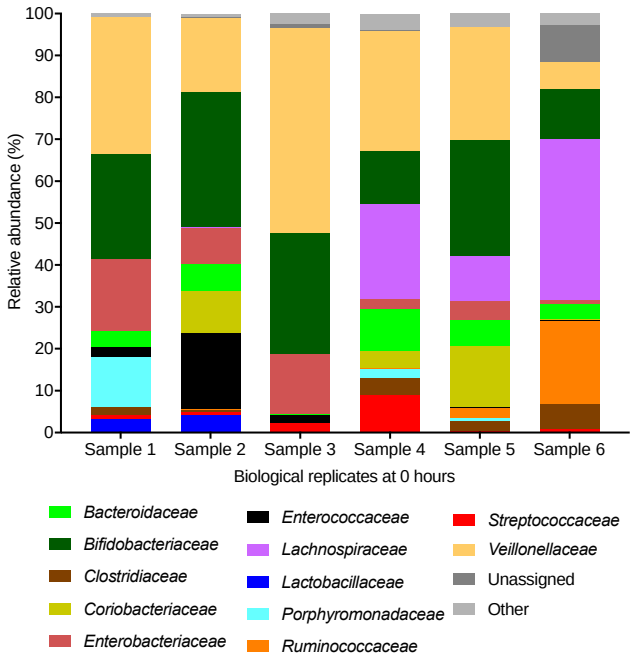

Figure S1 Family level taxonomic composition of the initial (at 0 hours) gut microbiota. The relative abundance at the family level was determined using QIIME and GraphPad Prism (V7). Bacterial identifications that were not assigned to a family are categorised as "Unassigned". Bacterial groups with less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

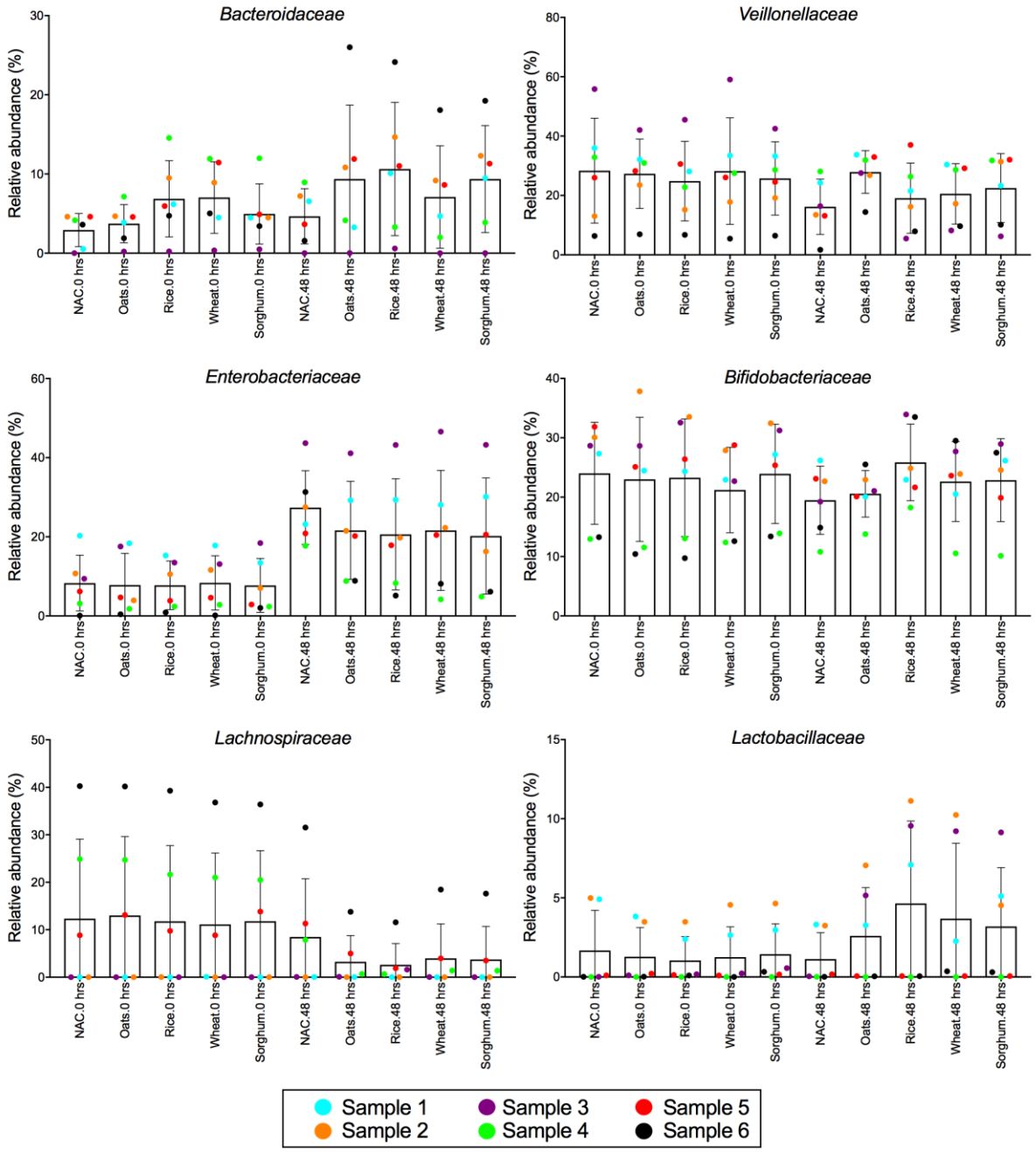

Figure S2 The relative abundance for bacterial families found to be significantly differentially abundant between treatments in at least three biological samples. Significance was determined using a Tukey's multiple comparisons test. Biological samples were analysed individually. The relative abundance 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.

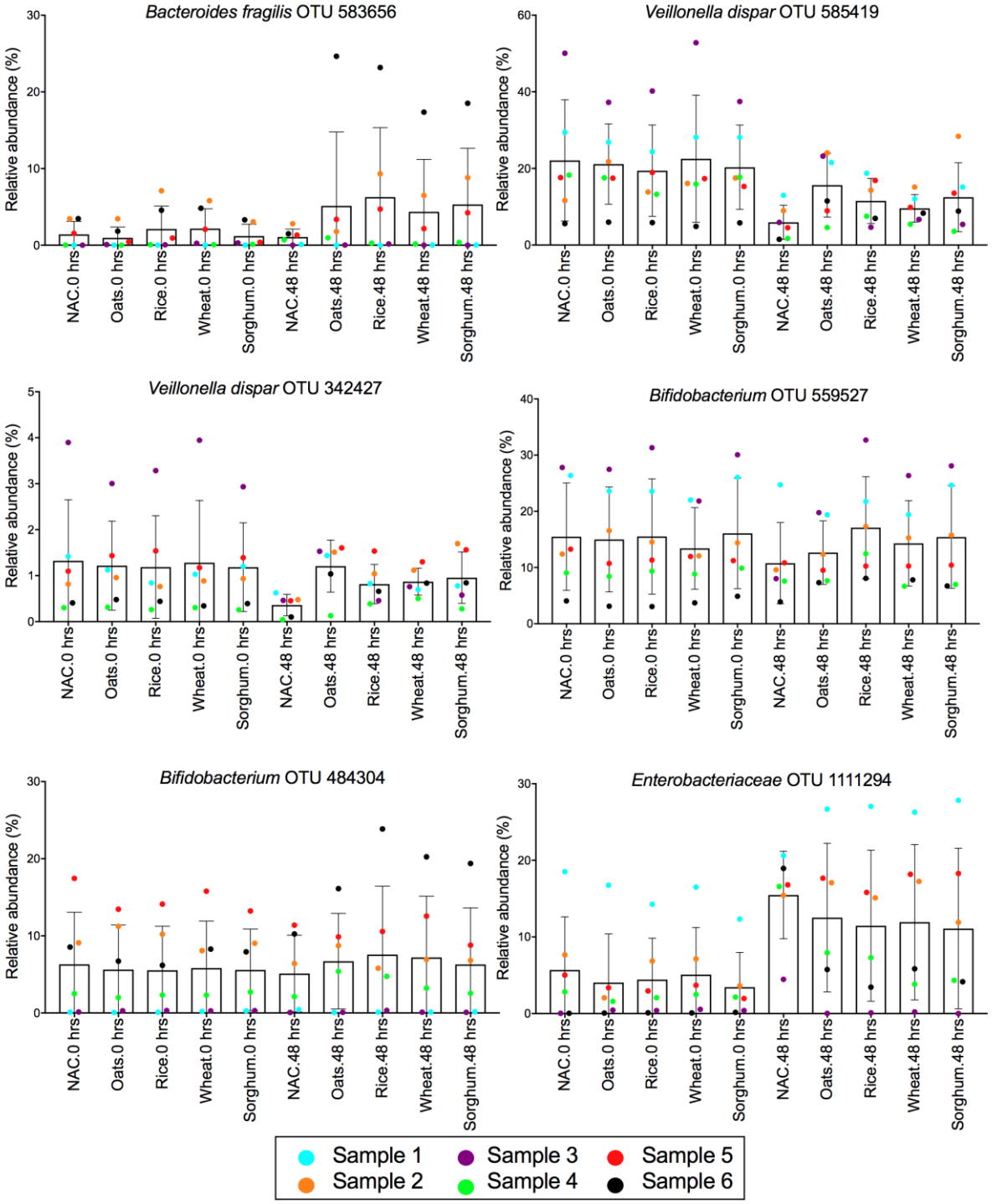

Figure S3 The relative abundance of OTUs found to be significantly differentially abundant between treatments in at least three biological samples. No added cereal control is abbreviated as NAC. Significance was determined using an ANOVA with Tukey's multiple comparisons test. Biological samples (sample 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.

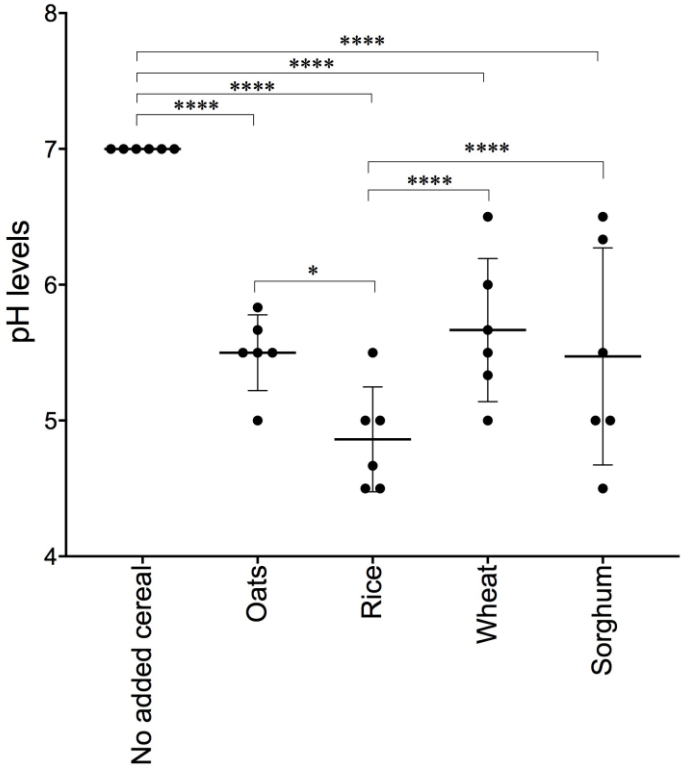

Figure S4 Measurements of pH for all cultures at 48 hours. Mean pH values for each of the three technical replicates in each of the six biological samples are indicated (dots). Bars represent the mean pH levels with ± SD for each treatment. Significance was determined using ANOVA with Tukey's multiple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

Supplementary tables (provided on the CD)

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 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 S5 The predicted relative abundance of KEGG Orthology pathways inferred using PICRUSt for each biological sample (sample 1-6) with different cereal additions. Mean ± SD for each treatment and time point (0 and 48 hours) 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.

CHAPTER 6

Conclusions and future directions

6.1 Conclusions

Diets low in microbiota-accessible carbohydrates significantly alter the gut microbiota, and lead to perturbation of the microbiota-host relationship [1]. This empirically shown association has initiated the concept of therapeutic modulation of the gut microbiota with the aim of treating or preventing gut microbiota-associated diseases. A variety of commercial supplements are currently available as dietary fibre or prebiotic products. However, only a limited number of these products have been scientifically investigated for their ability to alter the gut microbiota and lead to beneficial host health outcomes.

6.1.1 Fibre supplementation-mediated changes in gut microbiota community structure

We investigated the effect of specific commercially available dietary fibre products on the gut microbiota both *in vitro* and in C57BL/6J mice. *In vitro* fermentation of NutriKane™, Benefiber® and Macro Psyllium Husk with human adult gut microbiota dramatically shifted the overall microbial community structure and microbial diversity. Using a high fat diet fed mouse model we then investigated the potential for NutriKane and Benefiber dietary supplementation to ameliorate high fat diet-induced gut microbiota alterations. Neither fibre addition significantly shifted high fat diet-induced changes in the overall microbial community structure or loss in the gut microbial diversity, although changes in the abundance of specific OTUs were observed.

The combined effect of fibre supplementation (NutriKane and Benefiber) and weekly overnight fasting on the gut microbiota was also examined. Our results demonstrated clear shifts in the gut microbiota community structure upon fasting compared to the groups fed *ad libitum*. Fasting also resulted in a significant increase in the gut microbial diversity of mice fed a high fat diet or a high fat diet supplemented with NutriKane.

We then extended our studies to examine the effect of cereal products derived from wheat, sorghum, oats and rice on the infant gut microbiota using an *in vitro* infant gut microbiota

model system. Our results showed strong shifts in the microbiota community structure upon cereal addition, while the gut microbiota diversity increased only in specific individuals.

Overall, we demonstrated the potential of seven dietary fibre and cereal products in altering the gut microbial community structure. However, use of these fibre products as a part of the dietary fibre component had only a limited impact in improving high fat diet-induced alterations in the overall gut microbiota structure. We also demonstrated a significantly higher effect of fibre supplementation on the gut microbiota diversity upon combination with fasting.

6.1.2 Fibre product-specific effects on the gut microbiota

We examined whether dietary fibre or cereal product-mediated changes in the gut microbiota and metabolite production are similar between each product. Our results demonstrated significant product-specific alterations, suggesting that each product results in a unique gut microbiota composition and production of microbial metabolites.

Addition of NutriKane to the *in vitro* adult gut microbiota model system significantly promoted the growth of the OTUs in the genera *Coprococcus* and *Bifidobacterium,* whereas the relative abundance of the OTUs in the genera *Bacteroides, Parabacteroides* and *Faecalibacterium* significantly increased upon Benefiber addition. The abundance of many OTUs in the genus *Bacteroides* significantly increased with addition of Psyllium Husk. A similar product-specific increase was observed in the concentration of short chain fatty acids (SCFAs). These fibre product-dependent effects on the gut microbiota composition were mainly observed in the abundance of potential fibre-digesting bacteria and concentration of specific SCFAs.

Supplementation of high fat diet fed mice with NutriKane resulted in a significant increase in the abundance of the OTUs in the genera *Coprococcus, Lactobacillus, Oscillospira* and order *Clostridiales.* Similarly, supplementation with Benefiber promoted the abundance of the OTUs in the genera *Parabacteroides, Lactobacillus, Coprococcus* and family

Ruminococcaceae. While most of the high fat diet-induced reductions in the SCFA concentrations were not significantly increased with fibre addition, supplementation with Benefiber increased the production of propionate. This work confirmed the product-specific nature of impacts on the gut microbiota and metabolite production and provided a degree of *in vivo* support for the findings from the *in vitro* gut microbiota model system, with genera such as *Parabacteroides* and *Coprococcus* showing similar changes in abundance with specific fibre supplementation in the two systems.

Addition of cereal products to the *in vitro* infant gut microbiota model system resulted in common community shifts, as well as several product-specific changes. All tested cereals promoted the abundance of the families *Bacteroidaceae* and *Prevotellaceae*. Other families such as *Bifidobacteriaceae, Lactobacillaceae* and *Veillonellaceae* showed increased abundance due to specific cereal additions. However, many of the cereal addition-mediated changes in the abundance of specific gut bacteria and increase in the concentrations of SCFAs were individual-specific. This is likely due to the high degree of variability in the starting gut microbiota populations in the infants, and the varying ability of the microbiota to digest a range of polysaccharides.

Our results demonstrated product-dependent effects on the abundance of bacteria associated with fibre digestion. We observed product-specific increases in the abundance of bacteria in the genera *Bifidobacterium*, *Lactobacillus* and *Faecalibacterium,* which have been previously linked to beneficial host health outcomes. These product-specific impacts could be due to distinct variations in the ingredients and chemical structure of dietary fibre within each product. For example, NutriKane is derived from dried sugarcane stem and pectin, which could be rich in glucose, xyloglucans, xylans, glucomannan, arabinoxylan and glucuronoxylan. In comparison, Benefiber is derived from wheat dextrin, which is a polymer of D-glucose subunits. As most commercial fibre products have unique combinations of

ingredients, there is clearly a need for scientific evaluation of in the impact of specific products on the gut microbiota.

6.1.3 Can the effects of fibre addition on gut microbiota be linked to host health?

The bacterial family *Enterobacteriaceae* is commonly observed in pre-weaned infant gut microbiota, however it decreases in abundance upon weaning [2]. Our results demonstrated the potential of all four cereal products to reduce the abundance of this family in the infant gut microbiota. In addition, cereal supplementation promoted the growth of at least one bacterial group with known fibre-digesting ability and increased the concentration of SCFAs. These are characteristics of an adult gut microbiota, hence may indicate the potential of the tested cereal products to aid the establishment of an adult-like gut microbiota in infants.

In the adult human gut microbiota, a high abundance of the family *Enterobacteriaceae* has been previously linked with inflammation [3, 4]. In our *in vitro* study, the abundance of this family significantly reduced upon addition of NutriKane, Benefiber and Psyllium Husk. The abundance of the phylum *Proteobacteria* and many OTUs in the families *Clostridiaceae, Erysipelotrichaceae* and *Bacteroidaceae* significantly decreased upon supplementation with NutriKane or Benefiber. which were all observed to be highly abundant in the gut microbiota of high fat diet fed mice.

High fat diet fed mice demonstrated significantly altered host glucose tolerance and caecum mass, these were not significantly alleviated by inclusion of either NutriKane or Benefiber as a replacement for cellulose in the high fat diet. Suggesting that the specific fibre supplementations have a minimal effect on high fat diet-induced changes in the mouse physiology. Inclusion of these products in addition to the existing fibre component (cellulose) of the high fat diet may have beneficial effects on the host physiology. Mice fed a high fat diet with fasting showed similar glucose tolerance to mice fed normal chow, which may

suggest the potential of fasting in controlling high fat diet-induced impaired glucose clearance.

Overall, this work provides useful insight into the effect of seven commercially available dietary fibre and cereal products on the gut microbiota. Supplementation with each product demonstrated a product-dependent impact on the abundance of the gut bacteria related to fibre digestion. Fibre supplementation also mediated changes in the abundance of some gut bacteria with links to inflammation and high fat diet-induced gut microbiota alterations. We also demonstrated that these fibre products have a limited ability in ameliorating high fat diet-induced impaired glucose clearance and other changes in the host physiology. Our work highlights the importance of scientific evaluation of the impact of commercial fibre products on the gut microbiota and exploration of strategies to enhance the impact of fibre supplementation potentially through combining with other treatments or dietary modifications, such as fasting.

6.2 Future directions

6.2.1 Use of meta-omics techniques to study the gut microbiota

In the present work we used 16S rRNA amplicon sequencing to examine the effect of each treatment on the gut microbiota composition. This method is relatively economical, and therefore enables the investigation of larger numbers of samples but is limited to providing information about the taxonomic composition of the microbial community. Application of shotgun metagenomics would provide information on the full suite of genes within the metagenome and allow prediction of complete metabolic networks. In addition, use of metatranscriptomics would also benefit the present study through providing information on active gene expression of the gut microbiota.

We examined the effect of fibre and cereal products on the gut microbial metabolite production through quantification of three major SCFAs, acetate, butyrate and propionate as

they are the main end products from gut microbial-mediated fibre degradation. The gut microbiota is a major contributor in producing a range of metabolites that have well-established impacts on the host physiology. This includes SCFAs but also other metabolites such as bile acids, choline, vitamins, amino acids, lipids, phenol and phenyl derivatives. Therefore, examining the metabolic profile of the gut microbiota through metabolomics would provide useful information on the functional profile of the gut microbiota and may also facilitate the establishment of links between the gut microbiota and host.

We were limited to using 16S rRNA amplicon sequencing and quantification of only three SCFAs due to financial and time constraints. However, we believe the use of meta-omics techniques to examine the gut microbiota composition and functions would be useful in gaining further insight to the gut microbiota response towards the tested products.

6.2.2 Examining the host responses using omics-techniques

The host physiological responses to fibre supplementation were assessed in the present study through glucose tolerance tests, measurement of the body weight, weight of specific organs and tissues and feed intake. Ongoing work by the collaborators involved in the current study includes examination of the host colon epithelial cell transcriptomics, liver proteomics and colon mucin glycomics.

The gut microbiota and its metabolites have been previously linked with the regulation of host gene expression and production of specific proteins [5]. Furthermore, previous studies have also demonstrated an association between the mucin glycans and gut microbiota composition [6]. Information obtained through host trancriptomics, proteomics and glycomics will provide further insight into potential host physiological responses to dietary interventions and alterations in the gut microbiota.

6.2.3 *In vivo* experiments using human volunteers

Utilisation of *in vitro* gut microbiota and mouse model systems are useful in the gut microbiota studies as they provide useful insights while reducing issues in volunteer compliance, complications in ethical approval and variations in human host factors such as diet, environment and genetics [7, 8]. However, human clinical trials will be important in confirming the effect of these products on the human gut microbiota and on human health outcomes.

The experiments using NutriKane were initially planned to be conducted using studies involving human volunteers and clinical samples. The industry partner of this part of the project, Gratuk Technologies Pty Ltd was responsible for arranging such trials, however, due to several issues this was delayed and has not yet eventuated. To ensure timely completion of the current project, we conceived experiments using *in vitro* and mouse model systems. We foresee the importance and potential of testing these fibre products on human adult volunteers. These experiments could be conducted using individuals with diet-induced health issues such as impaired glucose tolerance and healthy volunteers as controls. Observations in the present work such as limitations in using these fibre products as a part of the dietary fibre component of a high fat diet and enhanced effect of fibre addition on the gut microbiota upon combination with fasting provide useful information for designing future studies using human volunteers.

Upon examination of the effect of cereal addition on the gut microbiota of infants *in vitro*, we observed several individual-specific responses. This could be due to the higher inter-individual variation of the initial gut microbiota composition in infants [9]. Human volunteer experiments to extend the findings in Chapter 5 would preferably be tested in infants spanning a narrower age range to minimise age related variations in the gut microbiota. For all experiments using human volunteers, it is recommended to closely monitor factors that can

influence the gut microbiota such as the normal diet, intake of antibiotics, age, gender and environment.

6.2.4 Investigating strategies to enhance fibre supplementation-mediated effects

In the present study we demonstrated the effect of fasting in altering the gut microbiota of high fat diet fed and fibre supplemented mice (Chapter 4). We think there is considerable potential to confirm these interesting findings and expand on this work through additional experimentation with a modified experimental design. The concept of combining strategies that have been previously shown to therapeutically modulate the gut microbiota (for example probiotics, prebiotics, time-restricted feeding) could hold promising potential in enhancing the beneficial effect on the gut microbiota. For instance, the use of synbiotics (combination of prebiotics and probiotics) has been reported for beneficially altering the gut microbiota [10].

Future experiments could be designed to investigate the impact of the tested fibre products on the gut microbiota in combination with specific feeding patterns (for example the 5:2 diet), probiotic microbial strains, dietary fibre from different sources and/or other non-carbohydrate compounds with prebiotic effects (polyphenols). *In vitro* gut mimicking model systems and/or mouse models can be useful for conducting proof of concept experiments which test the efficacy of such strategies. However, experiments using human volunteers would be essential in gaining further understanding of the effect of these strategies on the gut microbiota and host health related outcomes.

6.2.5 Standardising a framework for regulating the market

The concept of supplementation of dietary fibre for therapeutic modulation of the gut microbiota has fuelled a multi-billion-dollar global market, which in Australia alone is expected to reach US$60 million by 2020 [11]. However, a major limitation in this rapidly developing market is the lack of products with scientific evidence showing clear health and microbiota related outcomes (Table 6.1).

It is timely to recognise and promote products with scientific evidence for beneficial health outcomes and regulate mandatory scientific evidence to market products with prebiotic health claims. In parallel, it would be of significant benefit to the public if there were more robust scientific standards and governmental regulation related to the health benefits of these products. Well-designed and controlled clinical trials with clear outcomes on the gut microbiota and health could be made essential to market food products as medicines or functional foods. Additionally, government support for innovative scientific research is important for further development of the industry [12].

Table 6.1 A non-exhaustive list of dietary fibre available in the market with product names. Products with established gut microbiota accessible carbohydrates are indicated with an asterisk (*). Table adapted from Deehan and Walter 2016 [12] and modified (licence number 4310490379471).

Dietary fibres	Fibre products
Resistant starch	ActiStar® RM, Fibersym®* RW, pHi-MAIZE® 260*, PENFIBE® RS4
Arabinoxylan	Biofiber Gum , NAXUS®
β-Glucan	B-CANTM , PromOat® , Wellmune® , Yestimun®
Cellulose	GRINDSTED® MCC, MICROCEL, Solka-Floc®, Vitacel®
Inulin/oligofructose	Actilight®*, Frutalose® L90*, NUTRAFLORA®*, Oliggo-Fiber® DS2*, Orafti® Synergy1*
Galactooligosaccharide, xylooligosaccharide	Bimuno®, BIOLIGO® GL, Vivinal® GOS*, Longlive XOS*, NovaGreen XOS
Human milk oligosaccharides	Mum's Sweet Secret, Glycom
Polydextrose	STA-LITE®, Litesse® II*, NUTRIOSE® FB*
Wheat dextrin	Benefiber®*
Soluble corn fibre	PROMITOR®*
Alginate	Algogel™*, KIMICA ALGIN, Manugel DMB*
Pectin	Citrus Pectin USP, GENU® Pectin C74*, Unipectine®
Gum arabic/acacia gum	Agri-Spray Acacia®, EmulGold®*, Fibregum™*, Gum Arabic SD
Guar gum	GuarNT®, Ricol Rg-250, Viscogum™, Guar Guma
Fibre-rich raw materials	Best' Pea Fiber*, Corn Z-Trim®, Cranberry Fiber, Fibrex® Sugar Beet*, FIBRIM® Soy, Unicell® WF
Sugarcane fibre	NutriKaneD™*, Phytocell Kfibre, FutureBiotics Colesterole balance,
Psyllium husk	Macro Psyllium husk*, Meta Mucil®*, Nature's Way

There is a significant limitation in the functional food industry with regards to obtaining disease-oriented health certification for food ingredients or products even when scientific research provides support for product efficacy. The government policies in many countries need to be changed to enable dietary fibre products with clinical evaluations to promote the findings of such evaluations in a clear way. This could preferably be done by implementing policies specifically for foods, independent of drugs. Effective communication of scientifically validated outcomes of products to the society is crucial in increasing the public awareness of which products have substantiated claims. This will facilitate consumer choice, enabling them to identify products with clinically proven beneficial outcomes on the gut microbiota and host health.

6.2.6 Developing personalised-prebiotics

An accumulating amount of research and large-scale projects such as the NIH human microbiome project and the human food project aim to understand the role of the gut microbiota on host physiology and to improve human health through monitoring or manipulating the human gut microbiota [13]. The human gut microbiota composition is unique in different individuals. Hence many previous studies have highlighted the potential use of personalised-diets to improve the gut microbiota health.

Different gut bacteria have the potential to digest different types of dietary fibre, while some can digest a broader range of dietary fibre, some bacteria are more specific. Scientific knowledge regarding gut microbial dietary fibre specificity and mechanisms of fibre digestion is currently rapidly expanding [14, 15]. Due to the individualised nature of the gut microbiota and gut bacterial specificity of dietary fibre digestion, it could be possible to design fibre products (chemically synthesised or combinations of raw materials) to alter specific conditions of the gut microbiota of an individual. Hence, it is timely to invest scientific and manufacturing efforts in designing personalised-prebiotics to target the gut microbiota to improve human health.

6.3 References

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APPENDIX I

Ethics approval letters

MACQUARIE UNIVERSITY

Office of the Deputy Vice-Chancellor (Research)

Research Office
C5C Research HUB East, Level 3, Room 324
MACQUARIE UNIVERSITY NSW 2109
AUSTRALIA

Phone **+61 (0)2 9850 4194**
Fax +61 (0)2 9850 4465
Email ethics.secretariat@mq.edu.au

9 September 2014

Professor Ian Paulsen

Department of Chemistry and Biomolecular Sciences

Faculty of Science

Macquarie University NSW 2122

Dear Professor Paulsen

Reference No: 5201400595

Title: Analysis of changes in the gut microbiome due modification of diet with a food supplement

Thank you for submitting the above application for ethical and scientific review. Your application was considered by the Macquarie University Human Research Ethics Committee (HREC (Medical Sciences)) at its meeting on 24 July 2014 at which further information was requested to be reviewed by the HREC (Medical Sciences) Executive.

The requested information was received with correspondence on 4 September 2014.

The HREC (Medical Sciences) considered your responses at its meeting held on 8 September 2014.

I am pleased to advise that ethical and scientific approval has been granted for this project to be conducted at:

- Macquarie University

This research meets the requirements set out in the *National Statement on Ethical Conduct in Human Research* (2007 – Updated March 2014) (the *National Statement*).

Details of this approval are as follows:

Approval Date: 8 September 2014

The following documentation has been reviewed and approved by the HREC (Medical Sciences):

Documents reviewed	Version no.	Date
Macquarie University Ethics Application Form		
Correspondence from Hasinika Hewawasam Gamage responding to the issues raised by the HREC (Medical Sciences) – including attachments		Received 4/09/2014
MQ Participant Information Sheet	1	3/09/2014
Study Advertisement	2	4/09/2014
Metadata Sheet	N/A	undated

Statement on future use of data/biospecimens and N/A undated the forms.

This letter constitutes ethical and scientific approval only.

Standard Conditions of Approval:

1. Continuing compliance with the requirements of the *National Statement*, which is available at the following website:

   http://www.nhmrc.gov.au/book/national-statement-ethical-conduct-human-research

2. This approval is valid for five (5) years, subject to the submission of annual reports. Please submit your reports on the anniversary of the approval for this protocol.

3. All adverse events, including events which might affect the continued ethical and scientific acceptability of the project, must be reported to the HREC within 72 hours.

4. Proposed changes to the protocol must be submitted to the Committee for approval before implementation.

   It is the responsibility of the Chief investigator to retain a copy of all documentation related to this project and to forward a copy of this approval letter to all personnel listed on the project.

Should you have any queries regarding your project, please contact the Ethics Secretariat on 9850 4194 or by email ethics.secretariat@mq.edu.au

The HREC (Medical Sciences) Terms of Reference and Standard Operating Procedures are available from the Research Office website at:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_rese arch_ethics

The HREC (Medical Sciences) wishes you every success in your research.

Yours sincerely

Professor Tony Eyers
Chair, Macquarie University Human Research Ethics Committee (Medical Sciences)

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research* (2007) and the *CPMP/ICH Note for Guidance on Good Clinical Practice*.

THE UNIVERSITY OF SYDNEY

RESEARCH INTEGRITY
Animal Ethics Committee

Wednesday, 1 July 2015

Assoc Prof Anandwardhan Hardikar

School of Public Health: NH&MRC Clinical Trials Centre; Sydney Medical School
The University of Sydney

Email: anand.hardikar@sydney.edu.au

Dear Assoc Prof Hardikar

Project Title: Effects of changes in gut microbiota on development of obesity and diabetes.

Project Number: 2014/611

Your request to modify the above project submitted on **22 May 2015** was considered by the Animal Ethics Committee at its meeting on **18 June 2015.**

The Committee had no ethical objections to the modification(s) and has approved the project to proceed with change in protocol.

Details of the approval are as follows:

Authorised Personnel: Hardikar Anandwardhan; Joglekar Mugdha; Kristensen-Walker Holly; Satoor Sarang; Wong Wilson; Bucio-Noble Daniel; Chong Wei; Kautto Liisa; Gamage Hasinika;

Change to Study Procedures as outlined in the Modification to an Existing Approved Application Form in IRMA.

Documents Approved:

Date	Type	Document
22/05/2015	Other	Experimental plan

Animals Approved:

Please refer to the document at the end of this letter, which details your approved animal usage.

Conditions of Approval

Approval of this project is conditional upon your adherence to the conditions outlined in this letter and your continuing compliance with the Animal Research Act (1985 – Animal Research Regulation 2010) and the 8th Edition of the Australian code for the care and use of animals for scientific purposes (NHMRC 2013).

1. The Animal Ethics Committee (AEC) reviews and approves protocols for their compliance with the NSW Animal Research Act (and associated regulations) and the 8th Edition of the Australian code for the care and use of animals for scientific purposes (NHMRC, 2013).
2. This approval is in accordance with your original submission together with any additional information provided as part of the approval process.
3. Any changes to the protocol must be approved by the AEC before continuation of the study. This includes notifying the AEC of any changes to named personnel, source of animals, animal numbers, location of animals and experimental procedures.
4. Investigators should promptly notify the AEC of any unexpected **adverse events** that may impact on the wellbeing of an animal in their care, as per Clause 2.1.5 [v] [d] and 2.4.34 [ii] in the Australian code of practice (NHMRC, 2013). In the event that an unexpected adverse event occurs, please refer to the Animal Ethics website and log into IRMA to complete an Adverse Event form. For further information, please see the AEC Adverse Event Reporting Procedures (GL003) on the Animal Ethics website.

5. In the event an animal dies unexpectedly or requires euthanasia for welfare reasons, an autopsy should be performed by a person with appropriate qualifications and/or experience and the AEC should be notified promptly.

6. Animals must not be euthanised within sight or sound of other animals, in accordance with Clause 3.3.45 [vi] of the Australian code of practice (NHMRC, 2013).

7. Animals should not be housed singly unless otherwise approved by the AEC.

8. All animals must be provided with environmental enrichment appropriate for their species, unless otherwise approved by the AEC.

9. All pens, cages and containers used for holding animals must be clearly identified with chief investigator name, number of animals, DOB if provided and date of arrival, sex and strain.

10. A copy of this approval letter, together with all relevant monitoring records, must be kept in the facility where your animals are housed. These records must be updated regularly as breeding and husbandry events occur and current copies must be maintained in the animal house. Monitoring sheets must contain a section where expected post-operative effects are identified and observations recorded. Where relevant, the pens, cages and container number must be recorded on the monitoring sheet to ensure that affected animals can be easily located. Where electronic breeding

records are kept instead of records on cage cards, printed copies of the records should be placed in a folder in the relevant animal house, where they can be inspected by the AEC.

11. Data should be accurately recorded in a durable, indexed and retrievable form that complies with relevant legislation, policy and guidelines. Following completion of the study all data including consent forms must be retained in a secure location, such as a locked filing cabinet, at the University of Sydney for a period of at least seven (7) years.

12. The AEC will make regular announced inspections of all animal facilities and/or specific research protocols. The Animal Welfare Veterinarian will be conducting unannounced inspections of all animal facilities and/or specific research protocols.

13. All new investigators must successfully complete the Introduction to Animal Research (ITAR) course.

Please do not hesitate to contact the Research Integrity (Animal Ethics) Office at animal.ethics@sydney.edu.au should you require further information or clarification.

Yours sincerely

Professor David Allen

Chair

THE UNIVERSITY OF SYDNEY

Animal Ethics Committee

The AEC is constituted and operates in accordance with the NSW Animal Research Act (1985) and its associated Regulations, the 8th Edition Australian code for the care and use of animals for scientific purposes (NHMRC, 2013) and the Australian Code for the Responsible Conduct of Research (2007). All personnel named on the protocol should be conversant with these documents.

MACQUARIE University

HASINIKA KALHARI ARIYARATNE HEWAWASAM GAMAGE <hasinika-kalhari-ariyarat.h@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
PhD, Post Doctoral Fellow
ARC Training Centre for Molecular Technology in the Food Industry
Dept of Chemistry and Biomolecular Sciences
Level 3, E8C304
Macquarie University
NSW 2109 Australia

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

 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 onemonth 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 tothe 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 thatmay 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 guidelinesestablished 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

APPENDIX II

Biohazard risk assessment approval

SECTION A

MACQUARIE UNIVERSITY

Biohazard Risk Assessment Form – NON GMO

Notification Number:	*IAP310314BHA2*

Department	**Chemistry and Biomolecular Sciences**	**Date:**	**31/3/2014**
Chief investigator:	**Prof. Ian Paulsen**		
Contact number/email:	**x8152/ian.paulsen@mq.edu.au**		
Title of research/practical	**Metagenomic analyses of natural and human impacted environments**		
Is additional approval required?	**Animal Ethics** ☐ **Human Ethics** ☒ **Fieldwork Manager** ☐ **Other** ☐ **(state)**_____ Human Ethics may be required for some Group 2 samples		

Exact location(s) of research:

Primary location: E8A PC2 facility, lab 2; some work on coal seam samples will be conducted at CSIRO, North Ryde and work on uranium-contaminated soils will be conducted at the ANSTO Lucas Heights facility (see below).

Control measures: Eliminate risk ☐ **Substitute the hazard** ☐ **Isolate the hazard** ☒ **Implement engineering controls** ☒ **Administration** ☒ (e.g. Training) **PPE**☒
E.g. Eliminate by irradiation prior to use, isolation by class II biological safety cabinets, administration by following SWP as below, PPE as listed below.

Supporting documents which must be read in conjunction with this assessment. (e.g. Safe Working Procedures, Safety Data Sheets, Guidelines/Protocols)

MQU - A guide to Biological Risk Management: http://staff.mq.edu.au/human_resources/health_and_safety/policies-procedures-guidelines_forms/

What is the type of the biological material?

Bacteria ☒ **Fungi** ☒**Virus** ☐ **Cell Line** ☐ **Tissue** ☐ **Parasite** ☐ **Animal** ☐ **Plant** ☐ **Soil** ☒ **Toxin** ☐ **Prions** ☐ **Nucleic Acid**☒ **other** ☒ **microbial communities (mix of bacteria, fungi, archea, etc.)**

What is the name of the biological agent?

Microbial community samples from human faecal samples and animal faecal and gut samples for metagenomic and 16S rRNA sequence analyses
Microbial community samples from coal seams, aquatic, ocean and soil samples for metagenomics and 16S rRNA sequence analyses.

List the Personal Protective Equipment required:

Gloves ☒_____(e.g. chemical resistant) **Eye protection** ☒_____(e.g. safety glasses/goggles) **Clothing**_____ ☒ (e.g. button up lab coat/coveralls/apron)

Footwear ☒_____(e.g. Enclosed/Gumboots/overshoe covers) **Respiratory Protection** ☐_ (e.g.PF2 face mask) **Other** ☐_____

What are the risks associated with this Biological Agent. (Can be more than one risk group depending on method)			
Risk Group	**Details of Biohazards including risks associate with biological agent** http://www.absa.org/riskgroups/index.html	**Biosafety level**	**Risk Reduction Measures** (must be followed by the researcher)
Group 1- Low Individual and community risk (Microorganism that is unlikely to cause human, plant or animal disease)	Samples will consist of microbial communities collected from sites including: 1) coal seams*; 2) terrestrial aquatic environments (sink holes, underground caves etc.); 3) coastal and open ocean marine environments, or 4) soils including uranium contaminated soil** Microorganisms isolated from these environments are not likely to pose human health risks. However, the samples will be processed in our PC2 facility in accordance with PC2 standards (PPE: lab coat, gloves, closed footware). *Note: **Note: Samples from uranium-contaminated soils will be processed at the ANSTO Lucas Heights facility, following ANSTO guidelines for containment of radioactive hazards. Highly purified DNA may be used at Macquarie.	PC2	1 Standard laboratory procedures will be followed in accordance with Laboratory Microbiological Standards AS/NZ 2243:3:2010 and university guidelines (see supporting documents - Section A above) and include spillage and emergency response. 2 Investigator has attended university Biosafety training course (see 3) 3 Chief Investigator identified in Section A confirms that the researchers have received appropriate training and instruction or has adequate supervision and understands safe laboratory practice according to AS/NZ2243:3:2010 and university guidelines (see supporting documents - Section A above)
Group 2- Moderate individual risk, limited community risk (Microorganism that is unlikely to be a significant risk to laboratory workers, the community/livestock/environment. Laboratory exposures may cause infection but effective treatment and preventative measures are available and the risk of spread is limited).	Samples will consist of microbial communities collected from: 1) human or animal guts, or 2) human or animal faecal samples. Microorganisms isolated from these environments are pose moderate human health risks. The samples will be processed in our PC2 facility in accordance with PC2 standards, e.g., PPE will be used: lab coat, gloves, closed footware).	PC2	1 Standard laboratory procedures will be followed in accordance with Laboratory Microbiological Standards AS/NZ 2243:3:2010 and university guidelines which are appropriate for **Risk Group 2** (see supporting documents - Section A above) and include spillage and emergency response. 2 Researcher has attended university Biosafety training course (see 3) 3 Chief Investigator identified in Section A confirms that the researcher has received appropriate training and instruction or has adequate supervision and understands safe laboratory practice according to AS/NZ 2243:3:2010 and university guidelines (see supporting documents - Section A above)

Group 3 -High individual risk, limited community risk (Microorganisms that usually causes serious human or animal disease and may present a significant risk to laboratory workers. It could present a limited to moderate risk if spread in the community or the environment, but there are usually effective preventative measures or treatment available).	NA		1 Standard laboratory procedures will be followed in accordance with Laboratory Microbiological Standards AS/NZ 2243:3:2010 and university guidelines which are appropriate for **Risk Group 3** (see supporting documents - Section A above) and include spillage and emergency response. 2 Researcher has attended university Biosafety training course (see 3) 3 Chief Investigator identified in Section A confirms that the investigator has received appropriate training and instruction or has adequate supervision and understands safe laboratory practice according to AS/NZ 243:3:2010 and university guidelines (see supporting documents - Section A above)

Process and equipment to be used	You must include: - Brief description of work, control measures (including aerosols), sample storage, transport of samples, clean up procedures, disinfectant and waste disposal.

Brief description of work: Samples will be collected from field sites by suitably qualified professionals (field work risks will be outlined in separate risk assessments specific to each project). Sample processing will involve DNA isolation using extraction methods appropriate to the samples of interest (e.g. the MO BIO, PowerSoil® DNA isolation kit). Isolated DNA will be subject to shotgun sequencing using next-gen technologies.
The project associated with microbial analysis of human and animal biological samples contains two major aims 1) In vitro analysis of gut microbiome and 2) In vivo analysis of gut microbiome changes due to consumption of a food supplement. Samples for the first aim will be collected from human volunteers in Macquarie University (Human ethics approval form submitted). Human and animal biological samples for the second aim will be supplied by the industry partner of the project (Gratuk Technologies Pty Ltd). The location and the method of sample collection for Aim 2 is yet to be confirmed.

Control measures (including aerosols): All the experimental work will be carried out in biosafety cabinets in order to ensure aerosols produce do not contact the investigator or others. Tubes containing the samples will be opened only in safety cabinets until extracted DNA is obtained from the samples.Personal protective equipment will be worn in accordance with PC2 regulations (i.e. wearing gloves, lab coat and appropriate footware). All investigators will be trained in handling pathogenic bacteria using aseptic techniques to prevent contact with bacteria and will attend biosafety workshops.

Microbial analysis of human and animal biological samples- Samples for Aim 1 of this project will be collected from healthy volunteers (confirmed with an information sheet) hence will not be screened. This part of the project is subject to human ethic approval outcome. Human and animal biological samples collected for Aim 2 will be screened. All the samples screened/not screened will be treated as a sample with potential risks therefore precautions to avoid any adverse effect on the investigator or others in the laboratory will be taken.

Sample storage: Samples will be stored in a PC2 laboratory in clearly labelled unbreakable containers. Long term storage will be as -80C freezer stocks in designated freezers in a PC2 laboratory.

Transport of samples: Samples will be transported in double packaged unbreakable containers. If group 2 samples need to be transported they will be carried only by

All the experiments are to be carried out in PC2 labs.

Waste disposal: Waste from Group 1 samples will be autoclaved and disposed on in designated autoclave waste bins. Waste material from Group 2 samples will be disposed of as clinical waste.

SECTION B

MACQUARIE UNIVERSITY

Biohazard Safety Committee – Risk Assessment Decision

Important Information

For **non GMO** investigations email this assessment to biohazard@mq.edu.au for approval by the **Biohazard** Safety Committee.

Individual Responsibilities

By submitting this assessment the Chief Investigator identified in Section A, confirms that any supporting documents, training, guidance, instruction or protocols issued by the University will been followed so far as reasonably practicable to ensure the work is carried out without risk to health, safety or the environment. The Chief investigator is responsible for ensuring, so far as reasonably practicable the safety of researchers and others who may be affected by the work described within this document.

Decision to be completed by the Biohazard Safety Committee:

The Committee has agreed that this risk assessment is sufficient for investigations to commence? **Yes** √☐ **No** ☐ **Further action required** √☐

Further Action/Comments:
The committee has reviewed your application and it is now approved.

Name of Approver (Committee Rep):	J Cuomo
Date Approved:	8 th of August 2014

This Risk Assessment must be approved for work to commence