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The Impact of Dietary Components on Gut Microbiome Modulation and Health

Thesis presented by

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Author Contributions

Chapter 2: The microbiome modulating potential of superheated steam (SHS) treatment of dietary fibres: Carried out *in vitro* colonic fermentation via MicroMatrix^{TM} *in vitro* batch fermentation system. Performed DNA extractions. Prepared 16S rRNA gene compositional sequencing libraries. Performed SCFA sample preparation and carried out GC-FID (gas chromatography-flame ionisation detection) analysis. Performed statistical analysis and part of bioinformatics analysis. Drafted the manuscript. Dr. Stefano Renzetti and Dr. Martijn Noort performed superheated steam treatment on brans and performed physicochemical analysis of samples. Dr. Kiera Murphy performed part of the bioinformatics analysis of 16S rRNA gene sequencing data. Dr. Ivan Sugrue performed *in vitro* digestion and dialysis. Prof. R. Paul Ross and Prof. Catherine Stanton designed the study and acquired ethical approval.

Chapter 3: Biscuits for the gut: A symphony of FODMAPs and dietary fibre in gut microbiome for irritable bowel syndrome (IBS) management: Applied for ethical approval. Collected and prepared faecal slurry. Performed *in vitro* digestion, colonic fermentation, and DNA extractions. Prepared 16S rRNA gene sequencing libraries. Performed SCFA sample preparation and operated GC-FID. Performed bioinformatics analysis and statistical analysis. Drafted the manuscript. Prof. Elke Arendt, Dr. Aylin Sahin and Dr. Jonas Atzler provided biscuit samples. Prof. Paul Ross and Prof. Catherine Stanton designed the study.

Chapter 4: Impact of low FODMAP sourdough bread on gut microbiota using an *in vitro* colonic fermentation model: Applied for ethical approval. Collected and prepared faecal slurry. Performed *in vitro* digestion, colonic fermentation. Performed

DNA extractions. Prepared 16S rRNA gene sequencing libraries. Performed SCFA sample preparation and operated GC-FID. Performed bioinformatics analysis and statistical analysis. Drafted the manuscript. Prof. Aidan Coffey provided strains used in sourdough fermentation. Prof. Elke Arendt provided sourdough fermented breads. Prof. Paul Ross and Prof. Catherine Stanton designed the study.

Chapter 5: The Potential Prebiotic and Postbiotic Effects of Tarhana Produced from Different Flours on Human Gut Microbiome Using an *in vitro* Fermentation Model: Applied for ethical approval. Collected and prepared faecal slurry. Performed *in vitro* digestion, colonic fermentation and DNA extractions. Prepared 16S rRNA gene sequencing libraries. Performed SCFA sample preparation and operated GC-FID. Performed bioinformatics analysis and statistical analysis. Drafted the manuscript. Prof. Sine Ozmen Togay and Prof. Yasemin Sahan provided tarhana samples. Prof. Sine Ozmen Togay, Prof. Yasemin Sahan, Prof. Catherine Stanton and Prof. Paul Ross designed the study.

Chapter 6: Shaping Metabolic Health: The Role of *Lactobacillus mucosae* DPC6426 and Dietary Fibre in a Porcine Model of Metabolic Syndrome: Carried out metabolomics analysis at The Metabolomics Innovation Centre in University of Alberta. Prof. David Wishart, Dr. Rupasri Mandal, Mathew Johnson and Dr. Tammy Zheng created the metabolomics method and performed some of the analysis. Performed statistical analysis and generated graphics. Drafted the manuscript. Prof. Noel Caplice, Dr. Florence Herisson, Dr. Maria Antonia Llopis-Grimalt, and Dr. Aoife O'Donovan performed pig trials and provided faecal and blood samples. Prof. Noel Caplice, Prof. Catherine Stanton and Prof. Paul Ross designed the study and provided ethical approval.

Chapter 7.2: Gut Microbiome in Children with Congenital Heart Disease after Cardiopulmonary Bypass Surgery (GuMiBear Study): Performed DNA extraction. Prepared 16S rRNA gene sequencing libraries. Drafted the manuscript. Dr. Claire Magner, Dr. Sean Kelleher, Mong Tan, Molly O'Toole, Dominic Jenkins, Jordan Boyle, Marielle Lavelle and Niamh Maguire collected samples. Dr. Kiera Murphy performed statistical analysis and bioinformatics analysis. Dr. Claire Magner, Prof. Colin McMahon, Prof. Catherine Stanton and Prof. Paul Ross designed the study, acquired the ethical approval.

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Abstract

The human gut microbiome plays a critical role in maintaining overall health, influencing key physiological processes such as digestion, immune function, and behaviour. A deeper understanding of the complex interactions between the gut microbiome, diet, and disease is essential for advancing strategies to promote health and manage chronic conditions. This thesis explores the relationships between dietary components, the gut microbiome, and their implications for health, focusing on various dietary fibres, fermented foods, and microbiome interventions.

Chapter 1.1 reviews and discusses an overview of the gut microbiome, its role in human health, and the current models used to study it, including *in vitro* fermentation systems, organoid and animal models. These models allow for the examination of microbial dynamics, host-microbiome interactions, and the influence of diet and disease. *In vitro* models such as batch and continuous culture systems offer controlled environments for studying microbial interactions, while animal models especially pigs provide more physiologically relevant insights into human microbiome dynamics. This chapter highlights the importance of selecting the appropriate model for microbiome research to bridge the gap between bench and bedside.

Chapter 1.2 delves into the role of dietary fibre in shaping the gut microbiome. Despite recommendations to consume 25-38g/day of fibre, many individuals fall short, leading to imbalances in the gut microbiota and increased inflammation, which are linked to several non-communicable diseases. This chapter discusses the impact of different fibre types on gut health and underscores the importance of personalized dietary fibre strategies to optimize gut microbiome health based on individual microbiota profiles. Chapter 2 investigates the prebiotic potential of superheated steam (SHS)-treated

wheat and oat bran fibres. The results from *in vitro* digestion and fermentation study demonstrate that SHS treatment enhanced the fibres' ability to modulate the gut microbiota, with significant shifts in microbial composition, such as increased *Ruminococcus* and decreased *Escherichia-Shigella* in faecal samples fermented with SHS treated oat fibres. These findings suggest that SHS treatment may improve the prebiotic properties of dietary fibres and their potential to influence gut health. Chapter 3 explores the impact of low and high FODMAP biscuit models on gut microbiota, focusing on formulations such as Biscuit Flour Control (BiFC), Low-FODMAP Control (LFC), Low-FODMAP High-Fibre Prototype (LFP), and Wholemeal Flour Control (WMC). *In vitro* colonic fermentation revealed that LFP fermented faecal samples exhibited increased microbial diversity and a reduced relative abundance of *Escherichia-Shigella* compared to other formulations. The study suggests that manipulating the formulation of biscuits may offer a practical approach to modulating the gut microbiota and managing symptoms of irritable bowel syndrome (IBS).

Chapter 4 examines the development of whole-grain sourdough bread with reduced FODMAP content, using different lactic acid bacteria (LAB) strains, to influence gut microbiota. *In vitro* fermentation studies showed that sourdough fermentation with *Lactiplantibacillus plantarum* FST1.7 enhanced acetate production and positively correlated with beneficial bacteria such as *Bifidobacterium* following *in vitro* colonic fermentation of pre-digested bread. This chapter highlights the potential of sourdough fermentation as a tool for creating functional foods that support gut health, particularly for individuals with IBS.

Chapter 5 investigates tarhana, a traditional fermented food, and its impact on the gut microbiome, specifically focusing on the effects of different flour types and fermentation methods. Using *in vitro* colonic fermentation, the study demonstrates that

sourdough-fermented tarhana with purple potato and chickpea flour demonstrated significant reductions in *Veillonella* and *Escherichia-Shigella* while increasing acetate and propionate levels. These findings underscore the potential of tarhana as a personalized functional food to support gut health and potentially alleviate symptoms in individuals with IBS or other gut-related disorders.

Chapter 6 explores the potential of combining probiotics (*Lactobacillus mucosae* DPC6426) and dietary fibre as a synbiotic approach to modulate metabolic syndrome (MetS) in a porcine model. This study found that the synbiotic combination improved metabolic profiles, including lipid and amino acid metabolism, and enhanced insulin sensitivity. Faecal metabolomic analysis revealed increased levels of spermidine and 3-deoxyglucosone, while serum metabolomics showed elevated lysophospholipids, underscoring the potential of probiotics and fibre in addressing MetS and related cardiovascular risks.

Chapter 7 addresses the gut microbiome changes in infants with congenital heart disease (CHD) undergoing cardiopulmonary bypass surgery (CPB). Post-operative samples showed microbial shifts including elevated *Enterococcus*, *Akkermansia*, and *Staphylococcus*. One infant who developed post-operative necrotizing enterocolitis (NEC) exhibited a pre-operative dominance of *Enterococcus* (93%), identifying potential microbial biomarkers for NEC risk. The study reveals significant alterations in the gut microbiota pre- and post-surgery, highlighting the importance of understanding the microbiome's role in the health of vulnerable populations such as infants with CHD.

In conclusion, this thesis demonstrates how dietary components, fermented foods, and targeted probiotic interventions can modulate the gut microbiota to support health and

manage disease. Each chapter builds on a foundation of understanding the gut microbiome's complexities and its interactions with dietary components. Early chapters establish the role of dietary fibres and fermentation in shaping microbial communities, revealing significant potential for innovations like SHS-treated fibres, low-FODMAP biscuits, sourdough breads and tarhana with different production materials in modulating gut health, while the synbiotic approach in Chapter 6 illustrates a promising intervention for metabolic syndrome (MetS). The final chapter, GuMiBear, brings these insights into a translational context, focusing on vulnerable populations, such as infants with CHD. The observed microbial shifts, including biomarkers for NEC. underscore the critical need for microbiome-focused strategies in clinical care. These findings not only validate the preceding work on the importance of personalized dietary strategies but also extend its relevance to managing gut microbiome-related challenges in at-risk populations.

Publications

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1. Herisson, F.M., Cluzel, G.L., Llopis-Grimalt, M.A., O'Donovan, A.N., **Koc, F.**, Karnik, K., Laurie, I., Canene-Adams, K., Ross, R.P., Stanton, C. and Caplice, N.M., 2024. Targeting the Gut-Heart Axis Improves Cardiac Remodeling in a Clinical Scale Model of Cardiometabolic Syndrome. *JACC: Basic to Translational Science*.

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Abbreviations

ABG: arterial blood gas

ANCOMBC: Analysis of compositions of microbiomes with bias correction

ANOVA: Analysis of Variance

AOAC method: Association of Official Analytical Collaboration

APC: Alimentary Pharmabiotic centre

ASD: atrial septal defect

ASV: amplicon sequence variance

AVSD: atrioventricular septal defect

aw: water activity

BCFA: branched chain fatty acid

BH: Benjamin Hochberg

BiFC: Biscuit Flour Control

BMI: body mass index

CAZymes: carbohydrate-active enzymes

CHD: congenital heart disease

CHI: Children's Health Ireland

CLR: centred log-ratio

CPB: cardiopulmonary bypass surgery

CRF: case report form

CVD: cardiovascular disease

DESeq2: Differential expression of RNA-seq data

Dm: dry matter

DNA: Deoxyribonucleic acid

DTGA: D-transposition of the great arteries

DTGA-IVS: D-transposition of the great arteries with intact ventricular septum

EBD: epithelial barrier dysfunction

EBM: express breast milk

ECG: electrocardiogram

EDTA: Ethylenediaminetetraacetic acid

EFSA: European Food Safety Authority

EPS: exopolysaccharides

EU: European Union

F/B: Firmicutes/Bacteroidetes ratio

FDR: false discovery rate

FFM: faecal fermentation media

FODMAP: fermentable oligosaccharides, disaccharides, monosaccharides, and polyols

GC-FID: Gas chromatography- flame ionisation detector

GLP-1: glucagon-like peptide-1

GOS: galactooligosaccharides

GPR41: free fatty acid receptor 3

GPR43: free fatty acid receptor 2

GuMiBear: gut microbiota of infants with congenital heart disease undergoing cardiopulmonary bypass

HCl: hydrochloric acid

HFD: high fat, salt and sugar diet

HLHS: hypoplastic left heart syndrome

HLHS: hypoplastic left heart syndrome

HOMA-IR: Homeostatic Model Assessment for Insulin Resistance

HPAEC: high-performance anion exchange chromatography

HPLC: high performance liquid chromatography

HRHS: hypoplastic right heart syndrome

HSD: honestly significant difference

IBD: inflammatory bowel disease

IBS: irritable bowel syndrome

ICF: informed consent form

ICF: informed consent form

ICU: intensive care unit

IQR: interquartile range

LAB: lactic acid bacteria

LC-MS: liquid chromatography-mass spectrometry

LCOS: low cardiac output syndrome

LDA: Linear discriminant analysis

LEfSe: Linear discriminant analysis Effect Size

LFC: low FODMAP control biscuit

Lfc: log fold change

LFP: Low-FODMAP High-Fibre Prototype biscuit

LPS: lipopolysaccharide

LVOT: left ventricular outflow tract

MaAsLin2: multivariate associations with linear models

MetS: metabolic syndrome

NEC: necrotising enterocolitis

NSP: non-starch polysaccharides

OTUs: operational taxonomic unit

PBS: Phosphate buffered saline

PCA: Principal Component Analysis

PCoA: Principal Coordinate Analysis

PCR: polymerase chain reaction

PERMANOVA: Permutational analysis of variance

PICU: paediatric intensive care unit

PLS-DA: partial least squared-discriminate analysis

PPI: public and patient involvement

PS: pulmonary stenosis

PYY: peptide YY

QC: quality control

RBB: Repeated Bead Beating

rRNA: ribosomal ribonucleic acid

RVOT: right ventricular outflow tract

SACN: Scientific Advisory Committee on Nutrition

SCFA: short chain fatty acid

SD: Standard deviation

SD-Control: sourdough bread was prepared using baker's yeast

SD-FST1.7: Sourdough bread fermented with Lactobacillus plantarum FST1.7

SD-R3: Sourdough bread fermented with Lacticaseibacillus paracasei R3

SD-Rye106: Pediococcus pentosaceus RYE106

SDS: sodium dodecyl sulphate

SE: standard error

SHS: superheated steam treatment

TFA: trifluoracetic acid

TGA: Transposition of the Great Arteries

Treg cells: T regulatory cells

UK: United Kingdom

UV: ultraviolet

VSD: ventricular septal defect

WBC: water binding capacity

WEAX: Water-extractable arabinoxylan

WHO: World Health Organization

WMC: wholemeal flour control biscuit

β: beta

Units

˚C: Degree Celsius

µg: Microgram

µg/ml: Microgram per millilitre

µL: Microliter

bp: Base Pair

cm: Centimetre

Da: Dalton

g: Gram

g/d: gram per day

h: Hour

kDa: Kilodalton

kg/h: kg per hour

KU: kiloUnit

L: Litre

M: Molar

mg/L: Milligram per litre

mg ml^{-1}: Milligram per millilitre

min(s): Minute(s)

mm: Millimetre

mM: Millimolar

μM: micrometer

ng: Nanogram

nM: nanomolar

v/v: Volume per volume

V: Volt

w/v: Weight per volume

xg: Relative centrifugal force

Chapter 1.1

Models for Studying the Gut Microbiome: A Comprehensive Review of *in vitro* Systems, Animal Models and Advanced Technologies

1.1 Abstract

The gut microbiome is a crucial component of human health, influencing various physiological processes such as digestion, immune function, and behaviour. Research into the gut microbiome has significantly advanced, utilizing various models such as *in vitro* fermentation systems, organoid and animal models, as well as advanced technologies. Each model offers unique advantages and limitations, contributing to our understanding of the gut microbiome's complexity and its interactions with diet, disease, and treatment strategies. This review provides a comprehensive overview of these models, highlighting their contributions to gut microbiome research. *In vitro* models, including batch culture and continuous culture systems, offer controlled environments to study microbial interactions and the effects of different substrates but lack the complexity of a living host.

Animal models, particularly rodent and pig models provide more comprehensive insights into host-microbiome interactions, although interspecies differences can limit the extrapolation of findings to humans. Pig models are especially valuable due to their physiological and anatomical similarities to humans, making them ideal for studying gastrointestinal diseases and microbiome interventions. *In vitro* colonic fermentation models, such as the MicroMatrix™ *in vitro* fermentation system, have emerged as powerful tools for simulating the human colon environment, enabling high-throughput screening of microbial responses to dietary components and drugs. This review discusses the significance of these models, their methodological intricacies, and their contributions to gut microbiome research, emphasizing the balance between model simplicity and physiological relevance. Understanding the strengths and limitations of these models is crucial for designing robust studies that can translate findings from

bench to bedside, ultimately advancing our knowledge of the gut microbiome and its role in health and disease.

1.2 Introduction

The gut microbiome is a highly diverse and dynamic ecosystem of microorganisms comprising bacteria, viruses, fungi, and archaea residing in the human gastrointestinal tract (Jans and Vereecke, 2024). This microbial community plays a crucial role in maintaining host health by influencing a wide range of physiological functions, including nutrient metabolism, immune regulation, and protection against pathogens (Koç et al., 2020). Through metabolic activities, the gut microbiota contributes to the digestion of complex carbohydrates, the synthesis of essential vitamins, and the fermentation of dietary fibres into beneficial short-chain fatty acids (SCFAs), which have anti-inflammatory properties and contribute to gut health (Bull and Plummer, 2014, Zeng et al., 2024, Zabolotneva et al., 2024). Additionally, the microbiome supports the development of a functional immune system and plays a protective role by competing with pathogenic microorganisms for resources, thereby preventing infections (Wang et al., 2024, Dong et al., 2022).

Disruptions to the gut microbiome, known as dysbiosis, have been associated with a growing list of diseases, including inflammatory bowel disease (IBD), obesity, diabetes, cardiovascular diseases, metabolic syndrome, and even neuropsychiatric disorders (Turnbaugh et al., 2006, Lozupone et al., 2012, O'Donovan et al., 2020). These links highlight the importance of understanding the gut microbiome's composition, functional capacity, and its interactions with the host in the context of health and disease. By examining the gut microbiome, researchers aim to identify key microbial species, metabolic pathways, and host-microbe interactions that may serve as therapeutic targets for preventing or treating these diseases (Jabeen et al., 2023, Khattab et al., 2023, Vega-Sagardia et al., 2023).

Dietary components, particularly dietary fibres, play a significant role in shaping the gut microbiome (Sun et al., 2023). These complex carbohydrates, which are not digested by human enzymes, are fermented by gut bacteria to produce SCFAs, such as acetate, propionate, and butyrate (Mills et al., 2019). These SCFAs have been shown to enhance the growth of beneficial microbiota, modulate immune responses, reduce gut inflammation, and improve gut barrier function (Williams et al., 2021, Topping and Clifton, 2001). Additionally, the consumption of fibre-rich diets can lead to increased microbial diversity, which is often associated with a healthy gut microbiome (Rezende et al., 2021). However, the effects of different types of fibre and their interactions with the microbiome are still under investigation, requiring further research using appropriate experimental models.

Advances in sequencing technologies, including next-generation sequencing (NGS), and bioinformatics tools have greatly accelerated microbiome research (Qin et al., 2010). These technologies enable the profiling of microbial communities at exceptional resolution, facilitating the identification of previously unknown species and functional pathways within the microbiome (Carlino et al., 2024). Despite these advancements, studying the gut microbiome presents unique challenges. The complexity and dynamic nature of the microbiome make it difficult to assess its true diversity and functionality in a living organism (Weir, 2023). In addition, factors such as host genetics, diet, environment, and microbial interactions complicate efforts to determine cause-and-effect relationships between the microbiome and disease (Pedroza Matute and Iyavoo, 2023).

To overcome these challenges, researchers employ various models to study the gut microbiome. These models range from *in vitro* systems that simulate the gut environment to more complex animal models and clinical human trials (Van de Wiele

et al., 2015, Koc et al., 2022, Li et al., 2024b). *In vitro* models, such as batch culture and continuous culture systems, allow for controlled studies of microbial interactions and responses to specific dietary components but lack the full complexity of the host environment (Qi et al., 2023). However, these models can provide valuable insights into microbial behaviour and fermentation processes. Animal models, including rodents and pigs, are often used to study gut-microbiome interactions in a more integrated context, providing a better understanding of host physiology and the microbiome's impact on health. Pig models, in particular, offer a unique advantage due to their physiological and anatomical similarities to humans, making them valuable tools for translating microbiome research into human health applications (Zhang et al., 2013).

Furthermore, human clinical trials are critical for validating findings from preclinical models and understanding the direct implications of microbiome interventions in human health. The integration of emerging technologies, such as organoids and high-throughput screening systems, is enhancing the ability to study the microbiome in more physiologically relevant settings. Organoids, three-dimensional models derived from stem cells, can replicate key aspects of human gut function and provide a platform for studying host-microbiome interactions in a more dynamic and controlled environment (Ahn et al., 2023).

This review aims to provide an in-depth exploration of these diverse models, focusing on their strengths, limitations, and contributions to advancing our understanding of the gut microbiome. We place particular emphasis on the use of *in vitro* colonic fermentation models, such as the MicroMatrix™ system, which simulate the human colon's microbial environment, and the utility of pig studies in bridging the gap between animal models and human research. By understanding the unique

contributions of these models, we can optimize research strategies to better understand the gut microbiome's role in health and disease and identify potential interventions for improving gut health through diet, prebiotics, and other microbiome-targeted therapies.

1.3 *In Vitro* Models

In vitro models are laboratory-based systems used to study the gut microbiome under controlled conditions (Isenring et al., 2023). These models include batch culture systems, continuous culture systems, and advanced fermentation systems. Each type of *in vitro* model offers specific benefits for understanding microbial interactions and testing the effects of dietary components, pharmaceuticals, and other interventions. Table 1.1 summarizes the advantages of disadvantages of each system.

1.3.1 Batch culture systems

Batch culture systems involve growing microbial communities in a closed environment where no new nutrients are added after the initial setup. These systems are simple and cost-effective, making them suitable for preliminary studies and high-throughput screening (Miller and Wolin, 1981). The pH, temperature and dissolved oxygen levels are held constant during the operation (Lim and Shin, 2013). Batch cultures are particularly useful for studying microbial growth kinetics, metabolic pathways, and the impact of specific interventions such as antibiotics or prebiotics. Some of the advantages of batch culture systems are straightforward to set up and operate. They require minimal equipment and can be used to screen a large number of samples simultaneously. These systems are also highly reproducible, allowing for standardized experiments across different laboratories. On the other hand, these systems do not accurately replicate the dynamic environment of the human gut.

Nutrient depletion and waste accumulation can significantly alter microbial behaviour over time, leading to results that may not reflect *in vivo* conditions. Additionally, batch cultures cannot maintain a stable microbial community composition over extended periods, limiting their usefulness for long-term studies.

1.3.2 Continuous culture systems

Continuous culture systems, such as chemostats, maintain a constant environment by continuously supplying fresh nutrients and removing waste products. This approach allows for the study of microbial growth and metabolism under steady-state conditions, providing insights into community dynamics and microbial interactions (Hansen et al., 2012). Continuous culture systems are particularly valuable for investigating the stability and resilience of microbial communities under different environmental conditions. They can maintain a stable microbial community composition over extended periods, making them ideal for long-term studies. They allow for precise control of experimental variables, such as nutrient supply and growth rate, enabling detailed investigations of microbial physiology and ecology. However, they are more complex and expensive to maintain compared to batch culture systems (Ekkers et al., 2020). Besides, they require specialized equipment, such as peristaltic pumps and fermenters, and are more challenging to set up and operate. Additionally, the steady-state conditions in continuous cultures may not fully replicate the fluctuating environment of the human gut.

1.3.3 Advanced fermentation systems

Advanced fermentation systems, such as the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) and the TNO *in vitro* model of the colon (TIM-2), aim to replicate the human gut environment more accurately. These systems can simulate

different sections of the gastrointestinal tract, allowing for the study of regional microbial communities and their responses to various factors (Molly et al., 1993, Minekus et al., 1999).

1.3.3.1 SHIME

The SHIME model consists of multiple compartments representing different regions of the human gastrointestinal tract, including the stomach, small intestine, and colon. Each compartment can be independently controlled, allowing for the study of site-specific microbial communities and their metabolic activities. SHIME operates at 37^{o}C to mimic human gastrointestinal system (Van de Wiele et al., 2015). SHIME is particularly useful for investigating the impact of dietary components, such as fibre and polyphenols, on gut microbiota composition and function (Van den Abbeele et al., 2013).

1.3.3.2 TIM-2

The TIM-2 model focuses on the colon and is designed to replicate the conditions in the large intestine, including pH, temperature, and transit time. TIM-2 allows for the study of microbial fermentation processes and the production of metabolites, such as short-chain fatty acids (SCFAs), under conditions that closely mimic the human colon (Venema and van den Abbeele, 2013). Advanced fermentation systems offer greater physiological relevance compared to simpler *in vitro* models. They can simulate different regions of the gastrointestinal tract, providing a more comprehensive understanding of microbial interactions and metabolic activities. These systems also allow for the study of complex interventions, such as the effects of probiotics and prebiotics on gut microbiota composition and function. However, these systems require specialized equipment and expertise, making them more expensive and

challenging to operate. They are also more time-consuming to set up and maintain, limiting their use for high-throughput screening. Additionally, while these models provide a more accurate representation of the human gut environment, they still lack the complexity of host-microbiome interactions present *in vivo*.

1.3.3.3 MicroMatrix™

The MicroMatrix™ machine represents a cutting-edge approach in *in vitro* gut microbiome research. This high-throughput system allows for the parallel fermentation of multiple microbial communities under different conditions (Wiegmann et al., 2020). Each well of the MicroMatrix™ can be independently controlled, enabling precise manipulation of variables such as pH, temperature, and nutrient composition (O'Donnell et al., 2018). The MicroMatrix™ machine facilitates large-scale screening of microbial responses to dietary components, pharmaceuticals, and other interventions (Lynch et al., 2021, Koc et al., 2024, D'Ambrosio et al., 2021).

High-throughput capability of MicroMatrix™ allows for the simultaneous testing of multiple conditions (Koc et al., 2024), generating valuable data on microbial behaviour and interactions. The system's precise control over experimental variables enhances the reproducibility and reliability of results (Mathur et al., 2023). The initial cost of the MicroMatrix™ machine and the need for specialized training to operate it may limit its accessibility to some laboratories (O'Donnell et al., 2018). Additionally, while the MicroMatrix™ machine provides valuable insights into microbial responses, it still lacks the complexity of host-microbiome interactions present *in vivo*.

Table 1.1 Advantages and disadvantages of i*n vitro* models

Model	**Advantages**	**Disadvantages**
Batch Culture Systems	Simple, cost-effective, high throughput	Do not replicate dynamic gut environment
Continuous Culture Systems	Maintain steady-state conditions, study microbial dynamics	Complex, expensive
Advanced Fermentation Systems	Replicate different gut regions, greater physiological relevance	Specialized equipment, expertise required
MicroMatrix^{TM} Machine	High-throughput, precise control of variables	High initial cost, requires specialized training
SHIME	Simulate different gut regions, greater physiological relevance	Specialized equipment, expertise required
TIM-2	Simulate colon conditions, study microbial fermentation processes	Specialized equipment, expertise required

1.3.4 Cell culture models

Cell culture is a method to grow cells in an artificial environment (Segeritz and Vallier, 2017). Specific rich media is needed to support growth of cells, and it should provide nutrients, growth factors, hormones, vitamins and minerals (Holley, 1975). Cell culture experiments are mostly being used to see epithelial attachment (Venaille et al., 1989), toxicity (Baderna et al., 2019), epithelial barrier function (Sun et al., 2012) and disease models such as cancer cell lines (Cohen et al., 1999, Salucci et al., 2002).

Researchers have been using cell culture models such as Caco-2, HT-29 and CT26 to study prebiotic effects of substrate and probiotic effects of bacteria as well as to show

pathogenicity of bacteria such as *E. coli* (Fagundes et al., 2021, Lamers et al., 2003, Shang et al., 2020, Shil and Chichger, 2021). Caco-2 cells are being used to examine anti-inflammatory effects of bacteria and substrates as well as pathogenicity of *E. coli* (Mei and Chen, 2023, Toki et al., 2009). In addition to this, disease models such as irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD) models have been performed with Caco-2 cells (Weber et al., 2020, Andrews et al., 2018, Borgonetti et al., 2022). The advantages of cell culture experiments compared to *in vivo* studies include the absence of the need for ethical approval, faster results, greater consistency, and improved reproducibility as well as the ability to perform more replicates (Pearce et al., 2018, Arango et al., 2013). There are several limitations which cell culture can not reproduce an *in vivo* system, with the risk of contamination with bacteria or yeast, the cost is high and requires trained staff (Balon and Wiatrak, 2021).

1.3.5 Organoid models

Organoid models represent an innovative approach in gut microbiome research, as they mimic the human gastrointestinal tract's cellular structure and function (Ranganathan et al., 2020). Organoids are created from stem cells (Stelzner et al., 2012) or *ex vivo* cultures (Sato et al., 2011), allowing researchers to develop miniature, tissue-like structures that resemble various organs. These models are particularly valuable for studying the gut epithelium’s interaction with both beneficial (Pierzchalska et al., 2017) and pathogenic microorganisms (Ranganathan et al., 2019). For example, co-culturing *Lactobacillus rhamnosus* with intestinal organoids has been shown to promote cell proliferation and differentiation, providing insights into probiotic action mechanisms (Shaffiey et al., 2016). Additionally, pathogenic strains such as *Salmonella* and *Clostridium difficile* have been used with organoids to model infection and test therapeutic interventions (Zhang et al., 2014, Leslie et al., 2015).

The main advantage of organoid models is their ability to replicate complex tissue structures (Lukonin et al., 2020). However, creating and maintaining organoids are resource-intensive, and they do not capture the systemic host responses seen in animal models (Sugimoto and Sato, 2022).

1.4 Animal Models

Animal models are crucial tools in gut microbiome research, providing insights into host-microbiome interactions that cannot be captured by *in vitro* systems (Douglas, 2019). Commonly used animal models include rodents, pigs, and non-human primates, each offering unique advantages and limitations as summarized in Table 1.2.

1.5 Rodent Models

Rodent models, particularly mice and rats, are widely used in gut microbiome research due to their short generation times and well-established methodologies (Nguyen et al., 2015). Germ-free (GF) rodents, which lack a microbiome, and gnotobiotic rodents, which have a defined microbiome, are particularly valuable for studying host-microbiome interactions (Turnbaugh et al., 2009b). Rodent models are genetically well-characterized, allowing for the use of various genetic tools and techniques to study the gut microbiome (Wos-Oxley et al., 2012). Their short generation times and relatively low maintenance costs make them suitable for large-scale studies (Hugenholtz and de Vos, 2018). Additionally, the availability of germ-free and gnotobiotic rodents enable precise manipulation of the microbiome, facilitating the study of specific microbial taxa and their effects on the host (Manca et al., 2020, Jans and Vereecke, 2024, Darnaud et al., 2021). Researchers have been using mice and rat models to study different microbiome researches such as circadian rhythm, different

probiotic, prebiotic and dietary fibre interventions (Liu et al., 2021, Delannoy-Bruno et al., 2021, Lkhagva et al., 2021).

Despite their advantages, rodents have physiological and anatomical differences from humans that can limit the direct applicability of findings (Qi et al., 2023). For example, rodents have a different diet, gut morphology, and immune system compared to humans, which can influence microbiome composition and function (Skaggs et al., 2019, Nguyen et al., 2015). Additionally, the ethical considerations associated with animal experimentation must be carefully managed (Qi et al., 2023).

1.6 Pig Models

Pigs are increasingly recognized as valuable models for gut microbiome research due to their physiological and anatomical similarities to humans (Heinritz et al., 2016). These similarities include comparable digestive processes, gut morphology, and immune system function, making pigs particularly suitable for studying dietary interventions, probiotics, and gastrointestinal diseases (Heinritz et al., 2013, O'Donovan et al., 2020). Pigs have similar gastrointestinal tract structure and function to humans, allowing for more accurate extrapolation of findings (Turnbaugh et al., 2009a). The gut microbiome of pigs includes mainly Bacteroidota and Firmicutes phyla which are similar to humans (Leser et al., 2002). Their size and longer lifespan enable the study of chronic interventions and long-term health effects (Hoffe and Holahan, 2019). Pigs are also more responsive to human pathogens, making them suitable for studying infectious diseases and vaccine efficacy (Meurens et al., 2012). Some of the limitations of studying pigs can be the cost of trials, require more space and specialized facilities compared to rodent models (Pabst, 2020). Their longer generation times and larger size can limit the number of animals that can be studied

simultaneously. Additionally, ethical considerations and the need for specialized veterinary care must be addressed when using pigs in research.

1.7 Non-Human Primate Models

Non-human primates, such as macaques and baboons, are the closest models to humans in terms of genetic and physiological similarities. They are valuable for studying complex diseases, immune responses, and the effects of interventions on the gut microbiome (McKenna et al., 2008). Non-human primates share a high degree of genetic similarity with humans, allowing for the study of complex host-microbiome interactions and disease mechanisms (Grieneisen et al., 2020). Their immune system and gastrointestinal physiology closely resemble those of humans, providing valuable insights into the effects of interventions on gut health (Estes et al., 2018). The studies have limitations due to ethical considerations, high costs, and longer generation times (Fischer and Austad, 2011). Their use is often restricted to specific research questions that cannot be addressed using other models (Phillips et al., 2014). Additionally, maintaining non-human primates in a laboratory setting requires specialized facilities and expertise.

Table 1.2 Advantages and Disadvantages of Animal Models

Model	**Advantages**	**Disadvantages**
Rodent Models	Genetic tools, short generation times, low cost	Physiological differences from humans
Pig Models	Similar gut morphology, digestive processes to humans	Higher cost, larger space requirements
Non-Human Primate Models	Closest genetic and physiological similarity to humans	Ethical considerations, high cost, longer generation times

1.8 Human Clinical Trials

Human clinical trials provide the most direct insights into the gut microbiome's role in health and disease, although they are challenging due to inter-individual variability and ethical constraints (Swann et al., 2020). These trials often involve dietary interventions, probiotics, prebiotics, synbiotics, or other microbiota-targeted therapies to examine their impact on gut microbial composition, functional capacity, and host health (Yin et al., 2023, Huang et al., 2022, Barboi et al., 2022).

In infancy, early microbial colonization plays a crucial role in immune system development and metabolic programming. Clinical trials investigating probiotic and prebiotic supplementation in infants have demonstrated beneficial effects, such as increased colonization by *Bifidobacterium* and *Lactobacillus*, improved gut barrier function, and modulation of immune responses (Yin et al., 2023).

In adulthood, dietary interventions remain key in shaping the gut microbiome and influencing disease risk. Fibre-rich diets, for instance, have been linked to the promotion of beneficial bacteria, increased short-chain fatty acid (SCFA) production, and improved metabolic health (Gondalia et al., 2022). Advances in multi-omics

approaches, integrating microbiome, metabolome, and transcriptome data, have further elucidated mechanistic links between microbial metabolites and host health. For example, butyrate has been identified as a key regulator of epithelial integrity and immune modulation (Vanhoutvin et al., 2009).

Despite these advancements, challenges remain in translating clinical trial findings into practical applications. Inter-individual variability in microbiome responses, influenced by genetics, diet, medication use, and lifestyle factors, complicates the identification of universal therapeutic targets (Swann et al., 2020). Additionally, long-term studies are needed to assess the sustainability of microbiota changes and their clinical relevance. Addressing these limitations through robust trial designs, standardized methodologies, and larger cohorts will be critical to advancing the field.

1.9 Discussion

The use of different models in gut microbiome research is crucial for understanding the complex interactions between the host and its microbial inhabitants. Each model offers unique advantages and limitations, and the choice of model depends on the specific research question being addressed.

In vitro models provide a controlled environment for studying microbial interactions and the effects of different substrates (O'Donnell et al., 2018). Batch culture systems are suitable for preliminary studies and high-throughput screening, while continuous culture systems allow for the study of microbial dynamics under steady-state conditions (Lim and Shin, 2013). Advanced fermentation systems like SHIME and TIM-2 offer greater physiological relevance by simulating different sections of the gastrointestinal tract (Venema and van den Abbeele, 2013). The MicroMatrix^{TM} machine represents a significant advancement in *in vitro* gut microbiome research,

enabling high-throughput screening and precise control of experimental variables (Wiegmann et al., 2020).

Despite their advantages, *in vitro* models have limitations. They lack host-microbiome interactions and cannot fully replicate the complexity of the human gut environment. Therefore, findings from *in vitro* studies must be validated *in vivo* to ensure their relevance to human health (Qi et al., 2023).

Animal models are essential for studying host-microbiome interactions, disease mechanisms, and the effects of interventions. Rodent models are widely used due to their genetic similarity to humans, short generation times, and well-established methodologies (Wos-Oxley et al., 2012). However, their physiological differences from humans can limit the direct applicability of findings.

Pigs are valuable models for gut microbiome research due to their physiological and anatomical similarities to humans (Hou et al., 2022). These similarities include comparable digestive processes, gut morphology, and immune system function. Pig models are particularly suitable for studying dietary interventions, probiotics, and gastrointestinal diseases, providing more accurate extrapolation of findings to humans compared to rodent models (O'Donovan et al., 2020, Pabst, 2020, Heinritz et al., 2013). They also allow for the study of chronic interventions and long-term health effects, which are challenging to investigate in shorter-lived animal models (Jia et al., 2024). Additionally, pigs are more responsive to human pathogens, making them suitable for studying infectious diseases and vaccine efficacy (Meurens et al., 2012).

Non-human primates are the closest models to humans in terms of genetic and physiological similarities (Phillips et al., 2014). They are valuable for studying

complex diseases and immune responses but are limited by ethical considerations, high costs, and longer generation times.

While *in vitro* and animal models provide valuable insights, human studies remain the gold standard for understanding the gut microbiome's role in health and disease (Swann et al., 2020). These studies offer the most direct evidence but are inherently challenging due to inter-individual variability, environmental factors, and ethical constraints. Human studies often involve dietary interventions, probiotics, prebiotics, synbiotics, or other microbiota-targeted therapies to examine their impact on gut microbial composition, functional capacity, and host health (Swann et al., 2020, Tanihiro et al., 2024).

Dietary intervention studies have revealed how fibre-rich diets promote the growth of beneficial bacteria, leading to increased short-chain fatty acid (SCFA) production, which is associated with improved metabolic health and reduced inflammation (Gondalia et al., 2022, Li et al., 2024a). Prebiotic compounds like inulin have demonstrated their ability to selectively enrich beneficial microbes such as *Bifidobacterium*, improving gut barrier function and modulating immune responses (Rodriguez et al., 2022). Advances in multi-omics approaches, integrating microbiome, metabolome, and transcriptome data, have enhanced our understanding of the gut microbiome's systemic influence on health. Personalized nutrition strategies informed by microbiome profiling are also emerging, altering interventions to individual microbiome compositions and metabolic phenotypes.

Despite these advancements, translating findings from human trials into practical applications remains complex. Challenges such as inter-individual variability, the need for standardized methodologies, and the requirement for long-term studies highlight

the importance of integrating findings from *in vitro*, animal, and human studies to build a comprehensive understanding of the gut microbiome's role in health and disease.

1.10 Conclusion

Gut microbiome research has advanced significantly through the use of *in vitro* models, animal studies, and human clinical trials, each offering unique insights into the complex interactions between microbes and their host. *In vitro* colonic fermentation models, such as SHIME, TIM-2, and the MicroMatrix™, are essential tools for studying microbial fermentation processes and the effects of dietary components, drugs, and other interventions on the gut microbiome. The MicroMatrix™, in particular, represents a significant advancement in *in vitro* gut microbiome research, enabling high-throughput screening and precise control of experimental variables. While *in vitro* models provide controlled conditions to investigate microbial dynamics and substrate effects, and animal models allow for the study of host-microbiome interactions, human clinical trials remain the most definitive approach for understanding the microbiome's role in health and disease. Emerging technologies, such as advanced fermentation systems and multi-omics approaches, have bridged some of the gaps between model systems and human studies, offering more holistic perspectives on microbiome functions and their impacts on host health. Despite these advancements, challenges such as inter-individual variability, ethical considerations, and the translation of findings to clinical applications remain. By leveraging the complementary strengths of different models, researchers can advance our knowledge of the gut microbiome and its role in health and disease, ultimately leading to the development of novel therapeutic strategies for modulating the gut microbiome for health benefits.

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Chapter 1.2

The Public Health Rationale for Increasing Dietary Fibre: Health Benefits with a Focus on Gut Microbiota

Fatma Koc is first author of this review which is published in:

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1.1 Abstract

Dietary fibre can be considered an essential macronutrient which feeds our gut microbiota, thus enabling the optimal functioning of this 'virtual organ'. Recommended daily intakes range from 25 to 38 g/day. However, most individuals fail to reach this target and may consume <50% of the recommended amount. Poor dietary fibre intake is associated with an imbalanced gut microbiota leading to reduced diversity and increased abundance of non-beneficial microorganisms at the expense of beneficial ones. Inflammation is a recognised physiological effect of such a microbiome structure and is a common denominator in several non-communicable diseases associated with the Western lifestyle/developed world including obesity, cardiovascular disease, type 2 diabetes and colorectal cancer. Yet increasing dietary fibre intake can reduce risk factors associated with these diseases and may even improve prognosis. Several food manufacturers have now developed strategies to increase the fibre content of foods. However, research gaps still remain. In particular, dietary fibre is composed of various non-digestible carbohydrates and the exact effect of each of these on the gut microbiota is currently unknown. The links between dietary fibre and disease risk are generally associative. Understanding the biological mechanisms will require deeper insight into the impact of the gut microbiota on health. Furthermore, gut microbiota research has shown significant inter-individual variation meaning that some individuals may not respond to certain dietary fibres owing to the absence of specific microorganisms. Understanding how the different fibre types influence the microbiota, combined with knowledge of an individual's microbiota, should lead to fibre-led, microbiota-based dietary advice.

1.2 Introduction

The health benefits of plant-derived fibre have been acknowledged for over 200 years (Cummings and Engineer, 2018). In the late 1960s, the British surgeon, Denis Burkitt, built on existing research and proposed that low dietary fibre intakes increased the risk of various diseases including chronic heart disease, obesity, diabetes, large bowel issues, to name but a few (Cummings and Engineer, 2018). Today, scientists have begun to unlock some of the mechanisms by which dietary fibre influences health and disease, particularly through the gut microbiota.

The gut microbiota refers to the microbial populations found within the gastrointestinal tract, and it is increasingly appreciated for its effects on host health (Holmes et al., 2012). It is composed of bacteria, archaea, micro-eukaryotes and viruses and is often referred to as a virtual organ. It provides numerous physiological functions to its host including the production of bioactive metabolites and essential nutrients, protection against pathogen infection through colonisation resistance, maintenance of the mucosal barrier, regulation of the immune system, communication with the central nervous system and involvement in energy (Mills et al., 2019b).

One of the key functions of the gut microbiota is the breakdown and fermentation of dietary fibre, a task which is beyond the scope of human physiology, given that there are only 17 such enzymes encoded within the human genome (Cantarel et al., 2012). In contrast, the gut microbiota is rich in carbohydrate-active enzymes (CAZymes) which break down the complex carbohydrates/fibre into bacteria-fermentable monosaccharides (El Kaoutari et al., 2013). In the colon, the major fermentation site in humans, dietary fibre is fermented into various metabolites which have been associated with health (Hillman et al., 2017). Indeed, the resulting short-chain fatty

acids (SCFAs), acetate, propionate and butyrate, have multiple effects on host health (Zhang and Davies, 2016, Topping and Clifton, 2001).

In line with the observation of Burkitt, dietary patterns, particularly the Western diet or that of developed countries which lack sufficient dietary fibre, are associated with numerous non-communicable diseases including metabolic diseases (obesity, type 2 diabetes), immune-related diseases (e.g. inflammatory bowel disease (IBD), allergy) and colorectal cancer (Shin et al., 2015, Mosca et al., 2016).

In this review, we discuss the benefits of dietary fibre through the impact on the gut microbiota. We begin by looking at the definition of dietary fibre and public health guidelines for its daily intake. We then look at how dietary fibre promotes health through the gut microbiota, particularly in relation to SCFAs. We provide evidence that dietary fibre can benefit non-communicable diseases and briefly discuss strategies to increase fibre content in food. Finally, we discuss research gaps that need to be addressed to ensure that all individuals reap the benefits of dietary fibre.

1.2.1 The definition of dietary fibre

The dietary fibre definition has changed over time depending on what plant-derived substances are included (Buttriss and Stokes, 2008). In its Scientific Opinion on Dietary Reference Values for carbohydrates and dietary fibre, the European Food Safety Authority (EFSA) describes it as ‘non-digestible carbohydrates plus lignin, including non-starch polysaccharides’ (EFSA, 2010) and is measured by the approved AOAC method. It is found in many vegetables, cereals, fruits, berries and whole grains (Nutrition, 2015) (see Table 1.3). In the UK, dietary fibre was previously defined as non-starch polysaccharides (NSP), and this definition excluded resistant starches and lignin, but the definition was changed to be consistent with the EFSA definition by the

Scientific Advisory Committee on Nutrition (SACN) in their report on Carbohydrates and Health (Nutrition, 2015).

Dietary fibre can be classified according to its different properties such as solubility in water (Figure 1.1) or fermentability in an *in vitro* system (Tungland BC, 2002). Most of the well-fermented fibres are soluble, while partially or poorly fermented fibres are insoluble in water (Tungland BC, 2002). Soluble dietary fibre dissolves in water and is soluble in the gut, forming a gel-like substance. It can improve human health by reducing blood glucose (Weickert and Pfeiffer, 2008), improving insulin sensitivity (Robertson et al., 2003) and reducing cholesterol (Gunness and Gidley, 2010). Insoluble dietary fibre is not soluble in water and passes mostly through the digestive system. However, it aids bowel health and promotes regularity in bowel movements (Anderson et al., 2009b), delays gastric emptying (Maljaars et al., 2008) and can have a laxative effect (McRorie, 2015). Gums, pectin, β-glucan, mucilage, oligosaccharides, inulin and some hemicelluloses are described as soluble dietary fibres. Cellulose, lignin, resistant starch and some of the hemicelluloses are recognised as insoluble dietary fibres (Ciudad-Mulero et al., 2019). There is, however, a paucity of studies investigating the fermentability of different dietary fibres (Williams et al., 2017). Pectin, found in fruits, legumes, sugar beet and potato, is an example of a fermentable fibre (Dhingra et al., 2012).

1.2.2 Current guidelines

There are many recommendations about how much dietary fibre should be consumed daily. Dietary guidelines for Americans recommend that at least 25-38 g/day dietary fibre should be consumed by adults in order to promote health (McRae, 2018). According to EFSA, intakes greater than 25 g/day of dietary fibre are associated with

benefits to health (EFSA, 2010), while SACN recommended a dietary reference value of 30 g/day for the adult population in its 2015 Carbohydrates and Health report (Nutrition, 2015). Many studies have shown that daily consumption of 25 g or higher of dietary fibre has beneficial effects on health (EFSA, 2010, Eshak et al., 2010, Wang et al., 2019).

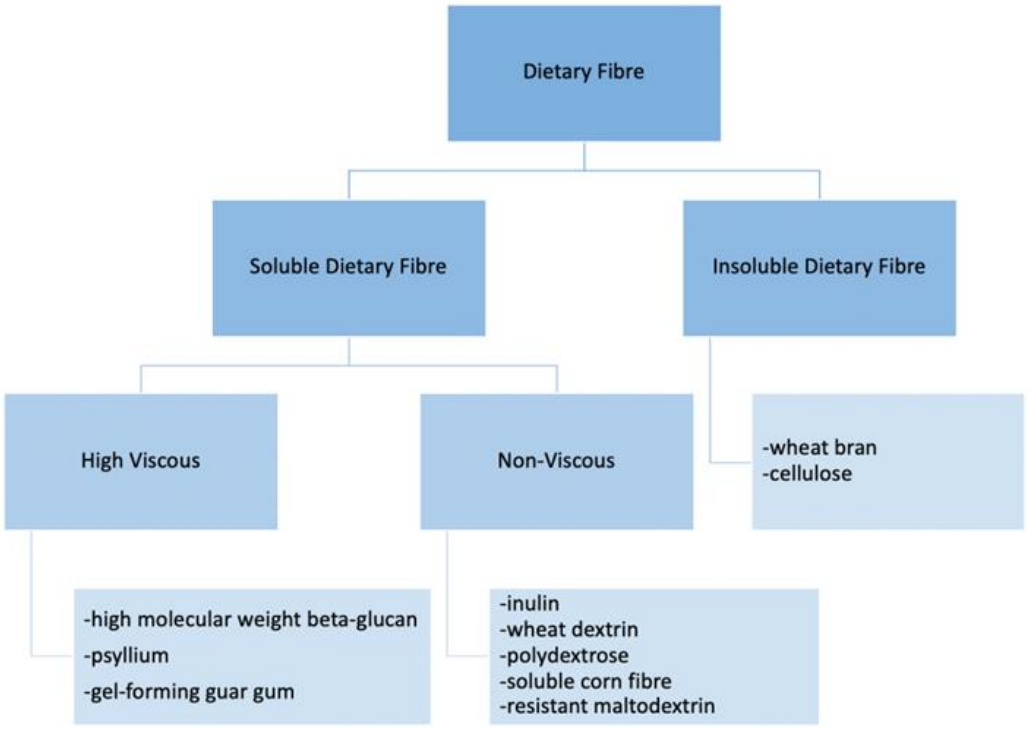

Figure 1.1 Dietary fibre classification

The Western diet is characterised by low dietary fibre intake in comparison to ancestral or non-industrialised diets, with many populations failing to achieve the recommended amounts. Indeed, our ancestors (100–50 × 103 years ago) are believed to have consumed up to 100 g fibre/day (Eaton, 2006). These days, average dietary fibre intakes among adults are estimated to be 18 g/day in the US (King et al., 2012), 16–28 g/day in the EU (EFSA, 2010) and 19 g/day in the UK (Roberts C, 2018, Bates B, 2019).

It is important to point out that these guidelines do not make the distinction between the various classes of dietary fibre, for example, soluble versus insoluble, fermentable versus non-fermentable and fermentable versus prebiotics [a prebiotic being defined as ‘a substrate that is selectively utilised by host microorganisms conferring a health benefit’ (Gibson, 2017)]. Thus, prebiotics result in specific changes to the composition and/or activity of the gut microbiota resulting in beneficial physiological effects, which have been validated with pre-clinical and clinical data (Holscher, 2017). The prebiotic definition will undoubtedly evolve as our understanding of the impact of the gut microbiota on health expands. The repertoire of recognised prebiotics will also grow as research uncovers specific beneficial impacts of fermentable fibres on the gut microbiota and host health. From a microbiome perspective, many people could theoretically be consuming in excess of the recommended amounts of dietary fibre but be consuming very little fermentable dietary fibre. In the absence of extensive data on the consumption of different types of dietary fibres, the available research suggests that a complex and varied diet including various plant-based foods should result in a diverse and complex gut microbiota, which is important in relation to the impact of fibre on host health as discussed below (Williams et al., 2017).

Table 1.3 Types of non-digestible carbohydrates and their sources

Types of non-digestible carbohydrates	Examples	Sources
Non-starch polysaccharides	Cellulose	Fruits and vegetables, whole-grain products, nuts and seeds
	Hemicelluloses	Whole grains, wheat bran
	Gums	Gum arabic, xanthan gum, guar gum, carrageenan gum - added to many types of foods
	Mucilages	Seeds such as flaxseeds, chia seeds, seaweed, figs, plantain, cassava
	Pectin	Fruits such as apples and pears, citrus fruits, bananas, vegetables such as peas, carrots, green beans, sweet potatoes
	Beta-glucan	Whole-grain barley and oats
	Inulin	Chicory, leeks, asparagus, onions, garlic, oats, wheat, soya beans
	Psyllium	Extract from psyllium plant, used as a supplement or added to some foods
Resistant oligosaccharides	Fructo-oligosaccharide	Wheat, rye, onions, leeks, garlic, broccoli, brussels sprouts, beetroot
	Galacto-oligosaccharide	Pulses such as kidney beans, chickpeas and lentils
	Inulin	Chicory, leeks, asparagus, onions, garlic, oats, wheat, soya beans
Resistant starches	Physically inaccessible	Coarsely ground, whole kernel grain products such as some breads, seeds
	Granular	Raw potatoes, green bananas, high amylose maize,
	Retrograded	Cooked, then cooled or cooled and reheated, starchy foods (*e.g*. Pasta, rice, stale bread)
	Chemically modified	Some commercially produced breads and cakes
	Amylose-lipid complex	Bread containing fat (*e.g*. Ciabatta)

1.2.3 The gut microbiome and fibre

Sequencing advances in the last decade have enabled scientists to gain in-depth snapshots of exactly who is there and what they are doing in the gut ecosystem at any one time. Studies have indicated that the gut microbiome has an impact on diseases such as obesity, type 2 diabetes, cancer, metabolic syndrome and cardiovascular disease (CVD) (Hur and Lee, 2015, Zitvogel et al., 2015, Tang et al., 2017). The healthy gut microbiome is composed predominantly of the phyla Firmicutes and Bacteroidetes, followed by Actinobacteria, Proteobacteria and Verrucomicrobia to a lesser extent (Li et al., 2014, Falony et al., 2016). The exact definition of a healthy gut microbiome has eluded scientists to date but it is generally agreed that gut microbiota diversity is an important factor. Microbiota composition is influenced by several factors such as human diet, antibiotic treatment and environmental factors (Mills et al., 2019b). A high-fibre diet promotes microbiota diversity, composition and functionality by increasing the abundance and activities of beneficial microorganisms (Simpson and Campbell, 2015). In particular, long-term intake of a variety of different types of dietary fibres from a diversity of sources (recorded as monthly intake of specific foods, particularly fruit, vegetables and grains) is most beneficial (Klimenko et al., 2018).

Furthermore, the gut microbiome is characterised by extensive inter-individual variation which is often greater than that introduced by diet (Salonen et al., 2014). Thus, the composition and functionality of an individual's baseline microbiome can influence the overall response to a fibre intervention resulting in 'responders' and 'non-responders' (Mills et al., 2019a). For example, a gut microbiota characterised by low numbers of ruminococci was observed in two obese men who failed to ferment significant amounts of resistant starch following a 10-week intervention (Walker et al.,

2011). Improved glucose tolerance in healthy subjects consuming barley kernel-based bread was associated with a gut microbiota enriched with *Prevotella copri* and these 'responders' exhibited a higher *Prevotella:Bacteroides* ratio after the intervention compared to 'non-responders,' the individuals who responded least to the intervention (Kovatcheva-Datchary et al., 2015).

1.2.4 Short-chain fatty acids

SCFA are the major end products of fibre degradation and fermentation by gut bacteria. Acetate is the major SCFA produced in the gut and is the major SCFA found in the blood (Thauer et al., 1989). Acetate serves as an energy substrate for glial cells in the brain (Brand et al., 1997) and as a substrate for cholesterol and lipid synthesis in the liver (Hotta and Chaikoff, 1952). It also serves as a ligand or signalling molecule for the cell surface G-coupled receptors GPR41 (free fatty acid receptor 3) and GPR43 (free fatty acid receptor 2) (Tang and Offermanns, 2017), that transmit signals to intracellular G-proteins that then convey signals to downstream intracellular effectors involved in energy regulation (Zhao et al., 2013). Acetate has been associated with many positive effects on host health (Zhang and Davies, 2016) such as anti-obesogenic effects (inhibits weight gain) (Hernandez et al., 2019). For example, a clinical study involving healthy volunteers (n = 6) reported that gastric infusions with acetate (12 mmol/h for 3 hours; calculated on the basis of continuous daily fermentation of 30 g of dietary fibre) decreased circulating free fatty acids (Laurent et al., 1995). In six overweight/obese men, distal infusions of sodium acetate (180 mmol/l) were found to promote fat oxidation, improve metabolic markers and increase fasting peptide YY (van der Beek et al., 2016).

Propionate is another SCFA produced by the gut microbiota (Zhang and Davies, 2016). Propionate serves as a substrate for gluconeogenesis, it also is a ligand for GPR41 and GPR43 (Tang and Offermanns, 2017). Daily intake of 10 g of an inulin-propionate ester for 24 weeks in a clinical study involving overweight individuals (n = 60) was shown to significantly reduce weight gain, reduce distribution of intra-abdominal adipose tissue and intra-hepatocellular lipid content and prevent the deterioration of insulin sensitivity which was observed in the control group (inulin only) (Chambers et al., 2019). The dose used represents a 2.5- fold increase in daily colonic propionate production, which the authors' state is difficult to achieve via a mixed fermentable fibre diet alone. In a later study (n = 12), administration of 20 g/day of an inulin-propionate ester to overweight/obese men for 42 days improved insulin sensitivity (Chambers et al., 2019). The authors also demonstrated that the inulin-propionate ester may play a role in attenuating reward-based eating behaviour to high-energy foods in non-obese men (n = 20) (Byrne et al., 2016).

Butyrate serves as the energy source for the colonocytes (Liu et al., 2018) and acts as a ligand for GPR41, GPR43 (Tang and Offermanns, 2017) and GPR109 (Thangaraju et al., 2009). Further, butyrate and propionate can alter gene expression by inhibiting histone deacetylase activity thus suppressing tumour formation and inflammatory pathways in several tissues (Zhang and Davies, 2016). Butyrate has been associated with many health-promoting activities such as anti-cancer (Blouin et al., 2011), anti-obesogenic (Gao et al., 2009), anti-inflammatory (Segain et al., 2000) and neuroprotective (Lanza et al., 2019) effects. In a clinical study, butyrate enemas (30 ml/d of 600 mmol/l for 30 days) improved recovery of tissue integrity in the colon/rectum of patients (n = 10) with inflammatory bowel disease, colorectal cancer or diverticulitis (Luceri et al., 2016). Supplementation with sodium butyrate capsules

has been shown to reduce diastolic blood pressure and increase glucagon-like peptide-1 in adults with type 2 diabetes (Roshanravan et al., 2017). Furthermore, administration of sodium butyrate (250 mg) with fumaric acid (100 mg), citric acid (60 mg), sorbic acid (50 mg) and malic acid (40 mg) can decrease the occurrence of traveller's diarrhoea in patients (Krokowicz et al., 2014).

Colonic infusions with mixtures of the three fatty acids (200 mmol/l) slightly higher than concentrations generally reached in the colon after fibre intake, in overweight/obese men (n = 12) increased fat oxidation, PYY and energy expenditure and decreased lipolysis (Canfora et al., 2017). The approximate molar ratio of acetate, propionate and butyrate in the colon and stool is 60:20:20 (den Besten et al., 2013) and they reach a combined concentration of ~50–150 mmol/l in the colon (GR, 1997). While the clinical studies presented above indicate that the SCFAs have a role to play in health, particularly metabolic health and obesity, the concentrations used do not necessarily reflect those achieved in the colon by consuming the daily recommended dose of fibre (~90 mmol/l acetate, 30 mmol/l propionate and 30 mmol/l butyrate). Thus, human intervention studies are required that relate dietary fibre intake to SCFA levels in the gut and the subsequent health outcomes of these SCFA concentrations.

Dietary fibre fermentation in the colon by gut bacteria also results in the production of other acid metabolites such as succinate and lactate (Oliphant and Allen-Vercoe, 2019). While the health effects of these metabolites are poorly understood, the reduction of luminal pH may promote resistance to gastrointestinal pathogens and may improve bone health by increasing mineral solubility and/or absorption. Microbiota derived succinate has been shown to improve glucose homoeostasis via intestinal gluconeogenesis in mice (De Vadder et al., 2016).

1.2.5 Mucus layer

The colonic mucus layer is composed of glycoproteins and is the first defence against pathogens. It is secreted by specialised epithelial cells called goblet cells. This glycoprotein-rich mucus layer is an alternative energy source for gut microorganisms, when dietary fibre is in low supply, with negative implications for host health. Reduced dietary fibre has been associated with a thinner colonic mucus layer (Brownlee et al., 2003, Hedemann et al., 2009). In order to show that fibre deficiency has an effect on the colonic mucus barrier and gut microbiome, researchers used a synthetic gut microbiota model. As a result, they found that intermittent and chronic fibre deficiency caused mucus erosion which led to intestinal barrier dysfunction (Desai et al., 2016).

1.2.6 Prebiotics

Fermentable dietary fibre may also be classed as a prebiotic, defined as 'a substrate that is selectively utilised by host microorganisms conferring a health benefit' (Gibson, 2017). Prebiotics are compounds that are not digested by the host (Gibson and Roberfroid, 1995) so they pass through the upper intestinal tract intact before reaching the colon where they are selectively fermented by certain species of the gut microbiota and thus confer health benefits on the host (Ansari et al., 2020). In order to classify dietary fibre as a prebiotic, it must fulfil the above criteria. Studies in humans and animals have indicated that prebiotics potentially have a number of different health effects such as improving mental health, including depression, anxiety, Alzheimer's disease and autism spectrum diseases, which is supported by clinical data (Ansari et al., 2020); reducing intestinal inflammation (Lomax and Calder, 2009) and increasing mineral absorption, which has been verified particularly for calcium in human

intervention studies (Whisner and Castillo, 2018). A number of randomised controlled trials have also reported beneficial effects of prebiotics on obesity and associated diseases such as reducing bodyweight and increasing satiety (Cerdo et al., 2019). These effects are mainly attributed to improved gut microbiota composition and functionality (Yasmin et al., 2015).

1.2.7 Western diet and dietary fibre intake

The Western diet is characterised by low fruit and vegetable intake and high consumption of animal-derived protein (meat and processed meat), saturated fats, refined grains, sugar, salt and alcohol (Mills et al., 2019b). A multitude of studies have shown that high-fat, low-fibre diets are linked to metabolic diseases such as obesity, CVD and metabolic syndrome (Mozaffarian et al., 2011, Shoelson et al., 2007). In contrast, high-fibre diets are associated with reduced risk of CVD and metabolic syndrome (Ribichini et al., 2019). The Western diet may cause dysbiosis in the gut microbiota (Statovci et al., 2017). Dysbiosis refers to changes in the structure of the gut microbiome, with decreased beneficial microorganisms while potentially pathogenic microorganisms increase (Petersen and Round, 2014). It is generally accepted that low microbial diversity is associated with inflammation and diseases such as inflammatory bowel disease and Clostridioides difficile infection, as examples (Petersen and Round, 2014, Kriss et al., 2018). We have already mentioned that Firmicutes and Bacteroidetes are the two major phyla in the gut microbiota. However, a high Firmicutes/Bacteroidetes (F/B) ratio is considered dysbiotic. Numerous studies have indicated a positive correlation between high F/B ratio and diseases such as obesity (Verdam et al., 2013), type 2 diabetes (Larsen et al., 2010), CVD (Yoshida et al., 2018) and hypertension (Yang et al., 2015). This is due to the reduced abundance of beneficial microorganisms such as *Lactobacillus* and *Bifidobacterium*, which are

known SCFA producers (Falony and Vuyst, 2009). In addition, the typical Western diet may cause an imbalance in the immune system by increasing potentially pathogenic microorganisms in the microbiota. The immune response must be balanced in order to prevent low-grade inflammation which may result in metabolic health conditions such as obesity. Though, it is important to note that not all studies support a direct link between the F/B ratio and disease states. Some research, including a meta-analysis (Magne et al., 2020), has found no consistent association between the F/B ratio and metabolic disorders, highlighting the complexity of gut microbiota composition and its relationship with health outcomes.

However, high-fibre diets can prevent dysbiosis by restoring intestinal barrier function (Chen et al., 2013), increasing mucus secretion (KA Lien, 2001) and modulating tight junctions (Zhong et al., 2009). Furthermore, production of butyrate and propionate by gut bacteria has inhibitory effects on low-grade inflammation via regulation of Treg cells (Petersen and Round, 2014). Administration of the probiotics [defined as 'live microorganisms, which when administered in adequate amounts confer a health benefit on the host' (Health and Organization), 2001)], *Lactobacillus paracasei* and *L. casei* have been shown to decrease the inflammatory cytokine IP-10 (IFN-γ inducible protein-10) with production of protease lactoceptin (von Schillde et al., 2012). Indeed, the butyrate-producing capacity of *Faecalibacterium prausnitzii* has been correlated with IL-10 production which is a known anti-inflammatory cytokine (Martin et al., 2017). Innate immune system activation has been associated with lipopolysaccharide (LPS) which exists on the outer membrane of Gram-negative bacteria. Thus, the Western diet may cause increased LPS production which results in increased colonic permeability (Myles et al., 2013) and systemic inflammation (Heinritz et al., 2016).

In regions of the world which have not yet adopted a Westernised diet/lifestyle, it is possible to see the benefits of a high-fibre diet through the microbiota. For example, inhabitants of a rural village in Burkino Faso in West Africa consume a high-fibre diet similar to that of early humans at the time agriculture was beginning to take hold. Comparisons of the gut microbiota composition of children from Burkino Faso with that of Italian children consuming a Western diet, showed a significantly higher biodiversity and richness in the gut microbiota of the Burkino Faso children (De Filippo et al., 2010). Firmicutes were depleted in the Burkino Faso children, while there was an enrichment of Bacteroidetes. In particular, the genera *Xylanibacter* and *Prevotella* (from Bacteroidetes) which metabolise cellulose and xylan were unique to the African children and were not found in the European children. In contrast, potentially pathogenic bacteria, *Shigella* and *Escherichia*, were significantly higher in the Italian children. Unsurprisingly, SCFAs were more significantly abundant in the Burkino Faso cohort. The gut microbiota of one of the last hunter-gatherer communities on the planet, the Hadza people of Tanzania is also higher in terms of microbial diversity and richness compared with an Italian cohort (Schnorr et al., 2014). Hadza hunter-gatherers live near Lake Eyasi in the central Rift Valley of Tanzania. The nutritional habits of the Hadza people change by season. They have wet (November to April) and dry (May to October) seasons. Their meat consumption increases during the dry season, on the other hand berry foraging and honey consumption are more frequent during the wet season (Smits et al., 2017). Fibre-rich tubers and baobab are consumed throughout all seasons. As a result, in the wet season, Bacteroidetes operational taxonomic units (OTUs) decrease significantly. However, the Firmicutes phylum has been found to be stable across all seasons, presumably a result of consuming tubers and baobab. Interestingly, there is no evidence of CVD risk

factors in this community (Raichlen et al., 2017), and older studies reported low rates of infectious diseases, nutritional deficiencies or metabolic diseases compared with settled groups in the surrounding regions (Bennett et al., 1973, Work et al., 1973, Blurton Jones et al., 1992).

1.3 Impact of Dietary Fibre on Non-Communicable Diseases

1.3.1 Cardiovascular disease

Cardiovascular disease (CVD) is a term used to describe a class of diseases including coronary heart disease, stroke, hypertension and heart failure and is one of the most common causes of mortality in developed countries (Anderson et al., 2009a, Leong et al., 2017). Diet has an important role to play in the prevention of CVD (Zampelas and Magriplis, 2020). Higher levels of dietary fibre intake are associated with lower incidence of CVD in prospective cohort studies (Liu et al., 1999, Liu et al., 2000, Merchant et al., 2003). SACN generated an extensive report on the role of dietary carbohydrates on health (Nutrition, 2015). The studies used to inform the report in relation to CVD included seven cohort studies (Liu et al., 2002, Bazzano et al., 2003, Eshak et al., 2010, Kokubo et al., 2011, Park et al., 2011, Wallstrom et al., 2012, Chuang et al., 2012) and an updated meta-analysis (Threapleton et al., 2013b) which included three later studies (Buyken et al., 2010, Akbaraly et al., 2011, Threapleton et al., 2013a). The meta-analysis of the prospective cohort studies, carried out by SACN, found an association between higher consumption of dietary fibre and reduced incidence of CVD (Nutrition, 2015). The SACN report concluded that, based on moderate evidence, there is a biologically relevant association between higher fibre consumption and reduced incidence of CVD (RR 0.91, 95% CI 0.88, 0.94. for each 7 g/d increase; $P < 0.001$). An association between insoluble but not soluble fibre was

also found (Nutrition, 2015). In a more recent prospective cohort study, researchers investigated whether there was an inverse association between dietary fibre intake and risk of peripheral artery disease. This study involved 26010 middle-aged men and over 20 years of follow-up. The results indicated that individuals who consumed the recommended intakes of fibre, fruit and vegetables had significantly lower risk of developing peripheral artery disease (Kulezic et al., 2019). In another study, the association between CVD risk and dietary fibre intake was examined in a cohort of women. The results revealed an inverse relationship not only for soluble fibre but also for insoluble fibre on CVD risk (Liu et al., 2002).

1.3.2 Diabetes

Diabetes is a condition in which the body does not produce sufficient insulin to regulate blood glucose levels and the insulin produced does not work effectively, leading to raised blood glucose levels. It is increasingly becoming a prevalent health issue and it has been estimated that by 2040, 600 million people will have this disease (Timpson et al., 2005). Dietary therapy has a significant effect on type 2 diabetes. The American Diabetes Association recommends consumption of 14 g/1000 kcal/day fibre both to prevent the development of type 2 diabetes and to improve glycaemic control (American Diabetes, 2007). A meta-analysis of ten cohort studies, carried out by SACN, found an association between higher consumption of dietary fibre and reduced incidence of type 2 diabetes, although meta-analyses of four and three RCTs found no effect on fasting glucose or fasting insulin levels, respectively (Nutrition, 2015). Associations between higher consumption of insoluble and soluble fibre and reduced incidence of type 2 diabetes were also found (Nutrition, 2015). The SACN report concluded that there is adequate evidence for a biologically relevant association

between higher dietary fibre intake and reduced incidence of type 2 diabetes (RR 0.94, 95% CI 0.90, 0.97 for each 7 g/day increase; $P < 0.001$).

Numerous short-term, randomised controlled studies indicate that high dietary fibre intake, especially soluble dietary fibre, plays an important role in controlling postprandial glycaemic and insulin responses in diabetic patients. In one study, researchers investigated the effect of soluble dietary fibre on postprandial plasma glucose and gastric emptying time. High soluble dietary fibre consumption delayed gastric emptying significantly in patients with type 2 diabetes (Fujii et al., 2013, Yu et al., 2014). Thus, dietary fibre may improve postprandial glycaemic response by delaying digestion and absorption of carbohydrates and increasing satiety (Post et al., 2012). Similarly, in another study, researchers developed a protein-enriched, dietary fibre-fortified bar as a pre-meal. The aim of this study was to investigate the postprandial glucose-lowering effects of pre-meal protein-enriched, dietary fibre-fortified bars with post-meal protein-enriched, dietary fibre-fortified bars in individuals with type 2 diabetes and individuals who have normal glucose responses. One of the important results of this study revealed that pre-meal protein-enriched, dietary fibre-fortified bars decreased postprandial glucose levels in type 2 diabetes patients (Bae et al., 2018).

In addition to delaying the digestion and absorption of carbohydrates, dietary fibre, by increasing the production of SCFAs in the intestine, may also enhance peripheral insulin sensitivity (Fujii et al., 2013) although the mechanisms involved are not fully understood (McNabney and Henagan, 2017). Despite this, dietary fibre has been shown to improve peripheral insulin resistance in subjects at increased risk of type 2 diabetes in a randomised, controlled cross-over, intervention study (Robertson et al., 2012). Whole-body insulin sensitivity was also improved following dietary fibre

intervention in overweight/obese subjects with normal glucose metabolism in a randomised, controlled, single-blind, cross-over study (Weickert et al., 2006).

In a double-blind and cross-over study, researchers showed that resistant starch can modulate insulin sensitivity in non-diabetic women. Indeed, it was found that consumption of 30 g/day high amylose-resistant starch improved insulin sensitivity in these women (Gower et al., 2016). In a cohort study of 4399 patients diagnosed with type 2 diabetes, increased dietary fibre intake was associated with better glycaemic control (Fujii et al., 2013).

Some types of dietary fibre (e.g. pectins, inulins and β-glucans, insoluble resistant starch and oligosaccharides) are readily fermentable and found in fruit, certain vegetables and some parts of barley and oats (Weickert and Pfeiffer, 2008). A review of prospective and intervention studies found that these kinds of foods reduced diabetes risk and insulin resistance by some 20–30% (Weickert and Pfeiffer, 2008). In an umbrella review of five meta-analyses, it was also found that the risk of developing type 2 diabetes was reduced by between 13–33% in people who consumed high-cereal fibre diets but there was no observed effect for those consuming the highest levels of fruit or vegetable fibre (McRae, 2018). McRae (2018) reviewed previously published meta-analyses and reported that when dietary fibre was separated into cereal, fruit and vegetable fibre groups, only cereal fibre significantly reduced the incidence of developing type 2 diabetes; by 33% in people who consumed high-cereal fibre diets. However, McRae warns that the positive results must be treated with caution given that statistically significant heterogeneity was noted across the meta-analyses which may be due to large variation in study design (numbers and characteristics of participants, amounts and compositions of dietary fibres consumed). Cereals made from oats and barley contain high amounts of water-soluble fibres such as β-glucan.

In the small intestine, such fibres form a viscous solution thus reducing the contact and mixing of macronutrients with digestive enzymes which delays glucose absorption (McRae, 2018). Furthermore, β-glucan is a fermentable dietary fibre.

1.3.3 Colorectal cancer

Colorectal cancer is one of the most commonly diagnosed and deadly cancers in the US, as well as in most European countries (Society, 2017, Vuik et al., 2019). Diet, physical activity levels and bodyweight have been associated with colorectal cancer risk. Dietary fibre intake is an important dietary component related inversely to this disease (Navarro et al., 2016). A meta-analysis of 11 cohort studies, carried out by SACN, found an association between higher consumption of dietary fibre and reduced incidence of colorectal cancer (Nutrition, 2015). An association between cereal fibre intake, but not fruit fibre or vegetable fibre intake and reduced incidence of colorectal cancer was also found (Nutrition, 2015). The SACN report therefore concluded that adequate evidence exists for a biologically relevant association between higher dietary fibre intake and reduced incidence of colorectal cancer (RR 0.92, 95% CI 0.87, 0.97 for each 7 g/d increase; P = 0.002) based on meta-analysis of 11 cohort studies (Nutrition, 2015). Butyrate is proposed to be involved in the anti-colorectal cancer properties of dietary fibre (Wu et al., 2018). Recent studies have highlighted that fibre-deficient diets promote an increase of some pathogenic bacteria which degrade host-secreted mucus glycoproteins resulting in aggressive colitis (Desai et al., 2016). Long-term low dietary fibre intake thus damages the mucus barrier and may increase the prevalence of mucin-degrading bacteria (Desai et al., 2016). The chronic inflammation associated with colitis is believed to contribute to the development of low- and high-grade dysplasia that can further transform into colitis-associated colorectal cancer

(Rogler, 2014). This may help explain how bacterial dysbiosis is associated with higher risk of colorectal cancer (Davis and Milner, 2009).

Many studies have shown that dietary fibre intake can reduce colorectal cancer risk through butyrate production (Ben et al., 2014, Flint et al., 2012, Kunzmann et al., 2015). Butyrate promotes the growth of the colonic epithelium but has an opposing effect on colonic cancer cells (Bergman, 1990, Goncalves and Martel, 2013). Butyrate also promotes production of antimicrobial peptides which increase bactericidal activities *in vivo* and *in vitro* (Schulthess et al., 2019). Furthermore, reduced levels of butyrate-producing bacteria have been found in the gut mucosa and faeces of colon cancer patients (Wang et al., 2012).

1.3.4 Obesity and bodyweight

Obesity is a major health problem characterised by low-grade inflammation, increased adipose tissue mass and is an increasing global epidemic (Bluher, 2019, Hosseini-Esfahani et al., 2017). Obesity is a considerable risk factor for type 2 diabetes, CVD, several metabolic diseases and different types of cancer (such as breast, ovarian, kidney and colon cancer) (Bluher, 2019, Sarker, 2017). Dietary fibre intake has been associated with lower bodyweight or less weight gain in many observational studies (Du et al., 2010, Liu et al., 2003, Ye et al., 2012), although SACN, based on limited evidence, found no association between dietary fibre intake and body fatness (Nutrition, 2015). The SACN report reviewed data from cohort studies that investigated (Nutrition, 2015) the association between dietary fibre intake and bodyweight change, and energy intake (Nutrition, 2015). With regards to bodyweight change, a review of five cohort studies concluded that there was moderate evidence to conclude that there was no evidence of a consistent association between bodyweight

change and dietary fibre intake. Likewise, moderate evidence, based on six randomised controlled trials, exists to conclude that there is no effect of dietary fibre on energy intake.

Nevertheless, dietary fibre could potentially affect bodyweight by a number of mechanisms. One mechanism is that feelings of satiety are increased with fibre-rich, low energy dense foods compared to low energy dense foods lacking fibre. Both long-term and short-term studies have indicated that dietary fibre can influence energy intake by affecting appetite (Poutanen et al., 2017). It can regulate satiety by releasing appetite-related hormones such as glucagon-like peptide 1 and cholecystokinin (Du et al., 2010). Besides, slower gastric emptying and slower absorption of carbohydrates may reduce the postprandial glucose response (DS, 2001).

Twelve weeks on an energy restricted diet with whole-grain wheat in post-menopausal women resulted in a greater reduction in percentage fat mass than was observed in the group consuming the same energy restricted diet but with refined wheat (Kristensen et al., 2012). Likewise, visceral fat area was significantly reduced in Japanese subjects following consumption of whole-grain wheat bread but this was not observed for those consuming refined wheat bread (Kikuchi et al., 2018). Waist circumference was also found to decrease in subjects following consumption of a whole-grain ready-to-eat oat cereal compared with subjects on energy-matched low-fibre foods (Maki et al., 2010).

1.3.5 Dietary fibre addition to foods

The health-promoting properties of food can be enhanced through the addition of fibre. Indeed, readily digestible carbohydrates in foods can be replaced with dietary fibre. To date, fibre has been mainly added to dairy products (e.g. inulin and oat bran), meat products and cereal products, or fibres can be used as additives to alter the viscosity

and texture of foods (Dhingra et al., 2012). In order for a food to be considered as a 'source of fibre' it should contain at least 3 g fibre/100 g or 1.5 g fibre/100 kcal (Commission, 2006). A 'high-fibre' food should contain at least 6 g fibre/100 g or 3 g fibre/100 kcal (Commission, 2006). The Codex Alimentarius (2009) states that high-fibre foods should contain at least 6 g fibre/100 g or 20% of the daily reference value per serving, while a 'source of fibre' food should contain 3 g fibre/100 g or 10% of the daily reference value per serving.

Processes for the extraction of fibre from foods include dry processing, wet processing, gravimetric, chemical, enzymatic, physical and microbial methods, or combinations thereof; however, the different methods have different impacts on dietary fibre structure (Bingley, 2020, Yang et al., 2017). Emerging technologies for fibre extraction include high pressure processing, microwave and ultrasound (Yang et al., 2017). Furthermore, insoluble dietary fibre can be converted to soluble dietary fibre by microbial fermentation methods, mechanical degradation, chemical treatment and enzymatic methods which are discussed in detail by Yang et al. (Yang et al., 2017).

Enriching dairy products with fibre can be relatively straightforward by using starter cultures which secrete exopolysaccharides (EPS). EPS are very important for the production of yogurts, certain cheeses, fermented cream and milk-based desserts where they contribute to taste perception, mouth-feel, texture and stability of the final products (London et al., 2015, Mills et al., 2010). However, certain EPS can also have prebiotic properties and have been associated with cholesterol-lowering (Nakajima et al., 1992, Korcz et al., 2018) and immune-modulating (Hosono et al., 1997, Ryan et al., 2015) activities. Kearney et al. (Kearney et al., 2011) manufactured yogurt with a genetically engineered β-glucan producing *Lactobacillus paracasei* strain as adjunct, which had no effect on the starter cultures or on the properties of the yogurt itself but

resulted in a two-fold increase in viscosity thus improving texture. β-glucan from oats and barley has been associated with numerous health benefits and there are EU approved health claims that it reduces blood cholesterol and contributes to the reduction of the blood glucose rise after a meal (EFSA NDA Panel (Panel on Dietetic Products, 2011, El Khoury et al., 2012).

The addition of fibre to pasta, bread, extruded snacks, cakes and muffins has been discussed in detail by (Foschia et al., 2013). These types of products are consumed daily by the majority of the global population. The addition of fibre to food is an important strategy to enhance consumer health and prevent disease.

1.4 Conclusions

Dietary fibre can be considered an essential macronutrient. Consumption of a variety of different types of dietary fibre from a diversity of food sources is necessary to feed our gut bacteria. Dietary fibre promotes microbiome diversity and functionality, ensuring optimal activity of this virtual organ. Western diets fall well below the recommended daily intakes of fibre which range from 25–38 g/day. Low intake of dietary fibre has been associated with reduced microbial diversity in the gut and an enrichment of non-beneficial, often pathogenic microorganisms - a microbiota structure associated with inflammation. This inflammation is considered a common denominator in several non-communicable diseases afflicting the developed world.

Several studies have highlighted the health benefits of increasing daily fibre intake including reducing the risk of CVD, type 2 diabetes and colorectal cancer. However, to date these are generally associations rather than causal effects. Our deeper understanding of the impact of the gut microbiota on host health, supported with evidence of biological mechanisms, should help to decipher causal links between

dietary fibre and health. Additionally, the human gut microbiota is highly individual-specific and hence not all individuals may carry the necessary microorganisms to ferment the consumed fibre. This has led to the concept of 'responders' and 'non-responders,' with the former representing individuals with the capacity to metabolise a particular fibre and benefit from it while the latter lacks these microorganisms and fails to reap the benefits. This has been discussed in detail by Mills et al. (Mills et al., 2019a). Fibre thus offers an opportunity for precision nutrition advice. In the meantime, the best advice is to consume a complex and varied diet with various plant-based foods.

Many food production companies have enriched the fibre content of foods. However, it is important to point out that EU food law does not include fibre in its list of mandatory back-of-pack nutrients and does not allow fibre to be included in the front-of-pack declaration - hindering opportunities to signal the fibre content of products.

Dietary fibre is a complex set of molecules which has undergone many definitions as research uncovers new properties and types. In this regard, it is essential that future research focuses on the health implications of the different fibre types, particularly in relation to the composition and functionality of the gut microbiota. Thus, as well as understanding the compositional changes, we need to fully understand the impact of the different fibre types on the gut metabolome and ultimately the consequences of these changes on host physiology. Moving forward, it is envisioned that understanding the effects on the gut microbiota of the different dietary fibre types and whole fibre extracts from plant foods, combined with knowledge of an individual's gut microbiome composition should lead to individual-specific, fibre-led, microbiota-based dietary advice.

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Chapter 2

The Microbiome Modulating Potential of Superheated Steam (SHS) Treatment of Dietary Fibres

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2.1 Abstract

Super-heated steam treatment (SHS) is a thermal processing technique that uses steam at temperatures above its boiling point to modify the physicochemical properties of food components, improving their functional and nutritional attributes. The SHS method has been used to inactivate oxidative enzymes as well as increase bioactive compounds in bran. In this study, wheat and oat bran fibres were used to investigate the potential beneficial prebiotic-enhancing effects of superheated steam (SHS) treatment. Following SHS treatment of fibres, *in vitro* simulated gastric and intestinal digestion followed by batch culture fermentation was performed. 16S rRNA gene sequencing and short chain fatty acid analysis were conducted, which demonstrated that SHS treatment was associated with significant differences in beta diversity and the relative abundances of several microbial taxa. Principal Coordinate Analysis (PCoA) revealed significant separation between SHS-treated oat and non-treated oat ($p=0.013$), and between SHS-treated wheat and non-treated wheat following *in vitro* batch fermentation ($p=0.042$). Relative abundances of *Ruminococcus* were found to be significantly higher by 1.09-log in SHS-treated oat when compared with non-treated oat while abundances of *Escherichia_Shigella*, and *Alistipes* were significantly lower by 1.47-log and 0.39-log, respectively in the SHS-treated oat samples than the non-treated oat samples ($p= 6.85E-06$, $p=0.002037$, $p=0.002458$; respectively). These data demonstrate that SHS treatment of dietary fibre enhanced its effectiveness to modulate the gut microbiome.

Industrial Relevance: Superheated steam (SHS) treatment has emerged as an effective method to improve the shelf-life of grains by reducing lipid hydrolysis and oxidation. Our results revealed that SHS treatment of wheat and oat brans led to increased physiological and chemical properties including higher water-binding capacity, water-

extractable arabinoxylan, and total phenolics as well as improvement in human gut microbiota. Our results suggest that this method could be implemented in the food industry in order to improve beneficial properties of oat and wheat brans.

2.1 Introduction

Dietary fibres are a complex mixture of carbohydrates that resist digestion in the small intestine and can be fermented by the microorganisms in the colon (Jones, 2014). Dietary fibre intake is associated with a variety of health effects including overall metabolic function (Barber et al., 2020, Cronin et al., 2021). According to European Food Safety Authority (EFSA) guidelines, an intake of 25 g/day or more of dietary fibre is associated with health benefits (EFSA, 2010). However, the daily intake of dietary fibre has been decreasing as countries adopt a westernized diet (Oliver et al., 2021), characterized by a high fat, high cholesterol, and low fibre intake (Statovci et al., 2017). The westernized diet has been associated with several diseases such as irritable bowel syndrome (IBS) (Uranga et al., 2016), cardiovascular disease (Zampelas and Magriplis, 2020), asthma (Leiria et al., 2015), obesity, and type 2 diabetes mellitus (Flint et al., 2015, Mayerhofer et al., 2020).

Many of the beneficial effects of dietary fibre have been attributed to its influence on the gut microbiome and the promotion of the production of several beneficial metabolites including short chain fatty acids (SCFAs) (Shah et al., 2020). Recently, a three-week high fibre intervention study in humans resulted in a 1.4-fold increased abundance of *Bifidobacterium* between pre and post-intervention periods (Oliver et al., 2021). This gut modulating effect may be attributed to the production of SCFAs at the end of the fermentation process. SCFAs reduce colonic pH and can inhibit the growth of pathogenic microorganisms including *Enterobacteriaceae* (den Besten et al., 2013,

Parada Venegas et al., 2019). One of the major SCFAs produced in the colon is acetate which has been shown to inhibit colorectal cell proliferation, induce apoptosis, and promote lysosomal membrane permeabilization (Ferro et al., 2016, Marques et al., 2013, Matthews et al., 2012). Propionate and butyrate are the other major SCFAs produced in the colon (Parada Venegas et al., 2019) which play significant roles in the intestinal lumen including suppressing tumour formation and inflammatory pathways through the inhibition of histone deacetylase activity (Zhang et al., 2016). Butyrate is also used by colonocytes as an energy source and is considered protective against colorectal disease (Encarnação et al., 2015).

Oat bran and wheat bran are examples of fibres that exert health benefits and have been shown to have a prebiotic effect (Koç et al., 2020). A dietary prebiotic is defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al., 2017). Oat is rich in β-glucan, a soluble fibre associated with several health benefits including cholesterol-lowering effects and improving blood glucose levels in patients with diabetes (Francelino Andrade et al., 2014). Wheat is one of the most important and most widely consumed crops in the world with many reported health benefits including reducing the risk of cardiovascular disease, cancer, and gastrointestinal disease (Hu et al., 2018, Reynolds et al., 2019). These health-promoting effects can be mainly attributed to the bran layer which contains antioxidants, fibre, vitamins, and minerals (P and Joye, 2020). However, lipid oxidation in wheat bran can result in reduced shelf life, functionality, and palatability. As a result, the development of methods to inactivate or stabilize enzymatic hydrolysis and extend shelf life is of great importance (Hu et al., 2018).

Several methods have been used to improve the shelf life of wheat bran including hot air heating, the addition of metal salts, and superheated steam (SHS) treatment. SHS treatment has emerged as a promising method and has been shown to be effective at increasing shelf life as well as increasing antioxidant activity of brans. SHS treatment creates an oxygen free environment that can reduce oxidation loss (Hu et al., 2018). A recent study investigated the effect of SHS treatment on the lipid stability of noodles during storage and found that SHS treatment significantly reduced lipid hydrolysis and oxidation compared with non-treatment during 12-weeks storage period (Jia et al., 2021). The stabilization effect of SHS was also found to be effective for oat bran (Head et al., 2010).

Physical pre-treatment of bran material may increase the extractability of soluble fibres and bioactives from the cellulosic material, and further enhances the bioavailability of bioactives by improving the fermentability of the bran (Liu et al., 2021, Merali et al., 2015). The use of SHS processing as a novel clean process route has been successfully tested to increase the extraction of arabinoxylan from cereal by-products (Ma et al., 2021) and is comparable or better than using existing industrial processes alone (Phillips, 2009). The increased extraction of soluble bran compounds by SHS treatment appears to be related to structural and morphological changes in bran structure. These structural changes alter the physical properties of bran, by increasing the water holding capacity as well as the capacity to bind (simulated) gastric fluid (Diaz, 2015). Therefore, these physical and structural changes may promote the fermentation process directly (binding and/or hosting microorganisms) or indirectly (improved accessibility of compounds).

To our knowledge, no previous research has investigated the effects of SHS-treated bran on the gut microbiome. This study aimed to investigate the prebiotic potential of

SHS-treated wheat and SHS-treated oat. An *in vitro* batch fermentation system was used to mimic colonic fermentation following *in vitro* simulated oral, gastric, and intestinal digestion of fibres. Samples were analysed using 16S rRNA gene sequencing and SCFA analysis to examine potential microbiome and metabolome modulation effects.

2.2 Materials and Methods

2.2.1 Raw materials

Wheat and oat brans were provided by industry partners from the JPI LONGLIFE project, inulin and cellulose were sourced from Sigma Aldrich (Ireland). Wheat bran (15.4% protein, 17% starch, 0.5% sugar, 45% dietary fibre and 4.3% fat) was provided by Ernst Böcker GmbH (Germany) and oat bran (18% protein, 31.5% starch, 1.5% sugar, 25% dietary fibre, of which 14% is β-glucan, and 9% fat) was provided by Barilla (Italy). The soluble dietary fibres were 3.6% and 14% for wheat and oat bran, respectively, based on AOAC 991.43. Both were subjected to SHS treatment at Wageningen Food and Biobased Research (WFBR, Netherlands) (Section 2.2 SHS Treatment Method). The soluble dietary fibre content of the SHS treated wheat and SHS treated oat bran were 5.9% and 15%, respectively. Brans were stored at −20 °C prior to INFOGEST processing.

2.2.2 Effect of SHS treatment on physicochemical properties of wheat and oat bran

The ability of SHS treatment to enhance the release of soluble material and bioactive compounds from wheat and oat brans was screened in order to select the treatment conditions for the *in vitro* digestion and fermentation studies. SHS treatments were

performed in a pilot apparatus designed and manufactured by WFBR, The Netherlands. Samples were prepared by hydrating the bran in water at 30% (w/v) concentration (on a dry matter basis). This condition was chosen based on previous experience in treating lignocellulosic materials. The wetted material was loaded into the SHS chamber and treated at temperatures of 120 °C, 140 °C and 160 °C. The treatment times were 10 min and 40 min for temperatures below 160 °C and a treatment time of 10 min was applied at 160 °C. For all treatments, steam flow was set at 150 kg/h. The pressure in the SHS chamber was adjusted to obtain a water activity (aw) of 0.7 for all samples. Samples were coded following on the treatment temperature and time combinations, e.g. W-120-10 for wheat bran at 120 °C for 10 min, and O-120-10 for oat bran at 120 °C for 10 min. Additionally, the effect of saturated steam (aw = 1) was also studied for the conditions of 120 °C and 40 min treatment as compared to an aw of 0.7. These samples are coded as W-120-40-1 and O-120-40-1. After treatment, SHS treated samples were frozen at −30 °C and freeze-dried. Following freeze-drying, the bran particles were loosened using a laboratory blender from Waring commercial (Stamford, CT, USA) for 15 s at high speed.

2.2.3 Chemical and physical characterization of native and SHS-treated wheat and oat bran

2.2.3.1 Total analysable carbohydrate content

Total analysable carbohydrate content of brans was performed according to a previously described protocol as follows: (i) free sugars from the water extract, and (ii) after hydrolysis with 2 M trifluoracetic acid (TFA) at 100 °C for 1 h (arabinoxylan) of water extract (Renzetti et al., 2021). For the water extracts, 1 g of sample was weighed in a 50 mL Greiner tube, and deionized water was added to reach 25 mL in

volume. The solution was then kept under stirring at room temperature for 2 h. After stirring, the solution was centrifuged at 16000g for 10 min, and the supernatant was recovered. For the water-extractable arabinoxylan (WEAX) content, 1 mL of 2 M TFA was added to 1 mL of water extract. The obtained monosaccharides were determined by high-performance anion exchange chromatography (HPAEC) using an ICS-3000 Ion Chromatography HPLC system equipped with a CarboPac PA-1 column (250 × 2 mm) in combination with a CarboPac PA guard column (25 × 2 mm) and a pulsed electrochemical detector in pulsed amperometric detection mode (Dionex, Sunnyvale, CA, USA) at 20 °C, according to a previously described protocol (Gilbert-López et al., 2015). Analyses were performed in duplicate. Arabinoxylan content was determined as the sum of the arabinose and xylose multiplied by the factor of 0.88 (after correcting free arabinose and xylose in the water extract before hydrolysis). The content of galactose and glucose generated from soluble polysaccharides (i.e. polymeric galactose and polymeric glucose) was determined from the TFA hydrolysis after correction before hydrolysis.

2.2.3.2 β-Glucan content in water extract

The water extract was obtained similarly as described for the total analysable carbohydrate content (Renzetti et al., 2021). The β-glucan content from the supernatant of the oat bran was measured using a mixed-linkages assay kit (AOAC Method 995.16, Megazyme, Bray, Ireland). Briefly, 100–120 mg of freeze-dried supernatant was treated sequentially with lichenase and β-glucosidase before determination of released glucose by means of the GOPOD reagent. Quantification of glucose was carried out on Varian Cary 50 Scan UV Visible Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

2.2.4 Soluble proteins

Protein content in the water extract obtained from the total analysable carbohydrate analysis was determined using a DC Protein assay kit in duplicate (Bio-Rad Laboratories Inc., USA).

2.2.5 Total phenolic content

Extraction of phenolic content was performed using 80% (v/v) methanol at 70 °C for 15 min at 1/10 (w/v) solid material to solvent ratio. After cooling, the supernatant was decanted, the residue was re-extracted twice, and supernatants were combined. The content of total phenolic compounds in the extracts was investigated using Folin and Ciocalteu's phenol reagent. (+)-Catechin was used as a standard. The results were expressed as mg catechin equivalent per g of dry bran.

2.2.5.1 Water-binding capacity and soluble solids

The water-binding capacity (WBC) of the bran fractions was determined in duplicate according to a modified version of the protocol (Zanoletti et al., 2017). The bran was soaked in demi-water with a bran/water weight ratio of 1/30. After mixing in a vortex, the samples were left to shake at room temperature for 2 h on a Multi Reax Vortex (Heidolph, Schwabach, Germany). Then, the samples were centrifuged at 5000 x g for 60 min using an Avanti J-26XP High Speed Centrifuge from Beckman Coulter (Indianapolis, IN, USA). The supernatant was collected and dried to determine the soluble solids per g of bran on dry matter basis. The pellet was drained for 15 min at an angle of 45° and then weighed. WBC was expressed as g water taken up per g bran on dry matter basis (dm).

2.2.5.2 Colour measurements

The colour of the brans was measured with a Minolta CR310 Colour Meter (L*, a*, b* colour space). Three measurements per variation were performed.

2.2.6 *In vitro* simulated gastric digestion, dialysis, and freeze drying

Samples were processed using the harmonized INFOGEST method to mimic oral, gastric, and intestinal digestion as previously described (Egger et al., 2016, Minekus et al., 2014). Fifty millilitre aliquots of the INFOGEST end products were placed in sterile 110 × 110 mm petri dishes and freeze-dried for 24 h using a previously described protocol (Patterson et al., 2019) prior to *in vitro* faecal fermentation.

2.2.7 Preparation of faecal fermentation medium

The faecal fermentation medium (FFM) was prepared according to the Fooks and Gibson's protocol (Fooks and Gibson, 2003) with the following composition: Tryptone water (2 g/L), yeast extract (2 g/L), cysteine HCl (1 g/L), bile salts (0.5 g/L), Tween 80 (2 mL/L), hemin (0.05 mg/L; dissolved in 3 drops of 1 M NaOH), vitamin K_1 (10 µL/L), NaCl (100 mg/L), KH_2PO_4 (40 mg/L), K_2HPO_4 (40 mg/L), $CaCl_2.6H_2O$ (40 mg/L), $MgSO_4.7H_2O$ (10 mg/L), $NaHCO_3$ (2 mg/L). The medium was autoclaved at 121 °C for 15 min and stored at 4 °C until use.

2.2.8 Preparation of faecal slurry

Stool samples from six healthy individuals (no antibiotic use in 6 months prior to sample donation) were collected, homogenized, dispensed into equal parts in an anaerobic chamber and kept frozen at −80 °C (up to six months) until use for batch fermentation (O'Donnell et al., 2016). The following section outlines the details of sample collection and homogenization: faecal samples were processed in an anaerobic

chamber (Don Whitley anaerobic workstation, West Yorkshire, UK) within 2–3 h of sample collection from six healthy individuals. Approximately 60 g sample per individual (n=6) was placed into a large stomacher bag with a 70-µM filter insert (Sparks lab supplies, Rathcoole, Co. Dublin). Four hundred millilitres of 50 mM phosphate buffer supplemented with 0.05% (w/v) L-cysteine hydrochloride (Sigma Aldrich-Ireland) was added into stomacher bag and the faecal sample was homogenized. The filtered slurry was centrifuged at 4000 x g for 25 min at 4 °C in a Sorvall SLA-3000 centrifuge (Thermofisher, MA, USA). The pellet was re-suspended in 400 mL 50 mM phosphate buffer solution containing 25% glycerol (v/v). The faecal slurry was divided into even sets of aliquots (30 mL for each aliquot) and kept at −80 °C until use for batch fermentation experiment. Participant recruitment and collection of stool samples were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (review ref. no: ECM 4 (gg) 11/02/20 & ECM (iiii) 22/02/2022).

2.2.9 *In vitro* faecal fermentation

The faecal fermentation was performed using the MicroMatrix™ (Applikon Biotechnology, Heertjeslaan 2, 2629 JG Delft, The Netherlands) 24-well cassette system. For each well, 1 mL of thawed faecal slurry was inoculated into 4 mL FFM in the anaerobic chamber and mixed with 0.25 g pre-digested fibre. Running parameters included nitrogen gas at 40%, orbiter at 250 rpm, temperature at 37 °C, dissolved oxygen (dO_2) at 0%, and pH of 6.8. NaOH (4 M) and CO_2 gas were used to adjust the pH. The fermentation was performed for 12 h with eight replicates per fibre. Samples were collected from each well into sterile 2 mL screw-cap tubes and centrifuged at 15000 x g for 15 min at 4 °C. Supernatant was collected for SCFA analysis and pellet was kept for DNA extraction. Both supernatant and pellet were frozen at −80 °C for further analysis.

2.2.10 SCFA analysis

SCFAs were analysed from supernatant according to a previously described protocol (Lynch et al., 2021). Briefly, thawed samples were mixed with acidified water (pH = 2–3), 1:1 (v/v) and syringe filtered (0.22 μM, Corning). A 270 μL sample was mixed with 30 μL 10 mM internal standard (IS) in duplicate. The samples were vortex mixed and centrifuged at 16000 x g for 10 min at 4 °C. Following centrifugation, 250 μL supernatant was transferred in glass inserts (Agilent, California, USA) and placed in amber glass 2 mL GC vials (Agilent, California, USA). A standard curve (acetate, propionate, butyrate, and valerate (0.1 mM–10 mM); isobutyrate and isovalerate (0.01-1 mM)) was created for quantification. All standards were purchased from Sigma-Merck (Missouri, USA).

The analysis of standards and samples was carried out by gas chromatography flame ionisation detection (GC-FID) using a Varian 3800 GC system DB-FFAP column (30 m -length, 0.32 mm -inner diameter, 0.25 μM -film thickness; Agilent, California, USA) and a flame ionisation detector. Standards and samples (0.2 μL splitless injection) were loaded with a CP-8400 autosampler (Varian, California, USA). Helium was used as carrier gas at an initial flow rate of 1.3 mL/min. The initial oven temperature was 50 °C, and was maintained for 0.5 min, ramped up to 140 °C at 10 °C/min and held for 0.5 min. The temperature was then increased to 240 °C at 20 °C/min and held for 5.0 min. The total run time was 20 min. The detector and oven temperatures were set at 300 °C and 240 °C, respectively. Peaks were integrated with Varian Star Chromatography Workstation Version 6.0.

2.2.11 DNA extraction

DNA was extracted from each pellet (from a 2 mL sample tube) using the repeated bead-beating DNA extraction method according to Yu and Morrison, 2004 (Yu and Morrison, 2004). The negative control for DNA extraction was processed alongside the samples, using the same chemicals and kit throughout the extraction process. All chemicals were purchased from Sigma-Merck (Missouri, USA) unless stated otherwise. Pellets were suspended using 1 mL lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH: 8.0, 50 mM EDTA, and 4% sodium dodecyl sulphate (SDS)). The suspension was transferred to a 2 mL screw-capped microtube (Sarstedt, Nümbrecht, Germany) containing different size and amounts of Zirconia/Silica beads (0.125 g of 0.1 mm, 0.125 g of 1.5 mm, a single 2.5 mm; Biospec, Oklahoma, USA). The mixture was homogenized in a bead beater (Biospec, Oklahoma, USA) at 4000 rpm for 3 min. Following homogenization, the tubes were incubated at 70 °C for 15 min and centrifuged at 16000 x g for 5 min at 4 °C. The supernatant was transferred in a sterile 2 mL Eppendorf tube and 300 µL lysis buffer was added to the pellet. Homogenization, incubation, and centrifugation steps were repeated. Supernatants were collected in sterile 2 mL Eppendorf tubes. A 260 µL of 7.5 M cold ammonium acetate solution was then added and incubated on ice for 5 min. Following incubation, tubes were centrifuged at 16000 x g for 10 min at 4 °C, then 650 µL supernatant was transferred into two 1.5 mL tubes, 650 µL isopropanol was added, and incubated at −20 °C overnight. Samples were centrifuged at 16000 x g for 15 min at 4 °C. The supernatant was discarded and the pellet was washed with 200 µL of 70% ethanol. The pellet was then resuspended in 100 µL Tris-EDTA buffer and 2 µL RNase (10 mg/mL; Thermofisher, MA, USA) was added and incubated at 37 °C for 15 min. Following incubation, 15 µL proteinase K (QIAamp DNA Stool Mini Kit, Qiagen, Germany) and

200 µL AL buffer (QIAamp DNA Stool Mini Kit, Qiagen, Germany) were added to samples and incubated at 70 °C for 10 min. 200 µL 100% ethanol was added to samples for precipitation. Samples were transferred to QIAamp mini columns (Qiagen, Germany) and washing and elution procedures were performed according to the QIAamp kit protocol. DNA was stored at −20 °C until 16S rRNA gene sequencing library preparation.

2.2.12 16S rRNA gene sequencing library preparation

DNA extracts were prepared according to Illumina 16S Metagenomics Sequencing Library Preparation guidelines. All PCR reactions were conducted in a 2720 thermal cycler (Life Technologies, MA, USA) using KAPA HiFi Hot Start Ready-mix Kapa Biosystems (MA, USA). The variable V3 and V4 regions of the 16S rRNA gene were amplified using 16S Amplicon PCR Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC, resulting in an expected fragment size of approximately 464 bp. The amplicon PCR program was; 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 5 min 72 °C for the elongation step. For index PCR, the Illumina Index primer set was used according to the manufacturer's protocol. All PCR amplicons were visualised on 1.5% agarose gel (Sigma-Merck) stained with SYBR safe (Thermofisher, MA, USA). PCR products were cleaned up using AMPure XP bead solution (Beckman Coulter, CA, USA) according to Illumina's guidelines. Before pooling, concentrations of final products were measured with Qubit dsDNA high-sensitivity kit (Thermofisher, MA, USA) and Qubit 3.0 fluorimeter (Thermofisher, MA, USA). Samples were sequenced at the Teagasc Next Generation

DNA Sequencing Facility using the Illumina MiSeq platform with a MiSeq Reagent Kit v3 (Illumina, Inc. San Diego, USA).

2.2.13 Bioinformatics and statistics

Sequence reads were filtered using DADA2, version 1.16 using standard filtering parameters; maxN = 0, truncQ = 2, rm.phix = TRUE, and maxEE = 2. Primers were trimmed using trimLeft = c (17, 21). Filtered reads were then de-replicated and de-noised using DADA2 default parameters. An amplicon sequence variant (ASV) table was constructed and chimeric sequences are identified and removed. Taxonomy was assigned to the sequence variants using the IdTaxa taxonomic classification method via the DECIPHER Bioconductor package and the SILVA SSU r138 database. Reads below than 5000 bp were filtered for further analysis.

MicrobiomeAnalyst was used to analyse the ASV table for alpha diversity, beta diversity, and differential abundance measures (Chong et al., 2020). The alpha-diversity analysis function was performed on reads >5000 bp data using Shannon and Simpson diversity measures. Data were subsequently filtered for abundance for those ASVs that had a minimum total count of 4, or less than 20% total abundance in all samples. The ASVs in the lowest 5% for variance based on the inner-quartile range were then removed. Lastly, a centred log-ratio (CLR) transformation was performed. Abundance profiles were visualised at phylum and genus levels using GraphPad Prism version 9.1.0. A Bray-Curtis dissimilarity matrix was calculated and visualised using Principal Coordinate analysis (PCoA). The PERMANOVA statistical test was used to determine if microbial communities of sample groups are significantly different. Differential analyses were used to assess potential differentially abundant taxa between negative control (cellulose) and test group (for each fibre) using DESeq2 on

untransformed data (Love et al., 2014). DESeq2 uses a negative binomial generalized linear model with a variance stabilizing transformation on abundances. Linear discriminant analysis effect size (LEfSe) was used to identify the most effective genus in the groups. False detection rates (FDR) were reported to limit false positive results (Dong et al., 2020). FDR adjusted p value (FDR = 0.05) was used to determine significance level of relative abundance. Statistical analysis of SCFAs, physical, and chemical properties of bran fibres were performed using IBM SPSS version 27.0. Differences among groups were determined by a one-way Anova test with Tukey's honestly significant difference (HSD) test at a 5% level of significance.

2.3 Results

2.3.1 Physical and chemical properties of native and SHS-treated brans

SHS treatments were performed at different temperature and time combinations in order to assess their effects on the chemical and physical properties of the brans. In particular, the solubilisation of bran components was studied as an indicator of opening up of the bran matrix and increased accessibility of compounds. A PCA analysis was performed on wheat and oat bran separately, in order to distinguish the effect of the SHS treatments on each bran source. The sample variations (score plot) and the chemical and physical properties (loading plot) are shown for wheat (Figure 2.1A and 2.1B, respectively) and oat brans (Figure 2.2A and 2.2B, respectively). The first two principal components accounted for 88.7% and 91.3% of the variance for wheat and oat bran samples, respectively. Therefore, PC1–PC2 plane can be considered as indicative of the differences between the samples.

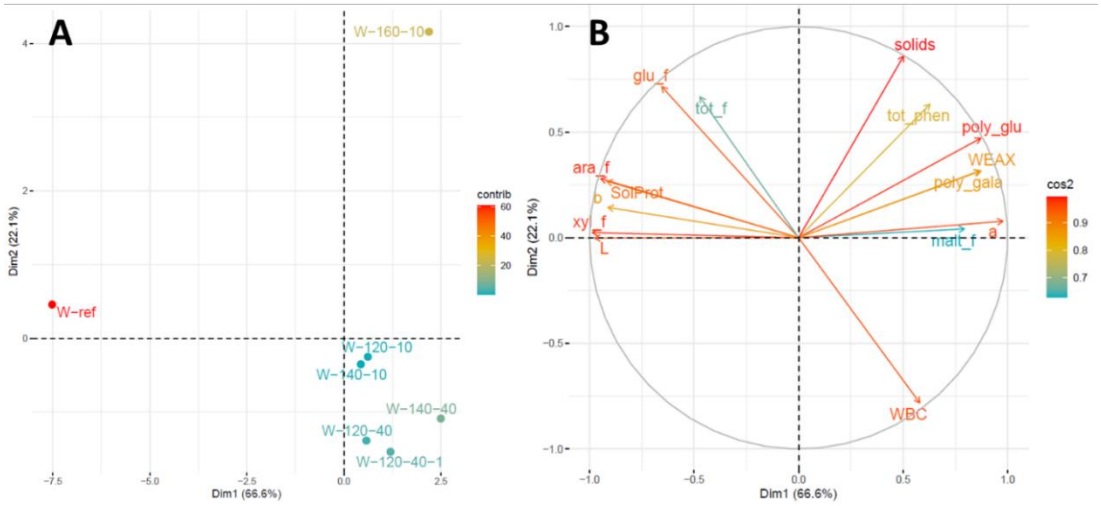

Figure 2.1 Principal component analysis (PCA) of wheat brans with samples score plot (A) and loading plot of compositional and physical properties (B). For the compositional and physical characteristics: ara_f=free arabinose, glu_f=free glucose, xyl_f=free xylose, malt_f=free maltose, tot_f=total free sugars, WEAX=water extractable arabinoxylans, ply_glu=polymeric glucose, poly_gala=polymeric galactose, tot_phen=total detectable phenolics, solids=soluble solids, WBC=water-binding capacity, L=lightness, a=a* in the CIELAB colour space, b=b* * in the CIELAB.

For both wheat and oat brans, all the SHS-treated samples were clearly separated from the native brans along the PC1 axis (Figure 2.1A and Fig. 2.2A, respectively). SHS-treated wheat brans showed considerably higher amounts of WEAX, polymeric glucose, polymeric galactose, and total phenolics compared to the native wheat bran (Figure 2.1B and Supplementary Table 2.1). On the contrary, free sugars (i.e. glucose, arabinose, and xylose) and soluble proteins were lower in the SHS-treated wheat samples compared to the native wheat bran. The SHS-treated samples were also darker and with an enhancement in the red colour space (i.e. a*), suggesting that the decrease in soluble proteins and free (reducing) sugars may be the result of Maillard reaction taking place. Overall, the measured soluble solids were correlated with the sum of individual compounds detected in the compositional analysis ($R2 = 0.882$, $p < 0.00$).

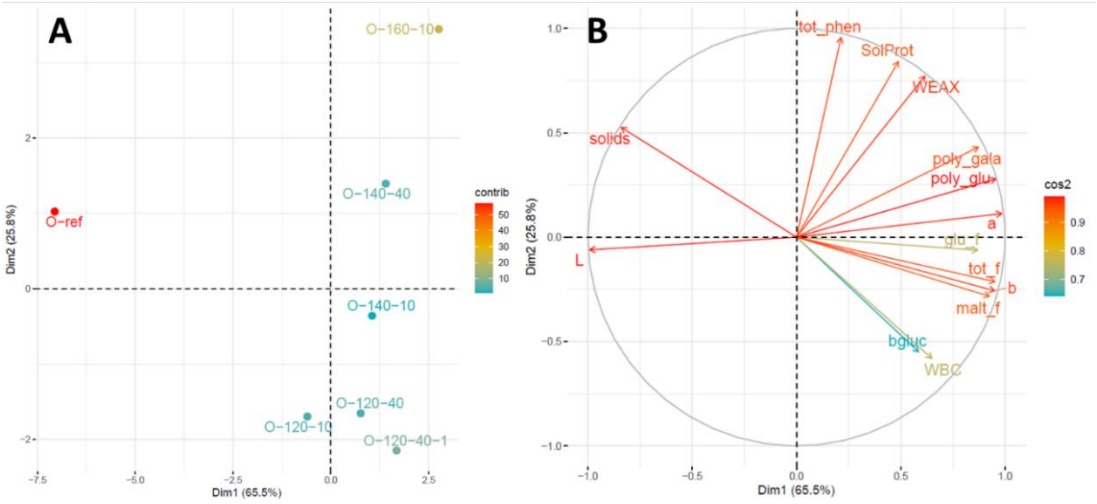

Figure 2.2 PCA of oat brans with samples score plot (A) and loading plot of compositional and physical properties (B). For the compositional and physical characteristics: ara_f=free arabinose, glu_f=free glucose, xyl_f=free xylose, malt_f=free maltose, tot_f=total free sugars, WEAX=water extractable arabinoxylans, ply_glu=polymeric glucose, poly_gala=polymeric galactose, tot_phen=total detectable phenolics, solids=soluble solids, WBC=water-binding capacity, L=lightness, a=a* in the CIELAB colour space, b=b* * in the CIELAB.

Among the SHS-treated wheat brans, sample W-160-10 could be clearly distinguished as the one with highest release of WEAX, polymeric glucose, and galactose, total phenolics and overall soluble solids (Supplementary Table 2.1).

For the SHS-treated oat bran, an increase in total free sugars, WEAX, polymeric glucose, total phenolics and soluble proteins was observed compared to the native oat bran (Figure 2.2B and Supplementary Table 2.2). Contrary to the wheat bran, a significant reduction in soluble solids was detected for the SHS-treated oat samples compared to the native oat bran. Concomitantly, an increase in WBC was observed. It seemed likely that the soluble solids might have been retained in the gel layered formed in the pellet during centrifugation. This hypothesis is supported by the fact that no correlation was observed between the measured soluble solids and the sum of individual compounds detected in the compositional analysis of Supplementary Table

2.2. Also for the oat brans, the treatment at 160 °C, i.e. sample O-160-10, showed the largest effects in enhancing the extraction of compounds, i.e. polymeric glucose, WEAX, and soluble proteins (Supplementary Table 2.2).

Overall, it can be suggested that the 160 °C-10 min SHS treatment resulted in wheat and oat brans with the highest accessibility of compounds. Therefore, samples W-160-10 and O-160-10 were selected for further investigation of the effect of the SHS treatment on *in vitro* digestion and gut microbiota modulation.

Several chemical and physical properties of SHS-treated oat (160 °C–10 min) and native oat differed significantly (Supplementary Table 2.2). Increased levels of maltose and total free sugars were found in SHS-treated oat compared with native oat ($p < 0.05$). Polymeric glucose, WEAX levels and WBC were higher in SHS-treated oat than native oat ($p < 0.05$). Soluble solids were found to be lower in SHS-treated oat than native oat ($p < 0.05$). Colour measurements were significantly different between SHS-treated oat and native oat ($p < 0.05$, Supplementary Table 2.2).

2.3.2 Alpha and beta diversity

Following *in vitro* oral, gastric, and upper intestinal digestion of SHS-treated wheat and oat brans and their native (non-SHS-treated) counterparts, *in vitro* faecal fermentation was performed to mimic colonic fermentation of the pre-digested fibres. The controls included inulin (positive control) and cellulose (negative control). At the endpoint of fermentation (T12), 16S rRNA gene sequencing was performed on samples. Shannon and Simpson diversity indices were used to assess the alpha diversity of samples. Both indices revealed a number of differences in diversity following fermentation in the presence of cellulose, resulting in the highest level of diversity among samples (Figure 2.3).

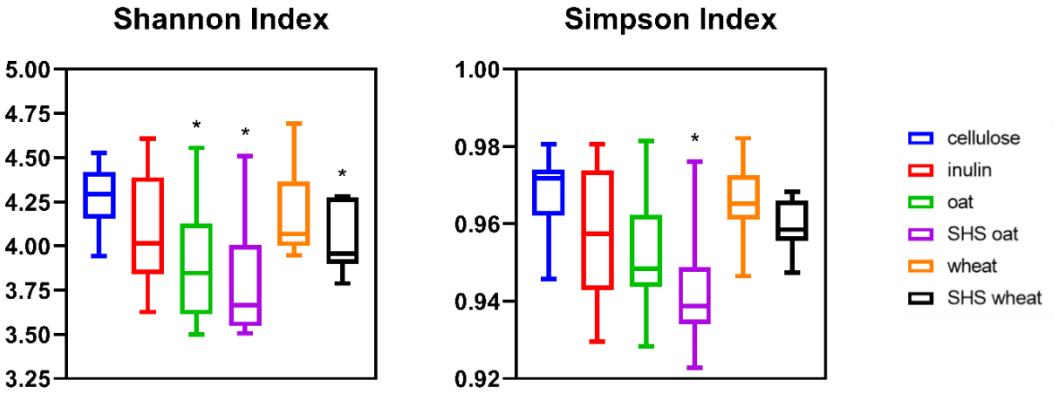

Figure 2.3 Box plots showing the Shannon and Simpson indices following 12 h fermentation. Mann-Whitney U test was used to compare groups to the negative control (*$p < 0.05$).

The Shannon index was significantly lower following fermentation in the presence of oat, SHS-treated oat, and SHS-treated wheat samples compared with cellulose ($p = 0.0379$, $p = 0.0147$, $p = 0.0498$; respectively). The Simpson index was significantly lower following fermentation in the presence of SHS-treated oat samples compared to cellulose ($p = 0.0147$) (Figure 2.3).

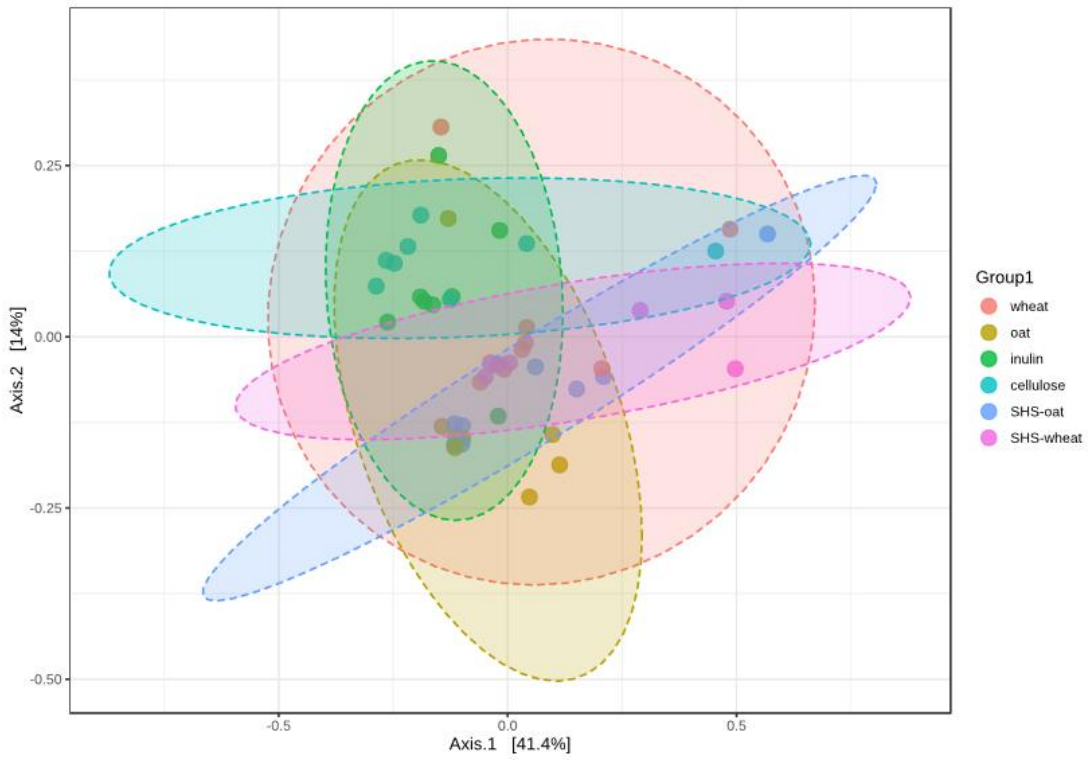

Figure 2.4 Principal Coordinate analysis (PCoA) using Bray-Curtis dissimilarity shows separation between groups. PERMANOVA reveals significant differences ($p < 0.05$) among groups. Eclipses indicate 95% confidence intervals around samples from each group.

Principal Coordinate analysis (PCoA) using Bray-Curtis distance was used to examine community compositional differences following fermentation in the presence of different fibre samples. The community composition following fermentation in presence of all fibre samples (with the exception of inulin) separated significantly from the negative control (cellulose) using permutational multivariate analysis of variance (PERMANOVA) (Figure 2.4). Moreover, a significant separation was observed in community composition between SHS-treated oat and non-treated oat ($p = 0.013$) as well as between SHS-treated wheat and non-treated wheat ($p = 0.042$) (Supplementary Table 2.3).

2.3.3 Taxonomic composition

At the phylum level, six phyla were detected in all groups following fermentation. As expected, Firmicutes and Bacteroidetes were the predominant phyla in all samples. DESeq2 was used to evaluate the differential abundance of bacteria at phylum and genus levels. Firmicutes was the dominant phylum following fermentation in the presence of SHS-treated oat where it had significantly higher relative abundance compared with the cellulose group (p = 2.00E-0.7, FDR = 1.40E-06; Supplementary Table 2.6) and oat samples (p = 1.65E-07, FDR = 1.15E-06; Supplementary Table 2.12).

Similarly, the relative abundances of Firmicutes following fermentation in the presence of wheat (p = 0.000878, FDR = 0.003073; Supplementary Table 2.8) and SHS-treated wheat (p = 1.49E-8, FDR = 1.04E-07; Supplementary Table 2.10) were significantly higher than in the presence of cellulose (Figure 2.5). The Bacteroidetes phylum had significantly higher relative abundance following fermentation in the presence of oat than in presence of cellulose (Figure 2.5) (p = 0.000595, FDR = 0.002427; Supplementary Table 2.4). Actinobacteria were detected at a significantly higher relative abundance following fermentation in presence of all fibres (with the exception of inulin) than in cellulose (Figure 2.5; Supplementary Table 2.4, 2.6, 2.8, and 2.10). Verrucomicrobiota was found at significantly lower relative abundance following fermentation of oat (p = 0.007589, FDR = 0.017708; Supplementary Table 2.4), SHS-treated oat (p = 0.003443, FDR = 0.008034; Supplementary Table 2.6), and wheat (p = 0.00435, FDR = 0.010149; Supplementary Table 2.8) compared with cellulose (Figure 2.5). However, it was significantly higher in the SHS-treated wheat group compared with cellulose (p = 0.010316, FDR = 0.018052; Supplementary Table 2.10).

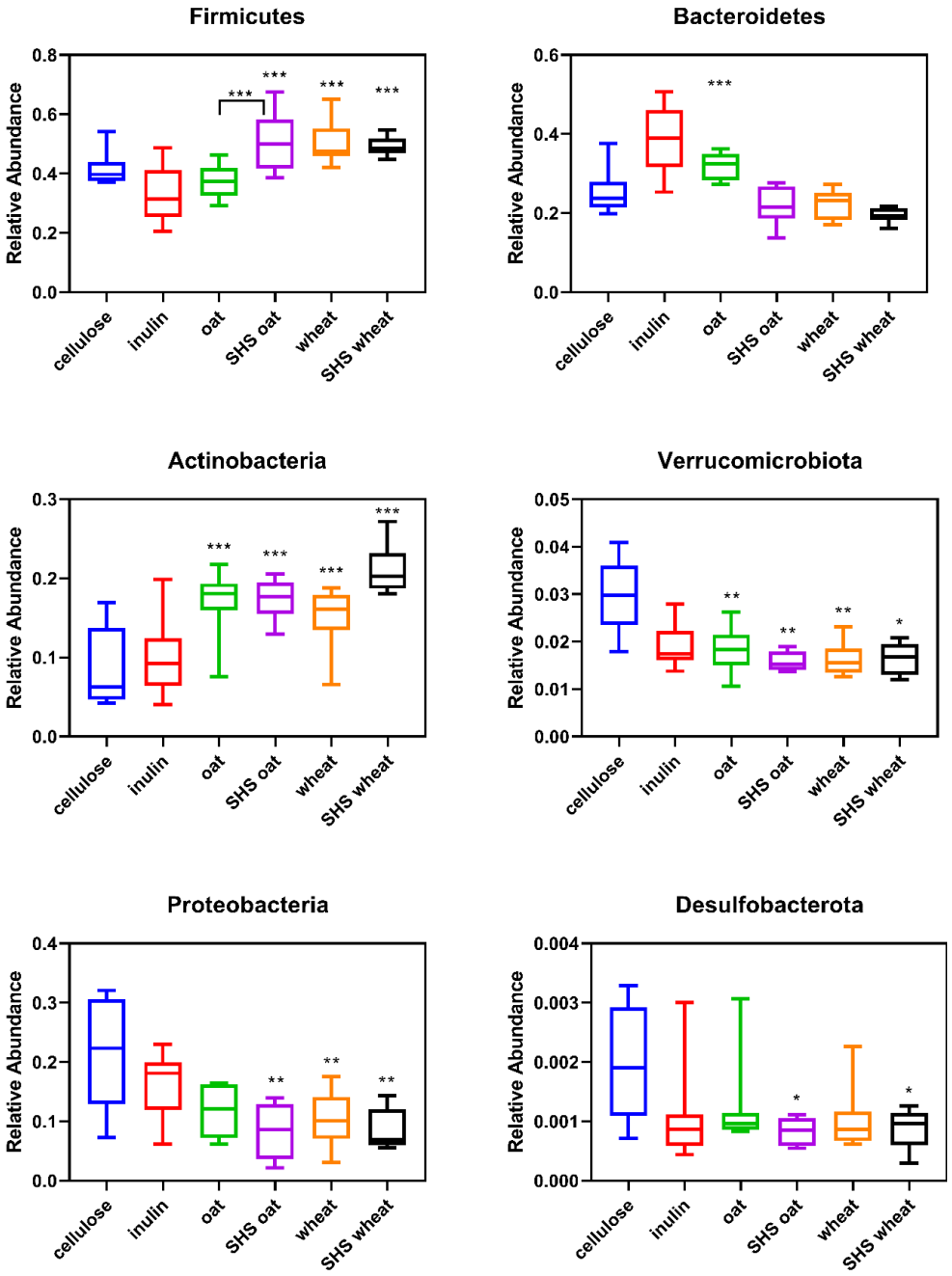

Figure 2.5 Relative abundance of Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobiota, Proteobacteria, and Desulfobacterota among groups. DESeq2 was used to evaluate differential abundance of bacterial phyla. Asterisks (*) above the bars indicate significance compared to the cellulose control (*p < 0.05, **p < 0.01, ***p < 0.001).

Proteobacteria was also found at significantly lower relative abundance in SHS-treated oat (p = 0.006064, FDR = 0.010613; Supplementary Table 2.6), wheat (p = 0.008122, FDR = 0.014214; Supplementary Table 2.8), and SHS-treated wheat (p = 0.002747,

FDR = 0.00641; Supplementary Table 2.10) following fermentation than in cellulose (Figure 2.5). Moreover, Desulfobacterota was found at lower relative abundance following fermentation in both SHS-treated oat (p = 0.034987, FDR = 0.048982; Supplementary Table 2.6) and SHS-treated wheat (p = 0.014161, FDR = 0.019825; Supplementary Table 2.10) compared with cellulose (Figure 2.5).

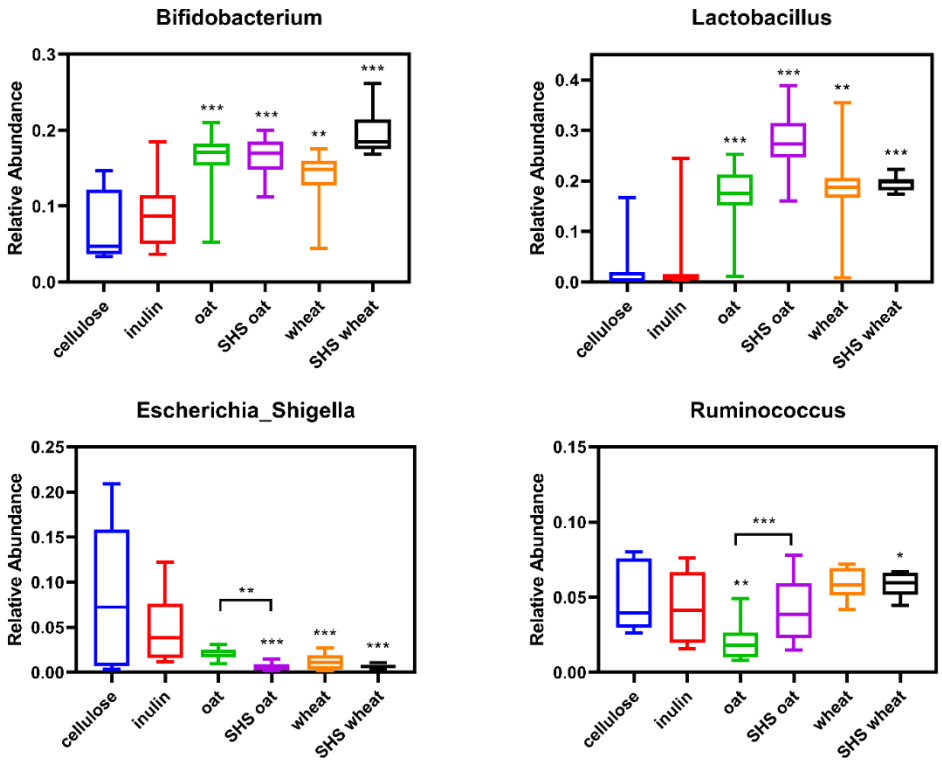

Figure 2.6 Relative abundances of *Bifidobacterium*, *Lactobacillus*, *Escherichia_Shigella*, and *Ruminococcus* among groups. DESeq2 was used to evaluate differential abundance of bacterial phyla. Asterisks (*) above the bars indicate significance compared to the cellulose control (*p < 0.05, **p < 0.01, ***p < 0.001).

A number of significant differences were revealed in community composition at genus level following fermentation in the presence of the different fibres. In total, 21 genera were found to be significantly different between oat and cellulose samples (Supplementary Table 2.5). Figure 2.6 shows that abundances of *Bifidobacterium* and

Lactobacillus were 1.94-fold (p = 7.06E-05, FDR = 0.0000261) and 3.55-fold (p = 0.00019, FDR = 0.003175) higher following fermentation in the presence of oat samples than in presence of cellulose, respectively.

Similarly, relative abundances of *Faecalibacterium* were 1.04-fold (p = 0.001564, FDR = 0.009434), *Coprobacter* 3.85-fold (p = 0.00169, FDR = 0.009434), and *Pediococcus* 3.85-fold (p = 0.002228, FDR = 0.009951) significantly increased in the oat samples compared with cellulose. *Megamonas* was 2.88-fold (p = 0.000248, FDR = 0.003322) significantly lower following fermentation in presence of oat than in presence of cellulose.

Abundances of nineteen genera were significantly altered following fermentation in the presence of SHS-treated oat and cellulose (Supplementary Table 2.7). Following fermentation in SHS-treated oat, *Escherichia-Shigella* was found to be 2.99-fold (p = 5.93E-05, FDR = 0.000519) significantly lower than in cellulose group while *Lactobacillus* was 3.81-fold (p = 3.24E-05, FDR = 0.000378) significantly higher in SHS-treated oat group compared with cellulose group (Figure 2.6). *Pediococcus* was significantly higher by 4.2-fold (p = 5.85E-05, FDR = 0.000519), *Bifidobacterium* by 1.87-fold (p = 9.85E-07, FDR = 3.95E-05), and *Faecalibacterium* by 1.35-fold (p = 3.11E-05, FDR = 0.000378) following fermentation in presence of SHS-treated oat than in presence of cellulose, respectively. In the presence of SHS-treated oat, significant decrease in relative abundance of *Megamonas* was observed by 3.03-fold when compared with cellulose (p = 0.000182, FDR = 0.001275).

The fermentation of wheat yielded significant differences of 21 genera compared to cellulose (Supplementary Table 2.9). The relative abundances of *Bifidobacterium* were significantly higher by 1.24-fold (p = 0.002073, FDR = 0.017042), *Lactobacillus* by

3.13-fold (p = 0.00101, FDR = 0.010675), and *Pediococcus* by 3.36-fold (p = 0.009684, FDR = 0.055126) following fermentation in presence of wheat than in presence of cellulose, respectively (Figure 2.6). Fermentation of wheat resulted in 3.33-fold reduction in the relative abundance of *Megamonas* and a 2.72-fold reduction of *Escherichia_Shigella* compared to cellulose (p = 2.69E-05, FDR = 0.0019875; p = 0.000226, FDR = 0.0048262; respectively).

Comparison of relative abundances at the genus level also revealed that 26 genera were significantly different following fermentation in presence of SHS-treated wheat and cellulose (Supplementary Table 2.11). In presence of the SHS-treated wheat, *Bifidobacterium* was significantly higher by 1.83-fold (p = 4.55E-08, FDR = 1.66E-06), *Lactobacillus* by 3.54-fold (p = 2.78E-05, FDR = 0.000406), and *Pediococcus* by 3.41-fold (p = 0.000713, FDR = 0.004003) than in presence of cellulose. *Escherichia_Shigella* was significantly lower by 3.21-fold (p = 9.71E-07, FDR = 1.77E-05), *Sutterella* by 2.07-fold (p = 1.15E-12, FDR = 8.40E-11), and *Megamonas* by 3.73-fold (p = 5.36E07, FDR = 1.30E-05) in SHS-treated wheat group when compared to cellulose, respectively.

Abundances of three genera were found to be significantly altered following fermentation in presence of SHS-treated oat and native oat (Supplementary Table 2.13). The abundance of *Escherichia_Shigella* was significantly lower by 1.47-fold (p = 0.002037, FDR = 0.056525) and *Alistipes* by 0.39-fold (p = 0.002458, FDR = 0.056525) in SHS-treated oat than non-treated oat groups, respectively. On the contrary, *Ruminococcus* was found to be 1.09-fold higher following fermentation in the presence of SHS-treated oat compared with non-treated oat (p = 6.65E-06, FDR = 0.00047276).

Fermentation of both SHS-treated oat and wheat resulted in significantly higher relative abundance of *Lachnospiraceae* NC2004 group than cellulose by 2.8-fold and 2.25-fold, respectively ($p = 0.009989$, FDR = 0.038844; $p = 0.005652$, FDR = 0.025789).

LEfSe analysis was used to determine the genera most likely to explain differences among groups (Figure 2.7). The most differentially abundant bacterial taxa in cellulose were *Escherichia_Shigella* and *Sutterella*. SHS-treated oat, oat and SHS-treated wheat were characterized by a preponderance of *Lactobacillus*.

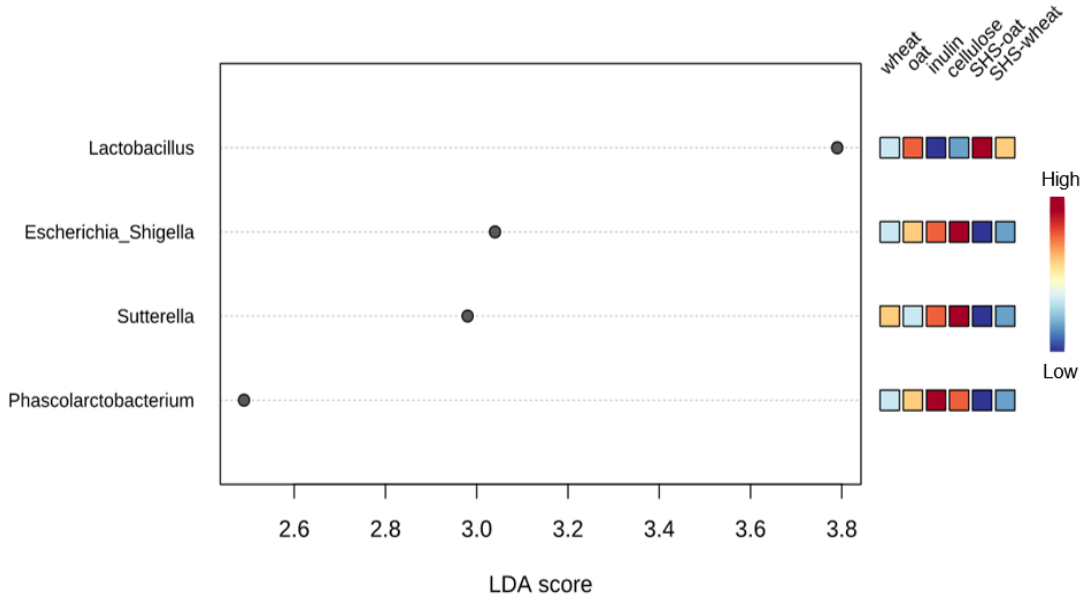

Figure 2.7 LEfSe analysis demonstrates the signature genera in each fibre group.

2.3.4 SCFA analysis

Acetate was significantly higher following fermentation in the presence of all fibre samples ($p < 0.001$) when compared with cellulose (negative control) (Figure 2.8). Acetate concentration was significantly higher in oat compared to all other fibre groups ($p < 0.001$).

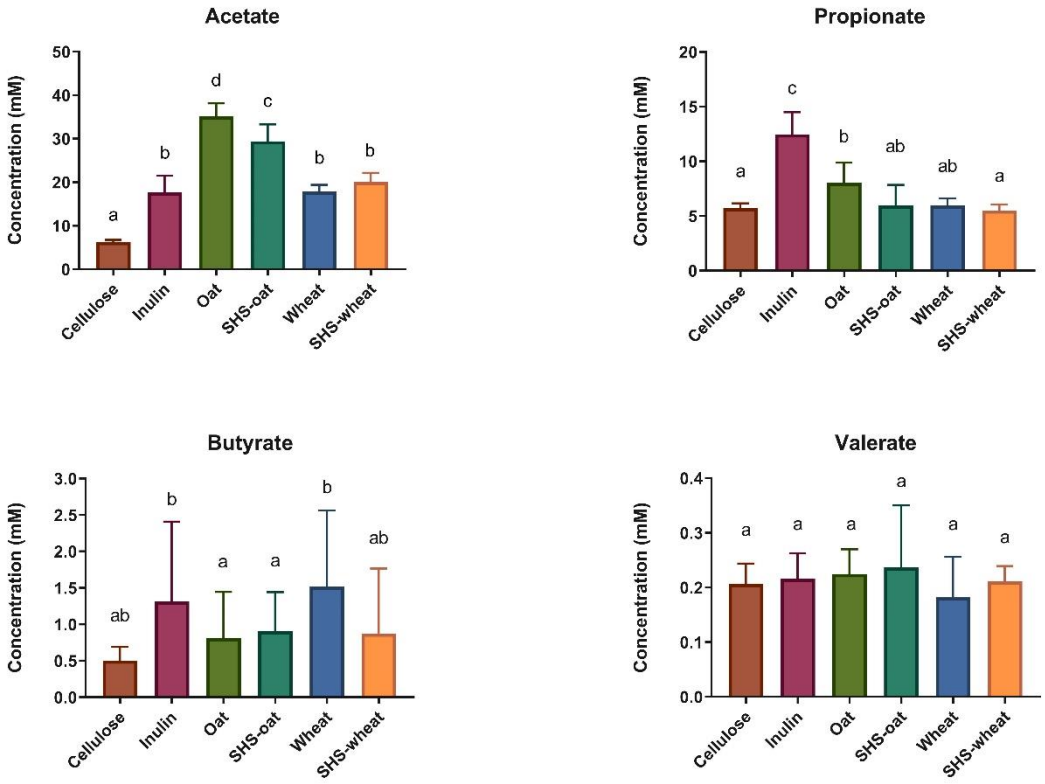

Figure 2.8 Concentrations of acetate, propionate, butyrate, and valerate among groups. A one-way ANOVA was performed with Tukey's test. Values with different letters indicate significant differences among the groups.

Propionate was significantly higher following fermentation in the presence of inulin when compared to all other fibre groups ($p < 0.001$) and was significantly higher following fermentation with oat samples when compared to cellulose and SHS-treated wheat ($p = 0.025$, $p = 014$; respectively) (Figure 2.8).

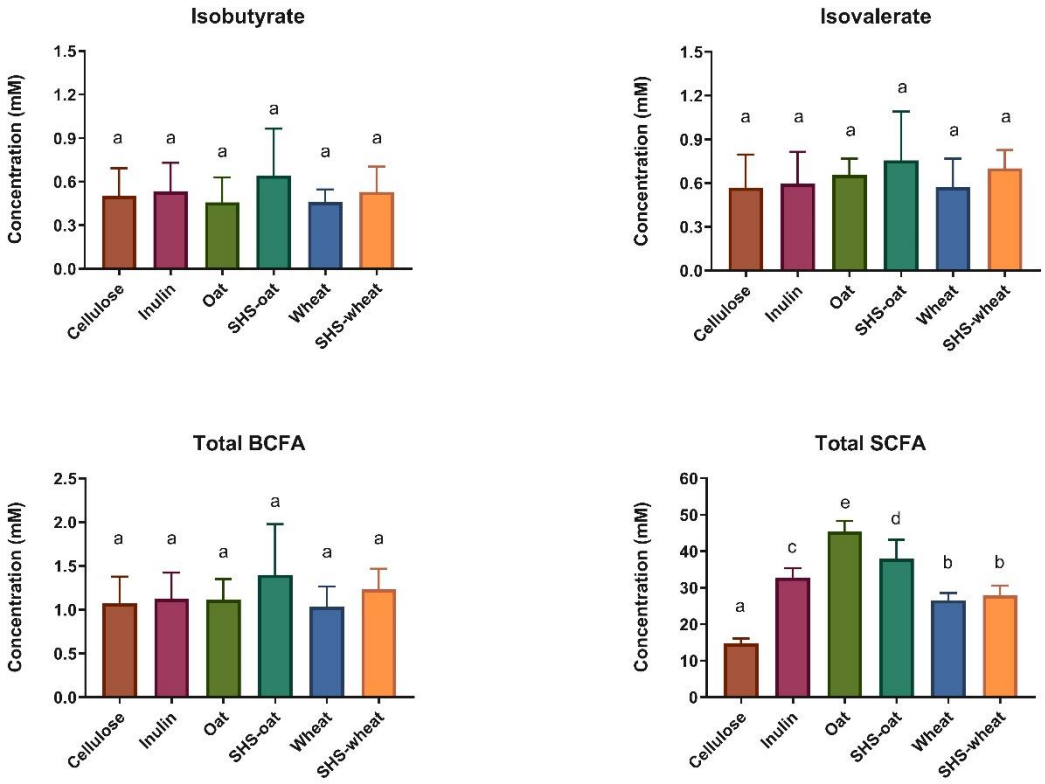

Figure 2.9 Concentrations of isobutyrate, isovalerate, total branched chain fatty acid (BCFA), and total short chain fatty acid (SCFA) among groups. A one-way ANOVA was performed with Tukey's test. Values with different letters indicate significant differences among the groups.

Butyrate levels were not significantly altered following fermentation in the presence of wheat compared to cellulose. Inulin, oat, SHS-treated oat, and SHS-treated wheat had reduced butyrate production compared to cellulose (although differences were not significant). Butyrate levels were significantly higher following fermentation in the presence of inulin compared to SHS-treated oat and native oat ($p = 0.004$, $p = 0.007$; respectively). Wheat had a significantly higher butyrate level than both native and SHS-treated oat ($p = 0.012$, $p = 0.008$; respectively).

Valerate levels were slightly higher following fermentation in the presence of all fibres, with the exception of wheat and SHS-treated wheat, when compared to

cellulose (not significant). No significant differences were observed in the concentrations of isobutyrate and isovalerate among the groups (Figure 2.9).

2.4 Discussion

SHS treatment of bran has been reported as a promising method to enhance its nutritional properties and/or make more readily available (Diaz, 2015). This study examined the modulating effects of SHS-treated dietary fibres on the human gut microbiota as well as their abilities to promote SCFA production.

Physical and chemical properties of brans showed a number of significant differences between SHS-treated and non-treated brans. SHS treatment of both oat and wheat resulted in increased level of maltose, polymeric glucose, and WEAX (Supplementary Tables 2.1 and 2.2). A 2019 study reported that increased total SCFA levels were found following *in vitro* fermentation of wheat and oat bran. It was also shown that during faecal fermentation, the beta glucan level was gradually decreased in both oat and wheat bran samples (Roye et al., 2019).

Analysis of alpha diversity following fermentation in the presence of SHS-treated oat, native (non-treated) oat, and SHS-treated wheat revealed that it was less diverse than following treatment with cellulose, which resulted in the highest diversity, using Shannon index. The Simpson index also revealed that fermentation in the presence of SHS-treated oat led to significantly less diverse microbiota compared with cellulose. PCoA analysis revealed significant separation of each fibre group from cellulose, with the exception of inulin. Of particular note was the observation that SHS-treated oat and SHS-treated wheat separated significantly from native oat and wheat, respectively ($p=0.013$ and $p=0.042$). This finding indicates that SHS treatment promotes altered gut microbiota composition compared to the untreated bran.

Fibre supplementation resulted in a significant increase in the relative abundance of Firmicutes, which is not surprising as this phylum is dominated by lactic acid bacteria including butyrate-producing bacteria (Flint et al., 2015, Tanaka and Nakayama, 2017). Similarly, Actinobacteria were found to be significantly higher in all fibre groups when compared with cellulose. A recent study demonstrated that the abundance of Firmicutes and Actinobacteria were significantly higher in young adults than in the elderly population (Li et al., 2021). This increase in abundance of Actinobacteria may be driven by the abundance of the *Bifidobacterium* genus (Li et al., 2021). Another study also showed that the abundance of several butyrate producing bacteria including *Eubacterium hallii* and *Ruminococcus obeum* et rel. were lower in elderly subjects (over 100 years old) than in younger subjects (Biagi et al., 2010). This reduction in Firmicutes phylum throughout ageing might be a result of reduced levels of butyrate producing bacteria.

Following fibre supplementation, significant increases in several potentially probiotic bacterial genera were observed. One of the most well-established probiotic bacteria is *Bifidobacterium* which is associated with the healthy human gut and is widely used as a probiotic supplement due to its health promoting effects (O'Callaghan and van Sinderen, 2016). Relative abundances of *Bifidobacterium* were significantly higher following fermentation in the presence of oat and wheat and their SHS-treated counterparts compared with cellulose. Studies have shown that both plant and host-derived carbohydrates can stimulate the growth of *Bifidobacterium* (O'Callaghan and van Sinderen, 2016). No significant differences were observed between the oat samples (i.e. SHS-treated oat and native oat) and the wheat samples (i.e. SHS-treated wheat and native wheat) in terms of relative abundances of *Bifidobacterium*. *Lactobacillus* abundances were also higher following fermentation in the presence of

oat, wheat, and their SHS-treated counterpart groups when compared to the negative control (cellulose). LEfSe analysis revealed that the SHS-treated oat group was shaped mostly by *Lactobacillus* which has been shown to have a pathogen-inhibiting effect via binding to epithelial cell receptors, producing bile salt hydrolase to survive in the small intestine and producing lactic acid which results in a reduction of pH (Er et al., 2019). Bile salts have been reported as toxic to bacterial cells via disrupting cell membrane (Succi et al., 2005). Reduction of pH inhibits the growth of pathogenic microorganisms including food-borne pathogens such as *Salmonella enterica* and *Listeria monocytogenes* (Lund et al., 2020).

Our study demonstrated a significant reduction in *Escherichia_Shigella* following fermentation in the presence of SHS-treated brans. LEfSe analysis revealed that *Escherichia_Shigella* was the signature genera of the negative control (cellulose) group. *Escherichia_Shigella* and *Alistipes* were found to be significantly lower following fermentation in the presence of SHS-treated oat compared with non-treated oat. *Escherichia* and *Shigella* are known as enteric pathogens which are associated with several gastric diseases (Devanga Ragupathi et al., 2018). While *Escherichia coli* might be considered as commensal bacteria in the healthy human gut, some strains have virulence factors (Kaper et al., 2004). A broad range of infections are attributed to different *Escherichia* strains such as urinary tract infection, meningitis/sepsis in children, and diarrhoea (James P. Nataro, 1998). Increases in abundance of *Escherichia* are commonly observed in gut microbiota dysbiosis such as small intestinal bacterial overgrowth or inflammatory bowel diseases (Leite et al., 2021, Siniagina et al., 2020).

Alistipes is a relatively new genus found in the human gut microbiota and belongs to the Bacteroidetes phylum. It is anaerobic and generally considered commensal bacteria

(Parker et al., 2020). This genus was first discovered in tissue samples of children diagnosed with appendicitis (Rautio et al., 2003). Some studies have revealed a link between *Alistipes* and a number of diseases including liver fibrosis, colorectal cancer, and cardiovascular disease (Moschen et al., 2016, Rau et al., 2018, Zuo et al., 2019). A recent study conducted in humans revealed a positive correlation between high systolic blood pressure and *Alistipes indistinctus* (Pearson correlation = 0.33, p = 0.0357; Spearman correlation = 0.28, p = 0.0818) and *Alistipes finegoldii* (Pearson correlation = 0.26, p = 0.069; Spearman correlation = 0.39, p = 0.0125) (Kim et al., 2018). Moreover, this genus has been implicated in inflammation and bacteraemia in gastrointestinal disorders (Parker et al., 2020).

Following fermentation in the presence of SHS-treated oat and wheat samples, both showed significantly increased relative abundances of *Lachnospiraceae* NC2004 group as compared to cellulose. Recently, a reduction of *Lachnospiraceae* NC2004 group in Chinese children with autism spectrum disorder was reported (Lun et al., 2019). *Lachnospiraceae* is known as one of the key butyrate producers in the gut (Pryde et al., 2002). Another genus that was found at significantly higher relative abundance in all fibre groups (with the exception of inulin) is *Pediococcus* with 4.2-fold increase in SHS-treated oat when compared to cellulose. *Pediococcus* is a member of the lactic acid bacteria group and is mainly used in the production of fermented products such as porridges, cooled gels, alcoholic, and non-alcoholic beverages (Coda et al., 2011, Nout, 2009). *Pediococcus* is also a pediocin producer, one of the most effective bacteriocins against *Listeria monocytogenes* (Porto et al., 2017). Interestingly, we observed that *Megamonas* was significantly lower in all fibre groups compared to cellulose. A 2015 study examined the faecal microbiota composition of patients diagnosed with major depressive disorder. *Megamonas*, *Alistipes*, and

Phascolarctobacterium were found to be more abundant in patients with major depressive disorder when compared with healthy controls (Jiang et al., 2015). In another study, *Megamonas* was also found to be 8.83-fold greater in subjects with pre-diabetes mellitus than in normal glucose tolerant subjects (Zhang et al., 2013). A number of studies have demonstrated that some strains of the *Megamonas* genus can ferment glucose to acetate and propionate (Chevrot et al., 2008, Sakon et al., 2008).

Faecalibacterium is another genus that contains butyrate-producing species such as *Faecalibacterium prausnitzii* (Zuo et al., 2019). The abundance of *Faecalibacterium* was significantly higher in relative abundance in all fibre groups (with the exception of inulin) compared with cellulose. A 2019 study investigating the heart-gut axis revealed that *Faecalibacterium* was lower in patients with atrial fibrillation than in healthy subjects (Zuo et al., 2019). Similarly, reduced abundances of *Faecalibacterium* have been reported in Crohn's disease patients (Takahashi et al., 2016).

Altogether, our results suggest that SHS treatment is more effective for oat bran than wheat bran. This effect might be attributed to the higher beta glucan content in oat samples. Following *in vitro* batch fermentation with fibre supplementation, a significant increase in relative abundance of Firmicutes and Actinobacteria was found. As the consumption of fibre has decreased over the years, it is important to produce novel foods to improve human health.

2.5 Conclusions

This study has demonstrated that SHS-treated oat and wheat bran can positively modulate gut microbiota composition generating differential effects when compared with the non-treated counterparts. SHS treatment can be useful in the food industry to

increase the functionality of dietary fibre to positively modify the gut microbiome. Even though this in vitro study showed the potential health promoting effects of SHS treatment, it is not certain that treated fibres have beneficial effects on human health. Animal studies and human clinical trials should be performed to confirm the beneficial effects *in vivo*.

2.6 Acknowledgements

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2.8 Supplementary Tables

Supplementary Table 2.1 Chemical and physical properties of native and SHS-treated wheat bran. Composition is expressed as milligrams of substance per grams of dry matter, unless otherwise stated. Different letters in the same row indicate statistically significant differences ($p < 0.05$).

	W-ref	W-120-10	W-120-40	W-140-10	W-140-40	W-160-10	W-120-40-1
Free sugars							
arabinose	5.8±0.1b	4.0±0.0a	3.6±0.1a	4.1±0.1a	3.3±0.1a	4.0±0.7a	3.4±0.0a
galactose	3.9±0.0b	2.6±0.0a	2.5±0.0a	2.6±0.0a	2.1±0.0a	2.5±0.4a	2.4±0.0a
glucose	31.1±1.3a	27.6±0.0a	26.6±0.0a	28.2±0.0a	24.9±0.3a	30.2±5.4a	26.2±0.3a
xylose	3.5±0.1e	1.8±0.0d	1.5±0.0c	1.7±0.0cd	0.9±0.0a	1.2±0.2b	1.5±0.1bc
maltose	9.7±0.4a	17.2±0.3b	13.4±0.2ab	13.5±0.1ab	15.2±0.4b	14.7±2.9b	13.2±0.0ab
Total free sugars	54.1±1.7a	53.3±0.2a	47.5±0.3a	50.0±0.2a	46.3±0.8a	52.5±9.6a	46.7±0.3a
Polysaccharides							

Polymeric galactose	1.3±0.0a	3.0±0.1ab	3.1±0.1ab	3.3±0.1b	2.9±1.0ab	4.4±0.9b	3.8±0.1b
Polymeric glucose	5.3±0.8a	27.7±0.6b	24.7±0.1b	25.6±0.7b	30.1±7.7b	38.0±8.0b	28.9±1.2b
WEAX	3.9±0.0a	13.6±0.1ab	15.3±0.4bc	16.4±0.1bc	26.0±5.3cd	28.0±4.9d	19.0±0.5bcd
Total phenolics	2.2±0.0	2.4±0.0	2.7±0.0	3.0±0.1	3.9±0.1	4.9±0.0	3.1±0.1
Soluble proteins	17.7±0.6d	5.4±0.1a	6.0±0.0ab	7.8±0.1bc	6.8±0.3abc	8.5±0.3c	6.6±0.1ab
Physical properties							
WBC (g/g dm)	2.4±0.0a	3.3±0.1b	3.3±0.0b	3.3±0.1b	3.4±0.3b	2.5±0.3a	3.7±0.1b
Soluble solids (%)	17.4±0.1a	18.7±0.3a	18.2±0.2a	18.4±0.4a	18.4±1.2a	21.7±1.4b	18.0±0.2a
L	63.6±20.e	53.1±0.3bc	53.6±0.4cd	52.2±0.1ab	51.6±0.1a	52.1±0.6ab	54.3±0.7d
a	3.9±0.1a	5.9±0.1b	5.8±0.1b	6.0±0.0b	6.5±0.1c	6.4±0.1c	5.6±0.2b
b	19.9±0.0d	15.3±0.4a	15.8±0.1bc	15.6±0.0ab	16.2±0.1c	16.1±0.2c	16.0±0.1bc

Supplementary Table 2.2 Chemical and physical properties of native and SHS-treated oat bran. Composition is expressed as milligrams of substance per grams of dry matter, unless otherwise stated. Different letters in the same row indicate statistically significant differences ($p < 0.05$).

	O-ref	O-120-10	O-120-40	O-140-10	O-140-40	O-160-10	O-120-40-1
Free sugars							
arabinose	<0.1	<0.1	<0.1	<0.1	0.2±0.0a	0.3±0.0b	<0.1
galactose	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
glucose	0.3±0.0a	0.5±0.2a	0.7±0.0a	0.7±0.0a	0.5±0.0a	0.7±0.4a	0.6±0.0a
xylose	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
maltose	1.0±0.0a	5.4±0.2b	5.4±0.1b	5.5±0.0b	5.2±0.2b	5.1±0.1b	5.2±0.1b
Total free sugars	1.3±0.0a	6.0±0.0b	6.3±0.1b	6.4±0.0b	6.0±0.2b	6.1±0.5b	6.0±0.1b
Soluble Polysaccharides							
Polymeric galactose	3.3±0.1a	3.5±0.0a	3.7±0.1a	3.7±0.1a	3.9±0.0a	4.0±0.0a	3.7±0.0a
Polymeric glucose	31.5±1.7a	62.6±5.1b	67.7±0.3bc	78.6±2.1cd	84.9±5.8ef	97.9±1.4f	78.6±6.4cd
WEAX	4.9±0.0a	5.7±0.3a	6.3±0.2bc	7.2±0.1bc	10.2±0.4cd	14.5±0.1d	6.9±0.2bc

β-glucans	5.6±0.1a	7.1±0.1a	7.2±0.2a	6.9±0.2a	7.1±0.7a	6.8±1.3a	9.7±0.3b
Total phenolics	1.1±0.0	1.0±0.0	0.9±0.0	1.0±0.0	1.3±0.0	1.6±0.0	1.0±0.0
Soluble proteins	3.3±0.6ab	2.8±0.3a	3.1±0.2ab	3.8±0.0ab	4.1±0.1bc	5.3±0.2c	3.4±0.3ab
Physical properties							
WBC (g/g dm)	2.9±0.0a	4.3±0.0cd	4.8±0.3de	3.9±0.1bc	3.5±0.3ab	4.2±0.0bcd	5.3±0.1e
Soluble solids (%)	12.9±0.3d	8.1±0.4b	7.4±0.2ab	7.7±0.3b	8.0±0.3b	9.2±0.3c	6.4±0.1a
L	84.1±0.0f	71.8±0.3e	71.2±0.2d	68.7±0.1c	67.9±0.1b	65.8±0.1a	68.6±0.2c
a	0.6±0.0a	2.5±0.0b	2.7±0.0c	3.0±0.0d	3.5±0.0f	3.6±0.0g	3.2±0.0e
b	11.0±0.0a	17.3±0.1b	17.5±0.1c	18.1±0.0e	17.3±0.0b	17.4±0.0bc	17.8±0.1d

Supplementary Table 2.3 PERMANOVA results show significant differences among groups ($p<0.05$).

Groups	F Value	R squared	P value
Oat and Cellulose	9.0991	0.39392	0.004
SHS-oat and Cellulose	15.23	0.52105	0.001
Wheat and Cellulose	7.4474	0.34724	0.006
SHS-wheat and Cellulose	14.952	0.51643	0.001
Oat and SHS-oat	3.4023	0.19551	0.013
Wheat and SHS-wheat	1.892	0.11905	0.042

Supplementary Table 2.4 DESeq2 analysis results show the significant differences between oat and cellulose at the phylum level.

Taxa_Phylum	log2 FC	Lfc SE	P value	FDR
Bacteroidetes	-0.69752	0.20312	0.000595	0.002427
Actinobacteria	-1.3181	0.38856	0.000693	0.002427
Verrucomicrobiota	0.2982	0.11169	0.007589	0.017708

Supplementary Table 2.5 DESeq2 analysis results show the significant differences between oat and cellulose at the genus level.

Taxa_Genus	log2 FC	Lfc SE	P value	FDR
Bifidobacterium	-1.9464	0.42156	3.89E-06	0.000261
Bacteroides	-1.1437	0.28777	7.06E-05	0.001933
Not_Assigned	0.45604	0.11617	8.65E-05	0.001933
Lactobacillus	-3.5552	0.95249	0.00019	0.003175
Megamonas	2.8828	0.78671	0.000248	0.003322
Holdemanella	1.047	0.30133	0.000511	0.00571
Succinivibrio	-1.7998	0.52643	0.000629	0.00576
Odoribacter	-0.70907	0.20889	0.000688	0.00576
Sutterella	1.1509	0.36005	0.001391	0.009434
Barnesiella	-1.8299	0.57417	0.001438	0.009434
Faecalibacterium	-1.0441	0.33014	0.001564	0.009434
Coprobacter	-3.8501	1.2262	0.00169	0.009434
Eubacterium_hallii_group	0.68206	0.21936	0.001875	0.009664
Negativibacillus	3.9562	1.2913	0.002186	0.009951

Pediococcus	-3.8934	1.2732	0.002228	0.009951
Alistipes	-0.86196	0.28838	0.002799	0.01172
Dialister	-0.414	0.1434	0.003889	0.015328
Ruminococcus	0.81234	0.29486	0.005869	0.021845
Acidaminococcus	1.2223	0.4897	0.012563	0.044302
UCG_002	0.79643	0.32576	0.014492	0.048548
Escherichia_Shigella	1.4744	0.65504	0.024393	0.077825

Supplementary Table 2.6 DESeq2 analysis results show the significant differences between SHS-oat and cellulose at the phylum level.

Taxa_Phylum	**log2 FC**	**Lfc SE**	**P value**	**FDR**
Firmicutes	0.79312	0.15253	2.00E-07	1.40E-06
Actinobacteria	1.481	0.32989	7.14E-06	2.50E-05
Verrucomicrobiota	-0.37165	0.12705	0.003443	0.008034
Proteobacteria	-1.0763	0.39221	0.006064	0.010613
Desulfobacterota	-0.79445	0.37678	0.034987	0.048982

Supplementary Table 2.7 DESeq2 analysis results show the significant differences between SHS-oat and cellulose at the genus level.

Taxa_Genus	**log2 FC**	**Lfc SE**	**P value**	**FDR**
Bifidobacterium	1.8726	0.3826	9.85E-07	3.95E-05
Sutterella	-1.7879	0.36729	1.13E-06	3.95E-05
Christensenellaceae_R_7_group	-0.64834	0.14199	4.97E-06	0.000116
Barnesiella	2.0075	0.46539	1.61E-05	0.000281
Faecalibacterium	1.3528	0.32478	3.11E-05	0.000378
Lactobacillus	3.8118	0.91724	3.24E-05	0.000378
Pediococcus	4.2065	1.0467	5.85E-05	0.000519
Escherichia_Shigella	-2.9932	0.74538	5.93E-05	0.000519
Anaerostipes	-0.71636	0.1849	0.000107	0.000832
Megamonas	-3.0373	0.81157	0.000182	0.001275
Holdemanella	-0.98863	0.26927	0.000241	0.001534
Parasutterella	-1.0911	0.30894	0.000413	0.002407
UCG_002	-0.81206	0.26434	0.002126	0.011447
Coprobacter	2.9917	0.98901	0.002487	0.012435

Not_Assigned	-0.30816	0.1125	0.006159	0.028743
Acidaminococcus	-1.2979	0.48807	0.007832	0.034264
Bilophila	-1.6261	0.63049	0.009907	0.038844
Lachnospiraceae_NC2004_group	2.8657	1.1124	0.009989	0.038844
Succinivibrio	1.326	0.56337	0.018591	0.068494

Supplementary Table 2.8 DESeq2 analysis results show the significant differences between wheat and cellulose at the phylum level.

Taxa_Phylum	**log2FC**	**lfcSE**	**P value**	**FDR**
Actinobacteria	-1.1404	0.31422	0.000284	0.00199
Firmicutes	-0.61115	0.1837	0.000878	0.003073
Verrucomicrobiota	0.53499	0.18761	0.00435	0.010149
Proteobacteria	0.79875	0.30176	0.008122	0.014214

Supplementary Table 2.9 DESeq2 analysis results show the significant differences between wheat and cellulose at the genus level

Taxa_Genus	**log2FC**	**lfcSE**	**P value**	**FDR**
Megamonas	3.3378	0.79497	2.69E-05	0.0019875
Subdoligranulum	0.62689	0.16692	0.000173	0.0048262
Escherichia_Shigella	2.7412	0.74334	0.000226	0.0048262
Christensenellaceae_R_7_group	0.59937	0.16415	0.000261	0.0048262
Sutterella	1.5167	0.43223	0.00045	0.0066566
Akkermansia	0.65962	0.19385	0.000667	0.0082287
Lactobacillus	-3.1302	0.95208	0.00101	0.010675
Phascolarctobacterium	0.95347	0.29797	0.001375	0.012716
Bifidobacterium	-1.236	0.40136	0.002073	0.017042
Oxalobacter	-5.3421	1.8146	0.00324	0.023975
Butyricimonas	0.92614	0.32753	0.004689	0.031542
Faecalibacterium	-0.84686	0.30828	0.006013	0.03708
Pediococcus	-3.3695	1.3025	0.009684	0.055126
Parabacteroides	0.72674	0.28945	0.012048	0.056769
Parasutterella	1.1054	0.44076	0.012146	0.056769
Barnesiella	-1.3999	0.55904	0.012274	0.056769
Succinivibrio	-1.1829	0.49716	0.017349	0.072035

UCG_002	0.38152	0.1606	0.017522	0.072035
Bilophila	1.4995	0.63724	0.01862	0.072519
Acidaminococcus	1.1526	0.50946	0.023669	0.087573
Slackia	0.74838	0.33896	0.027254	0.096038

Supplementary Table 2.10 DESeq2 analysis results show the significant differences between SHS-wheat and cellulose at the phylum level

Taxa_Phylum	**log2FC**	**lfcSE**	**P value**	**FDR**
Firmicutes	0.65421	0.11553	1.49E-08	1.04E-07
Actinobacteria	1.7293	0.32809	1.36E-07	4.76E-07
Proteobacteria	-0.95529	0.31899	0.002747	0.00641
Verrucomicrobiota	-0.43967	0.17141	0.010316	0.018052
Desulfobacterota	-0.68897	0.28085	0.014161	0.019825

Supplementary Table 2.11 DESeq2 analysis results show the significant differences between SHS-wheat and cellulose at the genus level

Taxa_Genus	**log2FC**	**lfcSE**	**P value**	**FDR**
Sutterella	-2.078	0.29221	1.15E-12	8.40E-11
Bifidobacterium	1.8337	0.33535	4.55E-08	1.66E-06
Megamonas	-3.7307	0.74419	5.36E-07	1.30E-05
Escherichia_Shigella	-3.2195	0.65737	9.71E-07	1.77E-05
Lactobacillus	3.542	0.84517	2.78E-05	0.000406
Parasutterella	-1.1433	0.28228	5.11E-05	0.000622
Parabacteroides	-1.0288	0.2585	6.89E-05	0.000718
Phascolarctobacterium	-0.94174	0.23958	8.47E-05	0.000773
Veillonella	-1.9574	0.535	0.000254	0.001954
Acidaminococcus	-1.583	0.43432	0.000268	0.001954
Butyricimonas	-0.98194	0.28113	0.000478	0.003171
Christensenellaceae_R_7_group	-0.4746	0.13946	0.000666	0.004003
Pediococcus	3.411	1.0078	0.000713	0.004003
Faecalibacterium	0.9011	0.30215	0.002861	0.014917
Barnesiella	1.5631	0.54723	0.004285	0.020851
Lachnospiraceae_NC2004_group	2.2585	0.81612	0.005652	0.025789
Subdoligranulum	-0.47742	0.1792	0.007719	0.032228

Eubacterium_ruminantium_group	3.7501	1.4166	0.008115	0.032228
Desulfovibrio	-0.9195	0.34882	0.008388	0.032228
Succinivibrio	1.2035	0.46162	0.00913	0.033305
Akkermansia	-0.5307	0.20486	0.009581	0.033305
Coprococcus	1.3338	0.52709	0.011389	0.037789
Family_XIII_AD3011_group	-0.34267	0.14574	0.018714	0.059396
Collinsella	0.61619	0.28167	0.028697	0.084068
Eubacterium_hallii_group	-0.60003	0.27445	0.02879	0.084068
Ruminococcus	0.56071	0.26096	0.03166	0.088893

Supplementary Table 2.12 DESeq2 analysis results show the significant differences between SHS-oat and oat at the phylum level

Taxa_Phylum	**log2FC**	**lfcSE**	**P value**	**FDR**
Firmicutes	0.62393	0.11918	1.65E-07	1.15E-06

Supplementary Table 2.13 DESeq2 analysis results show the significant differences between SHS-oat and oat at the genus level

Taxa_Genus	**log2FC**	**lfcSE**	**P value**	**FDR**
Ruminococcus	1.0968	0.24382	6.85E-06	0.00047276
Escherichia_Shigella	-1.4706	0.47672	0.002037	0.056525
Alistipes	-0.39441	0.13023	0.002458	0.056525

Chapter 3

Biscuits for the Gut: A Symphony of FODMAPs and Dietary Fibre in Gut Microbiome for Irritable Bowel Syndrome (IBS) Management

Fatma Koc is first author of this research paper which is published in:

Koç, F., Atzler, J., Sahin, A.W., Arendt, E., Ross, R. P., & Stanton, C. (2024). Biscuits for the gut: A symphony of FODMAPs and dietary fibre in gut microbiome for irritable bowel syndrome (IBS) management. Innovative Food Science and Emerging Technologies, 97. https://doi.org/10.1016/j.ifset.2024.103832

3.1 Abstract

Emerging evidence suggests gut microbiome alterations significantly influence irritable bowel syndrome (IBS) symptoms with fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) implicated in triggering symptoms. This study investigated the impact of low and high FODMAP biscuit models on gut microbiota including: Biscuit Flour Control (BiFC), Low-FODMAP Control (LFC), Low-FODMAP High-Fibre Prototype (LFP), and Wholemeal Flour Control (WMC). Biscuits were subjected to *in vitro* digestion which was followed by *in vitro* colonic fermentation. 16S rRNA gene sequencing and fatty acid analyses were performed at the end of fermentation. Alpha diversity analysis revealed samples fermented with LFP induced higher diversity compared to samples fermented with BiFC and LFC. The relative abundance of *Escherichia-Shigella* was significantly lower in LFP fermentates than BiFC fermentates. LEfSe analysis showed LFC fermentates were characterized by higher levels of *Citrobacter* and *Ligilactobacillus*. Significantly reduced acetate and butyrate levels were found in LFP fermentates compared to LFC fermentates. WMC fermentates had higher acetate and propionate levels compared to other biscuit fermentates. In conclusion, incorporating low-FODMAP, high-fibre food prototypes may enhance gut microbial diversity and reduce potentially pathogenic bacteria such as *Escherichia-Shigella*. These findings highlight the potential of personalized dietary interventions, such as the manipulation of biscuit formulations, in modulating gut microbiota and managing IBS symptoms.

Industrial relevance: The low FODMAP diet has been developed as an effective method to moderate the symptoms of IBS patients by reducing bloating and gas in the

colon. Our results revealed that the low-FODMAP, high-fibre biscuit model proved more effective than the low-FODMAP biscuit for generating a gut microbiota that could contribute to easing IBS symptoms. Future research should focus on improving this formulation and adapting it to treat the different forms of IBS. This study also demonstrates the efficacy of using colon fermentation models to devise food formulations for gut microbiome-associated diseases.

3.2 Introduction

Irritable bowel syndrome (IBS) is a chronic gastrointestinal disorder characterized by abdominal pain and altered bowel habits, including diarrhoea, constipation, or a combination of both (Canavan et al., 2014). The global prevalence of IBS varies substantially across different geographical regions and populations, with reported prevalence rates ranging from 1.1 % to 17.2 % (Bommelaer et al., 2004, Olafsdottir et al., 2012). These variations may be attributed to differences in diagnostic criteria, cultural factors, dietary habits, and genetic predisposition among populations (Canavan et al., 2014). Despite its high prevalence and significant impact on quality of life, the aetiology of IBS remains poorly understood (Enck et al., 2016). The condition is complex and multifactorial, with various factors implicated in its pathogenesis, including genetic predisposition, environmental triggers, altered gut motility, and visceral hypersensitivity (Nordin et al., 2024). Although the precise aetiology of IBS remains elusive, emerging evidence suggests that alterations in the gut microbiota composition and function may contribute to the pathophysiology of the condition (Jeffery et al., 2020).

The human gut microbiota comprises a diverse array of microorganisms, including bacteria, viruses, fungi, and archaea, which inhabit the gastrointestinal tract and play essential roles in host metabolism, immune regulation, and nutrient absorption (Lloyd-Price et al., 2016, Koç et al., 2020). Perturbations in the gut microbiota, termed dysbiosis, have been implicated in various gastrointestinal disorders, including IBS, inflammatory bowel disease (IBD), and functional dyspepsia (Jeffery et al., 2020).

Dietary factors have arisen as key determinants of gut microbiota composition and function, with growing evidence implicating dietary patterns in the development and exacerbation of IBS symptoms (Chey et al., 2021). Of particular interest are FODMAPs a group of short-chain carbohydrates that are poorly absorbed in the small intestine and fermented by colonic bacteria (Staudacher and Whelan, 2017). High FODMAP intake has been associated with increased intestinal osmotic load, enhanced bacterial fermentation, and the generation of gas leading to symptoms such as bloating, flatulence, abdominal pain, and altered bowel habits in susceptible individuals (Staudacher and Whelan, 2017).

A low-FODMAP diet developed by researchers at Monash University has gained widespread attention as a therapeutic approach for managing IBS symptoms (Gibson and Shepherd, 2010). This dietary intervention involves restricting high-FODMAP foods from the diet and gradually reintroducing them to identify individual triggers (Halmos et al., 2014). Clinical trials and observational studies have demonstrated the efficacy of the low-FODMAP diet in reducing IBS symptoms, particularly bloating, abdominal pain, and diarrhoea, in a significant proportion of patients (Halmos et al., 2014, Shaikh et al., 2023). However, approximately 30 % of patients do not experience symptom improvement with the low-FODMAP diet, highlighting the need for personalized dietary strategies and further research into alternative therapeutic approaches (Gibson, 2017).

In addition to FODMAPs, dietary fibre intake has been implicated in modulating gut microbiota composition and function and may also influence IBS symptoms (Slavin, 2013). Fibre-rich foods, such as fruits, vegetables, whole grains, and legumes, serve

as substrates for bacterial fermentation in the colon, leading to the production of SCFAs and promoting a healthy gut microbiota (Slavin, 2013). However, excessive fibre intake or the consumption of certain types of fibre, such as insoluble fibre, may exacerbate symptoms in some individuals with IBS, highlighting the importance of personalized dietary recommendations (Mullin et al., 2014).

Ensuring adequate dietary fibre intake while adhering to a low FODMAP diet poses a challenge for individuals with IBS as there are limited options available. Wheat is a good source of dietary fibre (P and Joye, 2020). However, it contains high levels of FODMAPs and is not suitable for individuals with IBS (Schmidt and Sciurba, 2020). Gluten-free bakery products, while popular, often lack dietary fibre as they substitute starch for flour (Sahin et al., 2023). This deficiency in fibre content presents a difficulty for patients striving to maintain a nutritious diet. Moreover, the increased focus on developing low-FODMAP food formulations reflects the ongoing effort to address dietary needs for individuals with sensitivities (Sahin et al., 2023). Many ready-to-eat products currently on the market contain high levels of FODMAPs, further complicating the dietary choices of individuals with IBS (Ispiryan et al., 2020). To address this gap, there is a necessity to develop new recipes that fortify low FODMAP bakery products with dietary fibre, offering patients more options to meet their nutritional needs while managing their condition.

Hence, understanding the intricate interplay between dietary fibre, FODMAPs, and gut microbiota in the context of IBS is essential for optimizing dietary interventions and improving patient outcomes. The aim of this study was to investigate the effects of four distinct biscuit recipes containing varying levels of FODMAPs and dietary fibre

on the human gut microbiota. Utilizing *in vitro* oral, gastric and intestinal digestion of the four biscuit types followed by *in vitro* colonic fermentation using the pre-digested food products, we analysed if any alterations in the gut microbiota occurred through 16S rRNA gene sequencing and short- and branched-chain fatty acid analyses.

3.3 Materials and Methods

3.3.1 Study design

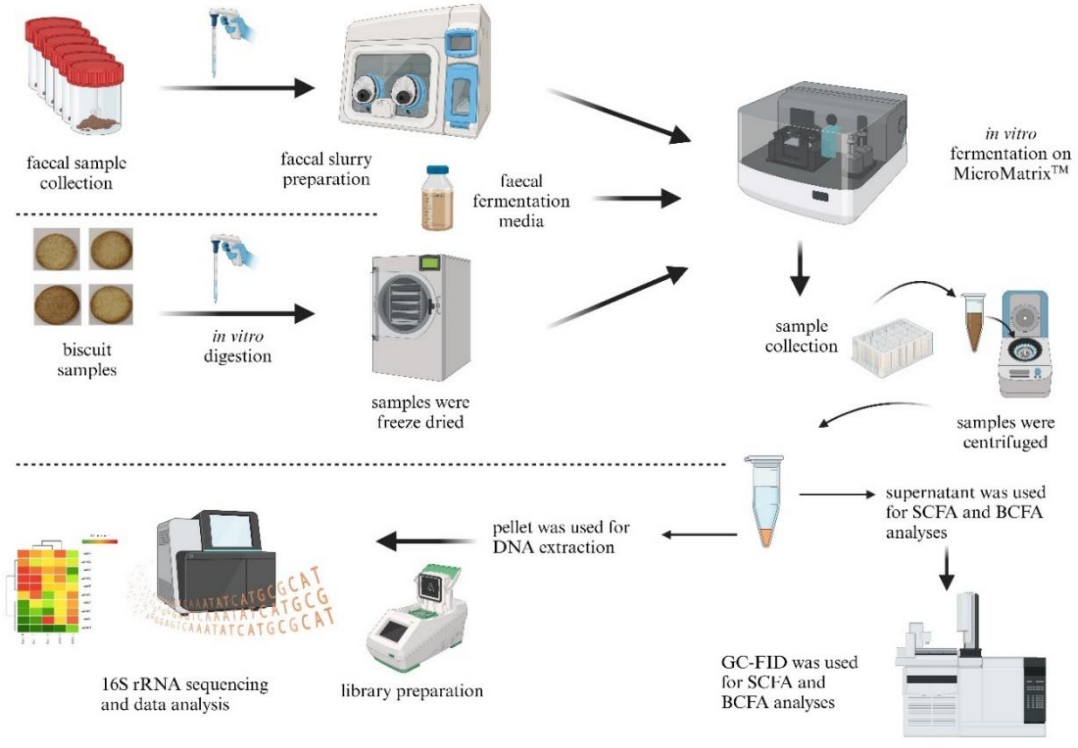

Figure 3.1 Illustration summarizes the study design

Four different biscuit recipes were developed by the Food Science Department at University College Cork, Ireland. The biscuits were subjected to *in vitro* digestion and *in vitro* colonic fermentation as described in a previously published protocol (Koc et al., 2022). Following *in vitro* colonic fermentation, samples were collected to carry out 16S rRNA gene sequencing, SCFA and branched-chain fatty acid (BCFA) analyses to

determine variations in gut microbiome and metabolome. The study summary is illustrated in Figure 3.1.

3.3.2 *In vitro* digestion and *in vitro* colonic fermentation

Biscuit samples were prepared from four different recipes which have been outlined in a previously published paper (Sahin et al., 2023). The biscuit ingredients are listed in Table 1. All ingredients were the same for each biscuit recipe except for the type of flour used. Biscuit flour control recipe (BiFC) contained biscuit flour, wholemeal flour control (WMC) consisted of wholemeal flour, low FODMAP control (LFC) included starch and gluten instead of flour, and the low-FODMAP high-fibre prototype (LFP) contained starch, gluten, resistant starch, cellulose and arabinoxylan. Cellulose and inulin served as negative and positive controls, respectively. *In vitro* digestion was performed according to the INFOGEST 2.0 method to mimic oral, gastric and intestinal phases of digestion on biscuit samples, cellulose (negative control) and inulin (positive control) (Brodkorb et al., 2019, Egger et al., 2016). After *in vitro* digestion, *in vitro* colonic fermentation was performed for 12 hours according to a previously published protocol (Koc et al., 2022). Biscuits were prepared in triplicate. Subsequently, samples underwent *in vitro* digestion and *in vitro* faecal fermentation in duplicate. Cellulose and inulin were used in triplicate for the processes. The faecal slurry was prepared using faecal samples collected from nine healthy individuals to perform *in vitro* batch fermentation using MicroMatrix^{TM} (Applikon Biotechnology, Heertjeslaan 2, 2629 JG Delft, Netherlands). Faecal samples were pooled in an anaerobic chamber following sample collection and were aliquoted in sterile falcons for further usage. The faecal slurry was prepared according to a previously described

protocol (O'Donnell et al., 2016). Participant recruitment and collection of stool samples were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (review ref. no: ECM 4 (gg) 11/02/20 & ECM 3 (iiii) 22/02/2022). Inclusion criteria of the study involved enrolling individuals aged 25 to 35 who were in good health and free of chronic conditions. Exclusion criteria included recent antibiotic use, regular medication use, and any acute or chronic diseases such as cardiovascular, immunological, or metabolic disorders, as well as any condition requiring medication.

Table 3.1 Biscuit Ingredients

	Biscuit Flour/ Wholemeal Flour		**Low FODMAP Control**		**Low FODMAP Prototype Biscuit**
Flour	100	**Starch**	90	**Starch**	37.7
Water	20	**Gluten**	10	**Gluten**	10
Shortening	44.4	**Water**	14.1	**Resistant Starch**	40
Sugar	40.4	**Shortening**	44.4	**Cellulose**	10
Baking Powder	0.5	**Sugar**	40.4	**Arabinoxylan**	2.3
DATEM	0.5	**Baking Powder**	0.5	**Water**	18.1
Salt	0.7	**DATEM**	0.5	**Shortening**	44.4
		Salt	0.7	**Sugar**	40.4
				Baking Powder	0.5
				DATEM	0.5
				Salt	0.7

3.3.3 Sample collection for DNA extraction and fatty acid analysis

Samples were collected from each MicroMatrix™ cassette well at the end of faecal fermentation (12th hour) to perform DNA extraction and SCFA and BCFA analysis. One millilitre of sample was collected into a sterile 2 mL screw cap tube (Sarstedt, Nümbrecht, Germany) and centrifuged at 16000 x g for 10 min at 4 °C. The

supernatant was transferred to a sterile new tube to be used for SCFA and BCFA analysis and the pellet was kept for DNA extraction. Both pellet and supernatant were stored at -80 °C for further analysis.

3.3.4 DNA extraction and 16S rRNA gene sequencing library preparation

The DNA extraction was performed from the pellet and 16S rRNA gene sequencing library preparation was performed according to a previously published paper with minor modifications (Koc et al., 2022). The variable V3 and V4 regions of the 16S rRNA genes were amplified using 16S Amplicon PCR Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA G and 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGT GTATAAGAGACAGGACTACHVGGGTATCTAATCC. All libraries were diluted and pooled at 4 nM final concentration before being sequenced by the NextSeq 2000 (Illumina, Inc. San Diego, USA) platform using the P1 600 cycle (2x300 bp) reagent kit (Illumina, Inc. San Diego, USA) at Teagasc Next Generation DNA Sequencing Facility in Teagasc Moorepark Research Centre, Cork, Ireland.

3.3.5 Short- and Branched-Chain Fatty Acid Analyses

Samples were prepared for short- and branched-chain fatty acid analyses according to a previously described protocol with minor modifications (Koc et al., 2022) using the supernatant. The standards and samples were analysed by GC-FID (gas chromatography - flame ionisation detector) system using an Agilent 8860 GC system (Agilent, California, United States) fitted with DB-FFAP column (30 m, 0.32 mm ID, 0.25 µM df, Agilent, California, United States) and flame ionisation detector. Samples

and standards were loaded (0.5 uL) with an autosampler using splitless injection. Helium was used as carrier gas at a constant flow rate of 1.3 mL/min. The initial GC oven temperature was set at 50^{o}C and it was maintained for 0.5 min, raised to 140^{o}C at 10^{o}C/min and held for 0.5 min, before being increased to 240^{o}C at 20^{o}C/min and held for 5 min. Total run time was 20 min. The temperatures of the detector and the injection port were set at 300^{o}C and 240^{o}C, respectively. Peaks were integrated using Agilent OpenLab version 2.0 (Agilent, California, United States). The concentrations were presented in milimolar (mM) of each compound.

3.3.6 Bioinformatics and statistical analysis

The raw sequencing data was processed using DADA2 pipeline (version 1.16) (Callahan et al., 2016a) using the following parameters; maxN=0, maxEE=c (2, 2), rm.phix=TRUE, truncQ=2, trimLeft=c (17, 21), truncLen=c (280,260) and maxN=0. The creation of the amplicon sequence variant (ASV) table and taxonomic assignment were performed according to a previously published protocol (Koc et al., 2022). A phyloseq object was created from metadata containing sample and group information, ASV table and taxonomic table (Callahan et al., 2016b). Alpha diversity, beta diversity, and relative abundance graphics were generated on phyloseq object and statistical tests were also performed on phyloseq object. Alpha diversity was measured using Observed, Chao1, Shannon, and Simpson indices. Beta diversity was calculated using Bray-Curtis distance matrix and principal coordinate analysis (PCoA). The Pairwise PERMANOVA (ADONIS) test was employed to determine if the separations across the groups were statistically significant, using the vegan package in R (version 4.3.2) (Oksanen et al., 2013). This test allows for the comparison of beta diversity

across multiple groups to assess whether differences in microbial community composition between the biscuit fermentates are meaningful. Wilcoxon-sum rank test was used to determine if the difference was significant in alpha diversity measurements as well as taxonomical differences at phylum and genus levels. Linear discriminant analysis Effect Size (LEfSe) analysis was performed to determine the signature genera of each group (Segata et al., 2011). $p < 0.05$ was considered to be statistically significant.

A one-way ANOVA with Tukey's HSD posthoc test was performed to determine significant differences of SCFA and BCFA levels among groups. Statistical tests were performed using ggpbur () package and box plots were generated using ggplot2 () package on R version 4.3.2.

3.4 Results

3.4.1 Microbiome analysis

The 16S rRNA gene sequencing analysis was performed on DNA extracted from each MicroMatrix^{TM} sample following *in vitro* colonic fermentation. Alpha diversity, beta diversity, and relative abundance analysis at phylum and genus levels were performed to explore if there were any gut microbiome alterations among groups. Furthermore, LEfSe analysis was performed to show significant genera for each group at the genus level.

3.4.2 Alpha and beta diversity analysis

Alpha diversity was calculated using Shannon, Simpson, Chao1, and Observed indices (Figure 3.2). Shannon and Simpson indices exhibited a significantly higher diversity

in the presence of WMC and inulin than in the presence of BiFC (p=0.009 and p=0.04). The inclusion of LFP during *in vitro* colonic fermentation caused a significantly higher diversity than inclusion of BiFC following the fermentation in both Shannon and Simpson indices (p=0.012, Figure 3.2A and 3.2B). Similarly, samples fermented with WMC had the highest diversity when compared with the other groups including the positive control in Observed and Chao1 indices (Figure 3.2C and 3.2D, not significant).

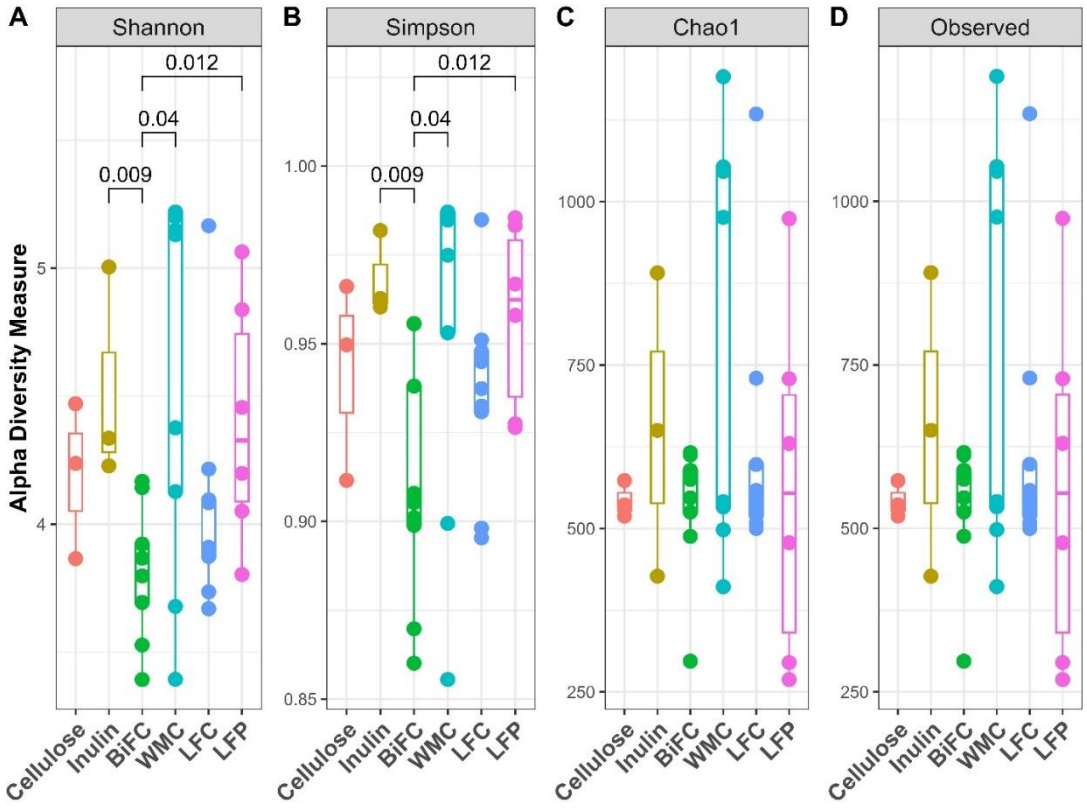

Figure 3.2 Alpha diversity was calculated using four indices including Shannon (A), Simpson (B), Chao1 (C) and Observed (D). Cellulose (negative control), inulin (positive control), BiFC (biscuit flour control biscuit), WMC (wholemeal flour biscuit), LFC (Low FODMAP control biscuit), LFP (low FODMAP high fibre prototype).

Beta diversity was calculated using Bray-Curtis distance index (Figure 3.3). Pairwise PERMANOVA (ADONIS) revealed no significant separation between the samples in the presence of different biscuits (Table 2) following *in vitro* colonic fermentation (FDR>0.05).

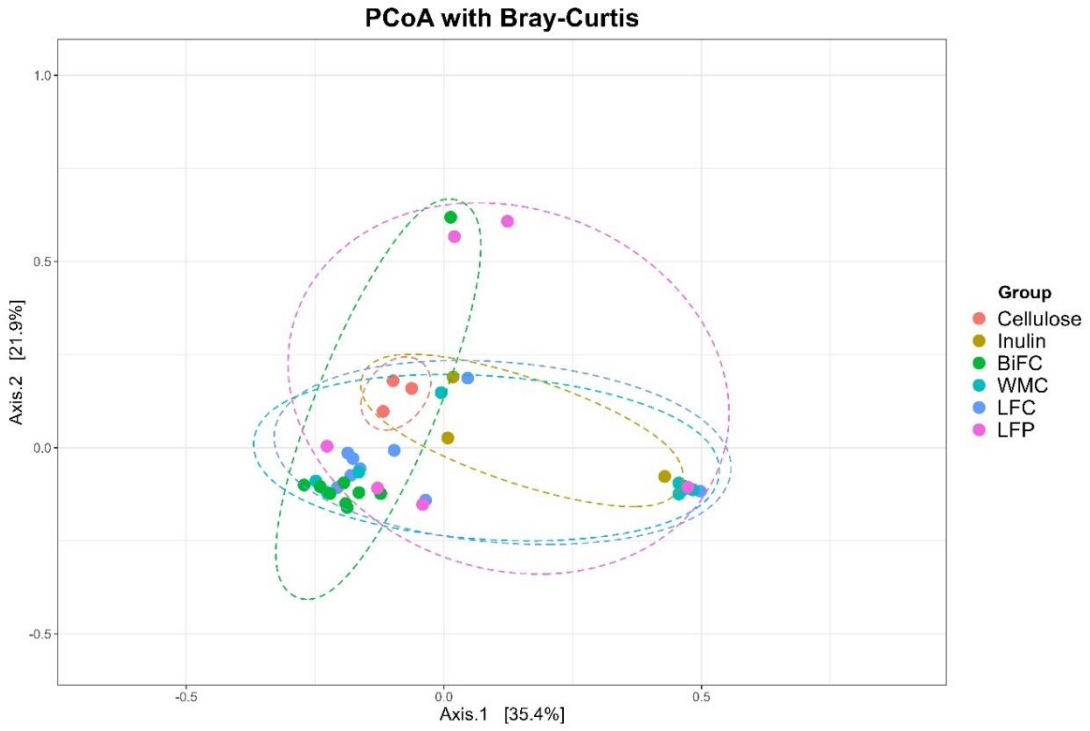

Figure 3.3 Principal coordinate analysis was performed using Bray-Curtis distance method to determine beta diversity among the groups.

3.4.3 Taxonomic analysis at phylum level

Following alpha and beta diversity analyses, relative abundances were computed in order to assess microbiome variations at the phylum and genus levels. Figure 3.4A illustrates the percentage of relative abundance average for each substrate at the phylum level, while Figure 3.4B depicts the relative abundance at the genus level. Bacillota, Pseudomonadota and Bacteroidota were found to be the three most dominant phyla in each fermentate following fermentation.

Table 3.2 Pairwise ADONIS results. The table presents the results of pairwise comparisons between six experimental groups (LFC, WMC, BiFC, LFP, Cellulose, and Inulin) based on the ADONIS test.

Comparison	F-model	R^2	P-value	Adjusted p-value (FDR)
LFC and WMC	2.486981	0.134526	0.096	1
LFC and BiFC	1.784669	0.100349	0.047	0.705
LFC and LFP	1.230474	0.086468	0.297	1
LFC and Cellulose	3.677621	0.268879	0.007	0.105
LFC and Inulin	2.136291	0.176025	0.06	0.9
WMC and BiFC	4.957724	0.236558	0.013	0.195
WMC and LFP	1.609825	0.110188	0.149	1
WMC and Cellulose	4.307582	0.30107	0.009	0.135
WMC and Inulin	1.134817	0.101916	0.218	1
BiFC and LFP	1.892707	0.12709	0.109	1
BiFC and Cellulose	5.73758	0.364578	0.011	0.165
BiFC and Inulin	4.180554	0.294809	0.015	0.225
LFP and Cellulose	2.205073	0.23955	0.087	1
LFP and Inulin	1.138529	0.139894	0.372	1
Cellulose and Inulin	2.388188	0.373844	0.1	1

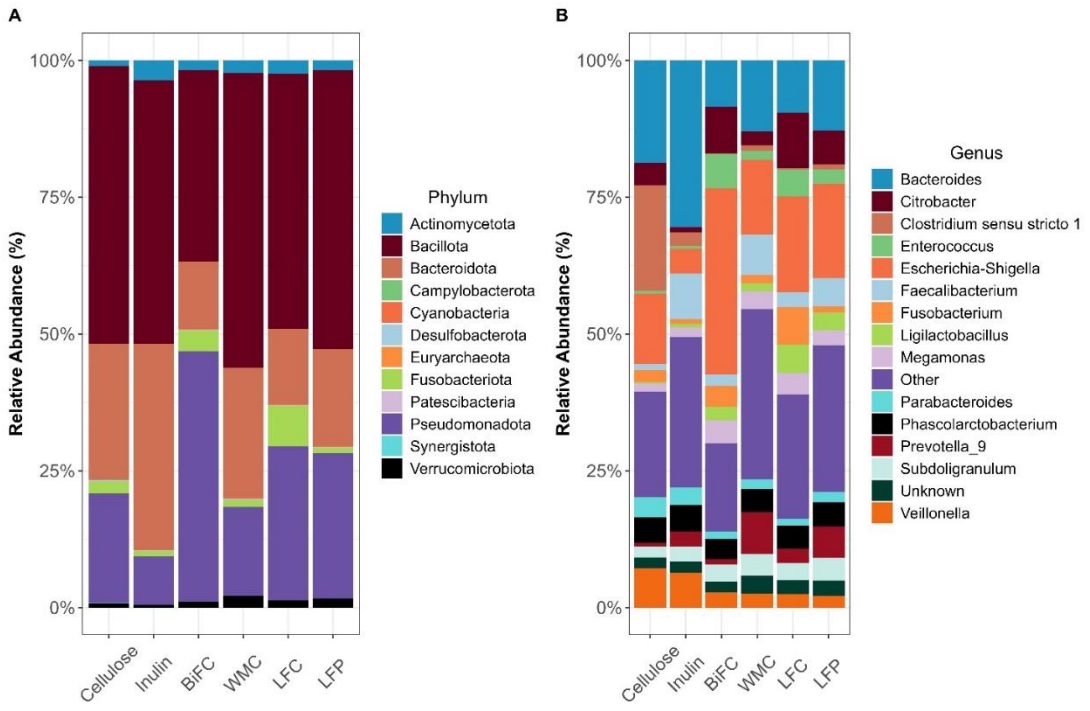

Figure 3.4 Bar charts represent relative abundance percentage of each biscuit model, positive and negative control following the fermentation at the phylum level (A) and Top 15 genera (B). Cellulose (negative control), inulin (positive control), BiFC (biscuit flour control biscuit), WMC (wholemeal flour biscuit), LFC (Low FODMAP control biscuit), LFP (low FODMAP high fibre prototype).

Actinomycetota (Figure 3.5A) relative abundance was significantly higher in samples fermented with inulin (3.34%) compared to samples fermented with BiFC (1.8%) and LFP (2.52%) ($p<0.05$). Conversely, cellulose fermentates had significantly lower Actinomycetota relative abundance (1.05%) compared to WMC (2.2%) and LFC (2.52%) ($p<0.05$). Pseudomonadota (Figure 3.5B) showed substantial variations across the biscuit fermentates, with BiFC samples exhibiting the highest relative abundance with 46.2%, significantly higher than inulin (8.32%), cellulose (20.17%), WMC (18.6%), LFC (30.7%), and LFP (26.4%) ($p<0.05$). In contrast, the relative abundance of Bacillota ranged from 51.6% to 33.8% across the samples, with WMC

fermentates exhibiting the highest abundance while BiFC fermentates had the lowest abundance (Figure 3.4A).

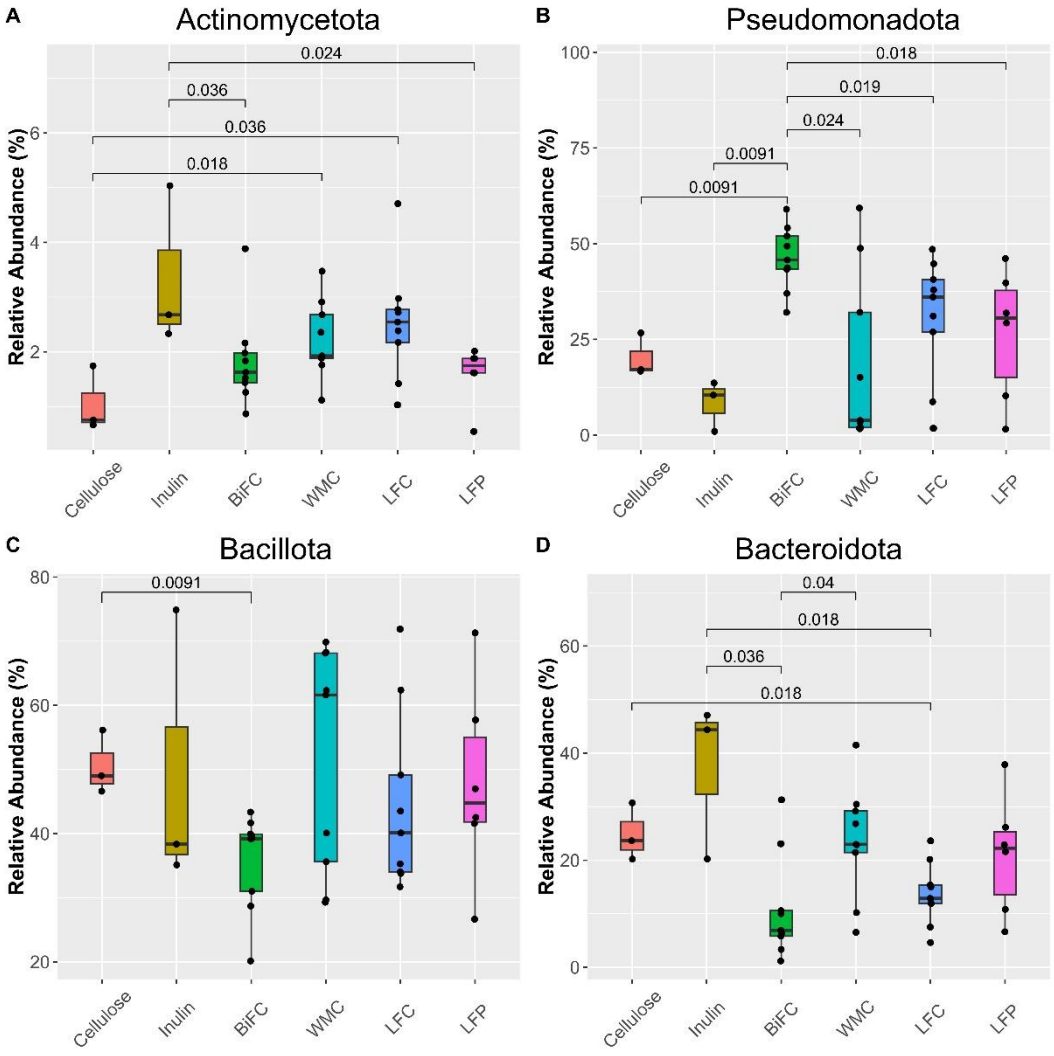

Figure 3.5 Box plots represent relative abundance of each group at the phylum level. Axis-x shows the groups and axis-y shows the relative abundance percentages. Cellulose (negative control), inulin (positive control), BiFC (biscuit flour control biscuit), WMC (wholemeal flour biscuit), LFC (Low FODMAP control biscuit), LFP (low FODMAP high fibre prototype).

The difference of Bacillota relative abundance between cellulose and BiFC was statistically significant (p=0.0091) (Figure 3.5C). Additionally, Bacteroidota (Figure

3.5D) was the most abundant phylum in the presence of inulin (37.2%) and the lowest in the presence of BiFC following the fermentation (10.9%) ($p=0.036$). Fermentation with LFC exhibited lower Bacteroidota abundance (13.7%) compared to inulin (37.2%) and cellulose (24.8%) ($p=0.018$ and $p=0.018$), while WMC fermentates had significantly higher Bacteroidota abundance compared to BiFC ($p=0.04$).

3.4.4 Taxonomic analysis at genus level

Figure 3.4B displays the relative abundance of the top 15 genera following the fermentation. *Bacteroides* (Figure 3.6A) represented a relative abundance of 30.4% in the presence of inulin which was significantly higher than BiFC (8.44%) and LFC (9.58%) ($p=0.036$ and $p=0.0036$). Similarly, samples fermented with cellulose also displayed significantly higher levels of *Bacteroides* (18.7%) compared to LFC ($p=0.0091$). *Citrobacter* was notably more abundant in samples fermented with BiFC (8.57%) compared to cellulose (4%), inulin (0.99%), and WMC (2.55%). Conversely, in the presence of LFC (10%), samples had significantly higher relative abundance of *Citrobacter* (Figure 3.6B) than WMC ($p<0.05$ for all). *Clostridium sensu stricto 1* was significantly more abundant in the samples fermented with cellulose (19%) than in all other biscuits except inulin (Figure 3.6C). Furthermore, in the presence of BiFC, samples exhibited the lowest relative abundance of *Clostridium sensu stricto 1* (0.1%) which was significantly lower than inulin (2.36%), WMC (1%), LFC (0.2%), and LFP (0.9%) ($p<0.05$).

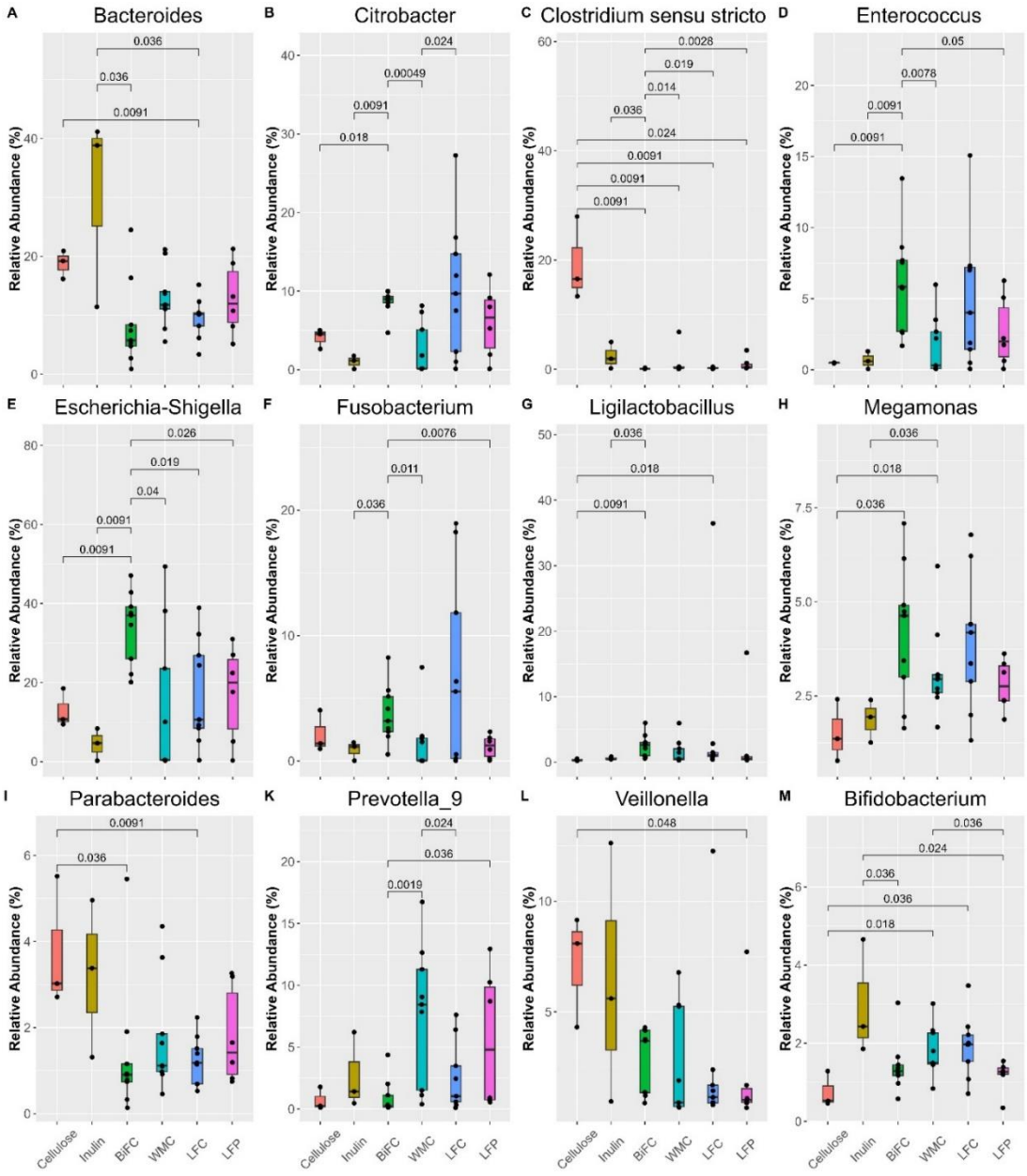

Figure 3.6 Each boxplot represents the relative abundance of the group and statistical differences among the groups. The Wilcoxon rank test was used to determine significant differences. Cellulose (negative control), inulin (positive control), BiFC (biscuit flour control biscuit), WMC (wholemeal flour biscuit), LFC (Low FODMAP control biscuit), LFP (low FODMAP high fibre prototype).

Enterococcus (Figure 3.6D) was significantly more abundant in the samples fermented with BiFC (6.2%) compared to cellulose (0.48%), inulin (0.65%), and WMC (1.68%)

(p=0.0091, p=0.0091, and p=0.0078). *Escherichia-Shigella* (Figure 3.6E) and *Fusobacterium* (Figure 3.6F) were found to be significantly higher in samples fermented with BiFC (34% and 3.75%) compared to inulin (4.4% and 0.8%, p=0.0091 and p=0.036), WMC (13.6% and 1.43%, p=0.04 and p=0.011), and LFP (17.2% and 1.14%, p=0.026 and p=0.0076). Moreover, *Ligilactobacillus* and *Megamonas* were significantly more abundant in the presence of BiFC (2.55% and 4.17%) compared to cellulose (0.33% and 1.5%) (p=0.0091 and p=0.036). Additionally, *Ligilactobacillus* (Figure 3.6G) abundance was significantly lower in the presence of cellulose (0.33%) compared to LFC (5.11%), while the abundance was significantly higher in the presence of BiFC than in the presence of inulin (0.57%) (p=0.018 and p=0.036). The relative abundance of *Megamonas* (Figure 3.6H) was also significantly higher in the samples fermented with WMC (3.16%) than cellulose (1.5%) and inulin (1.86%) (p=0.018 and p=0.036).

Fermentation of cellulose exhibited significantly higher levels of *Parabacteroides* (3.75%) compared to BiFC (1.37%) and LFC (1.24%) (p=0.036 and p=0.0091, Figure 3.6I). *Prevotella_9* was significantly more abundant in WMC fermentates (7.67%) compared to BiFC (0.95%) and LFC (2.5%) (p=0.0019 and p=0.024, Figure 3.6K). Samples fermented with cellulose exhibited significantly higher levels of *Veillonella* (7.18%) compared to those fermented with LFP (2.17%), while showing lower relative abundances of *Bifidobacterium* (0.75%) compared to samples fermented with WMC (1.79%) and LFC (1.92%) (p=0.048, p=0.018, and p=0.036). Additionally, samples demonstrated significantly higher relative abundances of *Bifidobacterium* (2.98%) in the presence of inulin compared to BiFC (1.4%) and LFP (1.16%) (p=0.036 and p=0.024). Furthermore, WMC fermentates exhibited a higher relative abundance of

Bifidobacterium (1.79%) compared to LFP (1.16%) ($p=0.036$, Figure 3.6M). The relative abundance of *Lactobacillus* was also higher in samples fermented with WMC, BiFC and LFC compared to those fermented with cellulose ($p<0.05$ for all).

3.4.1 Linear discriminant analysis Effect Size (LEfSe) results

LEfSe analysis was performed to detect signature genera for each substrate following the fermentations (Figure 3.7). The findings revealed distinct microbial signatures, with notable genera exhibiting significance across various fermentates. Specifically, *Sphingomonas* and *Microbacterium* emerged as significant genera characterizing LFP fermentates, while *Prevotella*_9 demonstrated significance within the WMC fermentates. Notably, *Bacteroides* and *Bifidobacterium* emerged as significant genera associated with inulin samples, while *Clostridium sensu stricto 1* exhibited significance within the cellulose samples following the fermentation. Additionally, *Escherichia-Shigella* and *Enterococcus* were identified as significant genera characterizing samples fermented with BiFC.

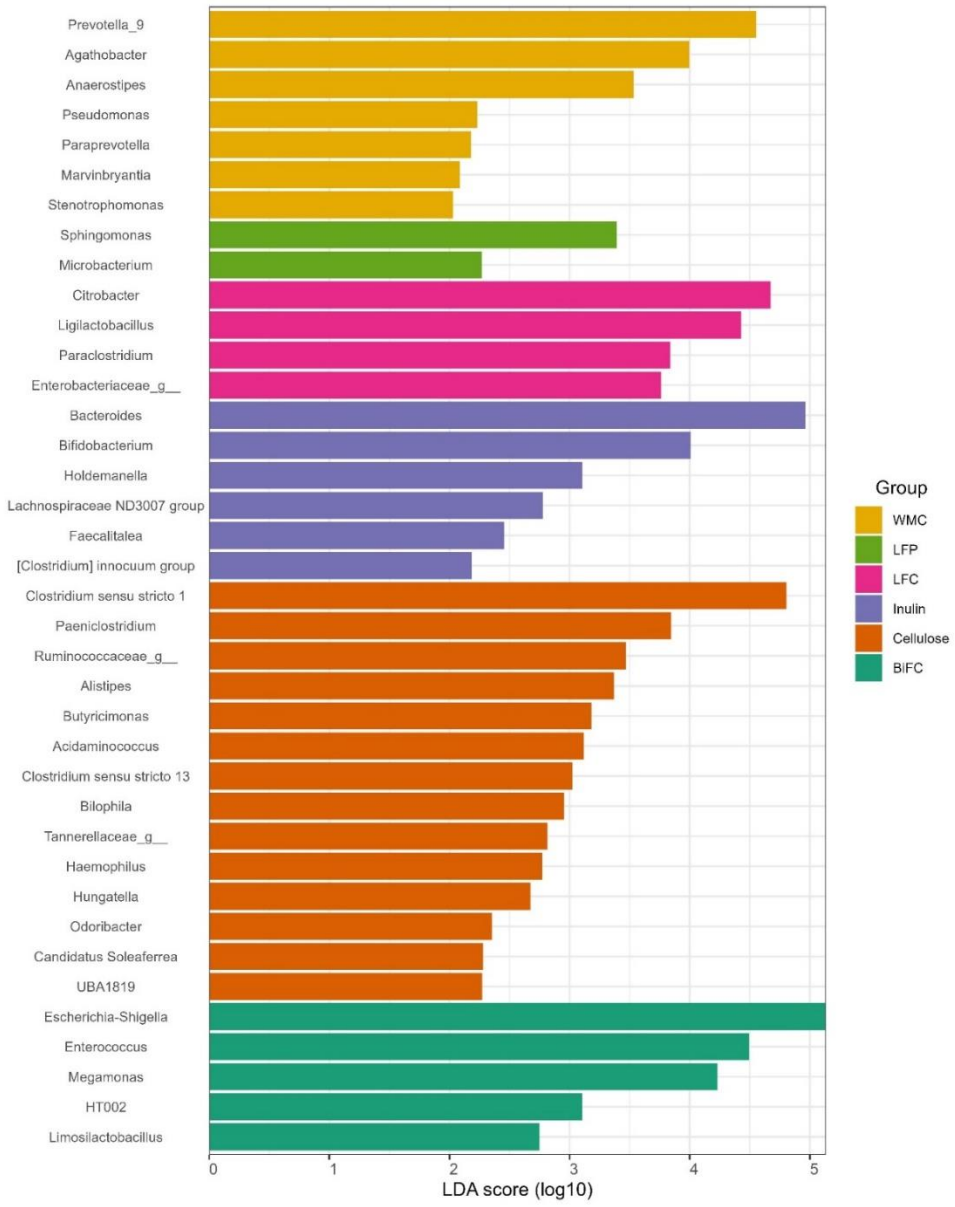

Figure 3.7 LEfSe (Linear discriminant analysis) demonstrates the signature genera of each group

3.4.2 Short- and Branched-Chain Fatty Acid Analyses

Following *in vitro* colonic fermentation, SCFA and BCFA analyses were performed using the Agilent GC-FID system on all biscuit samples as well as negative and positive controls. The results revealed a number of significant differences among the

samples fermented with different substrates. WMC fermentates exhibited significantly higher levels of acetate and propionate compared to the other substrates ($p<0.05$, Figure 3.8A and 3.8C). Inclusion of inulin was associated with significantly higher levels of butyrate and propionate compared to BiFC ($p=0.0091$), LFC ($p=0.0091$), and LFP ($p=0.0091$). Fermentation with LFC showed higher levels of acetate and butyrate compared to LFP ($p=0.05$ and $p=0.004$). Valerate levels were the lowest in samples fermented with LFP and the highest in samples fermented with BiFC, although the difference was not significant (Figure 3.8D). Isobutyrate levels were higher in the presence of inulin and cellulose compared to BiFC ($p=0.036$ and $p=0.005$), LFC ($p=0.042$ and $p=0.012$), and LFP ($p=0.036$ and $p=0.0053$). Isovalerate levels were higher in the BiFC fermentates compared to cellulose ($p=0.035$), inulin ($p=0.0066$), WMC ($p=0.028$), and LFC fermentates ($p=0.043$). Samples fermented with LFC also exhibited significantly higher levels of isovalerate compared to those fermented with cellulose ($p=0.023$). There were no significant differences between the biscuit fermentates and control fermentates for total branched-chain fatty acids (BCFAs). Total SCFA levels were significantly higher in the presence of WMC compared to other biscuits and controls ($p<0.05$, Figure 3.8H). LFP fermentates exhibited lower total SCFA levels than LFC and inulin ($p=0.0093$ and $p=0.049$).

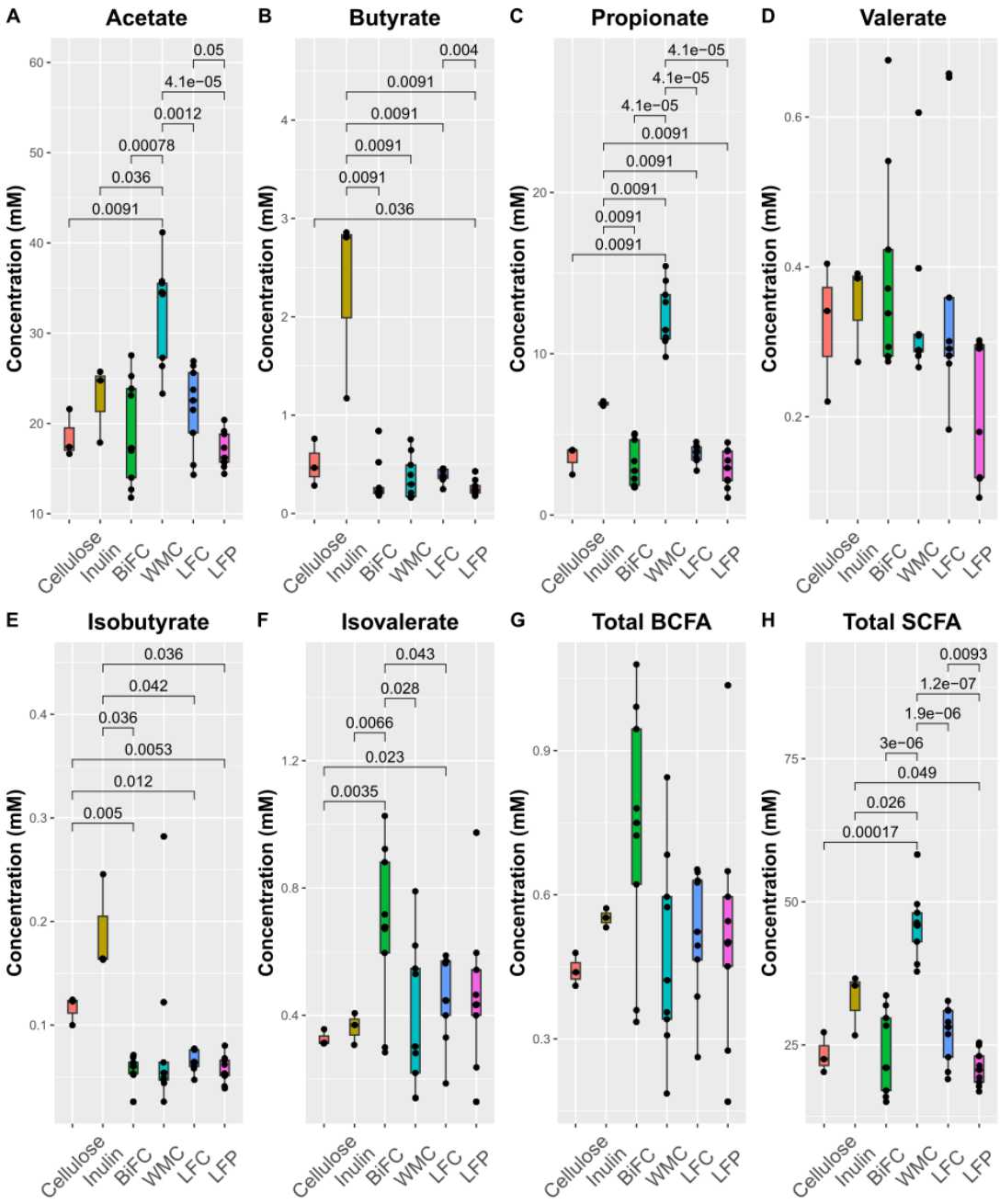

Figure 3.8 Boxplots show short- and branched-chain fatty acid levels.

3.5 Discussion

The present study aimed to elucidate the impact of biscuit formulations containing varying levels of FODMAPs and dietary fibre on the human gut microbiome by leveraging *in vitro* digestion and *in vitro* colonic fermentation models followed by 16S rRNA gene sequencing and SCFA and BCFA analyses. The findings may help to explain the potential of dietary interventions to modulate the gut microbiota and alleviate IBS symptoms and demonstrate the efficacy of using colonic fermentations to develop suitable food formulations for gut microbiome-associated conditions.

Alpha diversity analysis was performed using Shannon, Simpson, Observed, and Chao1 indices. The results from Shannon and Simpson indices revealed differences in microbial richness and evenness among groups following the fermentation. Samples fermented with LFP exhibited higher diversity compared to the samples fermented with BiFC. These findings may suggest that dietary manipulation, particularly reducing FODMAPs while increasing fibre content, may promote a more diverse and balanced gut microbiota, which is associated with gastrointestinal health (Jeffery et al., 2020). Beta diversity analysis did not reveal any statistically significant differences when comparing the microbial community compositions across the different groups.

Further investigation into specific taxa revealed noteworthy trends. The most abundant three phyla were Bacillota, Pseudomonadota and Bacteroidota. Samples fermented with BiFC were dominated by Pseudomonadota. The increase of Pseudomonadota can be a result of the high abundance of *Escherichia-Shigella* in these samples. The increase in Pseudomonadota abundance raises questions about the potential implications for gut health and underscores the importance of carefully selecting

dietary fibres in IBS management as Pseudomonadota include several pathogenic genera, such as *Escherichia* and *Shigella*, which have been associated with gastrointestinal disorders (Rizzatti et al., 2017). LEfSe analysis revealed samples fermented with BiFC were discriminated by *Escherichia-Shigella* and *Enterococcus*. On the contrary, samples fermented with LFC and LFP had significantly lower relative abundance of *Escherichia-Shigella* compared to BiFC. Some *Escherichia* and *Shigella* strains have been associated with IBS symptoms (Dogan et al., 2018, Sobieszczanska et al., 2007). Similarly, significantly higher levels of *Enterococcus* have been found in IBS patients compared with healthy subjects (Wei et al., 2020). Another study investigated the duodenal aspirate samples to detect small intestinal bacterial overgrowth in IBS patients and found higher levels of *Enterococcus, Escherichia coli,* and *Klebsiella* (Pyleris et al., 2012). A 2006 study reported that IBS patients administered a probiotic combination containing *Bifidobacterium, Lactobacillus* and *Enterococcus* showed improvement of IBS symptoms. Surprisingly, the *Enterococcus* level was found to be lower following the treatment (Fan et al., 2006). The role of *Enterococcus* in IBS symptoms is not clear.

The fermentates of LFP exhibited significantly reduced *Veillonella* levels compared to cellulose. *Veillonella* overgrowth is associated with oral diseases (Cortez et al., 2020), endocarditis (Clarisse Rovery, 2005), and ulcerative colitis (Brook, 1996). Elevated *Veillonella* levels have been observed in IBS cohorts in Japan and Finland (Tana et al., 2010, Malinen et al., 2005), as well as in colonic mucosa samples from IBS patients (Masoodi et al., 2020). The lipopolysaccharide of *Veillonella parvula*, a pathogenic species, increases colon permeability and induces inflammation by activating macrophages (Zhan et al., 2022). The pathogenicity of *Veillonella* may involve

reducing interleukin-22 expression in the colon, which plays a role in tissue response during inflammation (Geva-Zatorsky et al., 2017, Sonnenberg et al., 2011). Our findings suggest that LFP biscuits may alleviate IBS symptoms by reducing *Veillonella* levels.

Similar to our findings, recent studies also showed that the low FODMAP diet resulted in lower abundance of *Bifidobacterium* (McIntosh et al., 2017, Staudacher et al., 2012, Halmos et al., 2015, Staudacher et al., 2021) which can be a result of fewer fructooligosaccharides in this diet which are considered "bifidogenic" (Mahalak et al., 2022). Yet, the influence of reduced *Bifidobacterium* abundance on IBS symptoms is not clear (Staudacher et al., 2017). In our study, the signature genera of inulin fermentates were *Bacteroides* and *Bifidobacterium* which are known as carbohydrate-utilizing bacteria (Yin et al., 2023). Inulin is a nondigestible oligosaccharide which has been associated with modulating the gut microbiome, intestinal transit time, and stool consistency (Koç et al., 2020, Barboi et al., 2022). The efficiency of inulin in treating IBS patients has been demonstrated in several studies, emphasizing the significance of including inulin in a daily diet, particularly for individuals experiencing constipation (Dahl and Stewart, 2015, Micka et al., 2017, Barboi et al., 2022). Inulin can lead to an increase in the level of *Bacteroides* which can be both pathogenic and commensal (Sonnenburg et al., 2010). Studies have reported various results on the levels of *Bacteroides* in IBS patients (Rajilic-Stojanovic et al., 2011, Ponnusamy et al., 2011, Jeffery et al., 2012, Parkes et al., 2012) with some reporting high levels of *Bacteroides* in IBS patients (Liu et al., 2016, Zhong et al., 2019, Shukla et al., 2015, Tap et al., 2017). The *Bacteroides* level was also found to be higher in samples fermented with LFC, LFP and WMC compared to samples fermented with BiFC (not significant). A

recent clinical trial reported that the *Bacteroides* level was higher in a low FODMAP diet group compared with the sham-diet group (Staudacher et al., 2021). *Bacteroides* has been associated with animal protein, amino acids and fatty acids (Wu et al., 2011). A recent study examining the impact of faecal microbiota transplantation on IBS symptoms observed increased *Bacteroides* levels one year following the transplant. This increase was linked to a reduction in IBS symptoms (El-Salhy et al., 2022). Another study reported that *Bacteroides fragilis* exerted anti-inflammatory protection on its host (Mazmanian et al., 2008). Thus, the role of *Bacteroides* in IBS symptom development is not clear. *Bifidobacterium* on the other hand has been associated with modulating the gut microbiome, inhibiting the growth of pathogenic bacteria, reducing inflammation and promoting health (Hidalgo-Cantabrana et al., 2017, Rezende et al., 2021). *Bifidobacterium* levels were found to be lower in IBS patients compared to healthy controls while *Bacteroides* levels were higher in IBS patients than in healthy subjects (Zhuang et al., 2017, Parkes et al., 2012).

WMC fermentates exhibited a significantly higher relative abundance of *Lactobacillus* compared to LFP and cellulose whereas samples fermented with LFP had a similar abundance of *Lactobacillus* to the inulin, which was higher than the cellulose, but was lower than other biscuit models. A previous study by our group reported that whole grains led to an increase in *Lactobacillus* compared to cellulose following an *in vitro* fermentation (Koc et al., 2022). LEfSe analysis revealed samples fermented with WMC were mainly driven by *Prevotella*_9 and *Agathobacter* genera. *Agathobacter* and *Prevotella* were previously associated with high carbohydrate intake (Wu et al., 2011, Li et al., 2024). The main fermentation products of *Agathobacter* are acetate, butyrate, lactate and hydrogen (Rosero et al., 2016). Dietary intervention studies found

significantly higher levels of *Prevotella* and *Lactobacillus* following wholegrain consumption (Cui et al., 2022, Vitaglione et al., 2015, Costabile et al., 2008). *Prevotella* has been associated with a healthy gut and high fibre intake (Prasoodanan et al., 2021). Additionally, *Prevotella* can improve glucose metabolism of the host, may reduce cholesterol levels and may lead to weight loss (Zheng et al., 2021, Xu et al., 2021).

The signature genera for LFC fermentates were *Citrobacter* and *Ligilactobacillus*. A number of *Microbacterium*, *Fusobacterium*, and *Citrobacter* species were found to be associated with colorectal cancer, bacteraemia and urinal tract infection (Kharrat et al., 2019, Jabeen et al., 2023). *Fusobacterium* was also found to be higher in samples fermented with BiFC compared to those fermented with inulin, WMC and LFP. Increased levels of *Fusobacterium nucleatum* were detected in IBS-D patients which can increase IBS pathogenesis via microbial dysbiosis and increasing visceral hypersensitivity (Gu et al., 2020). Another study reported a correlation between *F. nucleatum* and IBD (Strauss et al., 2011). An increase of *F. nucleatum* was noted in liver metastatic colorectal cancer patients compared to healthy subjects (Khattab et al., 2023). *Citrobacter* can ferment glucose and produce gas as well as acid (Jabeen et al., 2023). Overgrowth of *Citrobacter* species was reported to increase bloating in IBS patients (Dibaise et al., 2006, Collins and Lin, 2010). *Ligilactobacillus* is one of the lactic acid bacteria (Petitfils et al., 2023). Some of the strains have been used for probiotic supplementation due to their ability to modulate the gut microbiota (Shen et al., 2024, Yang et al., 2023).

Sphingomonas and *Microbacterium* were signature genera for LFP samples following fermentation. *Sphingomonas* has been reported as a biomarker for IBS-D patients (Zhu et al., 2021). A recent study where faecal microbiota transplantation was investigated in combination with a low-FODMAP diet in IBS-D patients reported *Sphingomonas* as one of the signature genera (Huang et al., 2022). To our knowledge, no study revealed a relationship between IBS-C and *Sphingomonas*. Given the fact that LFP biscuits caused an increase in the relative abundance of *Sphingomonas*, this biscuit type might not be an option for patients who have IBS-D. The findings for *Microbacterium* levels in IBS patients varied across different studies. Increased levels of *Microbacterium* were found to be a signature genera in an IBS-C group in one study (Matsumoto et al., 2021) however another study showed low levels of *Microbacterium* in IBS-C patients compared to healthy subjects (Ng et al., 2013).

The signature genera for samples fermented with cellulose were mostly pathogenic bacteria such as *Clostridium sensu stricto 1*, *Paeniclostridium*, *Alistipes*, and *Acidaminococcus.* This might be the result of the absence of dietary fibre, which is necessary to promote potential probiotic and beneficial bacteria in the gut. These beneficial bacteria can inhibit the growth of pathogenic bacteria (Raheem et al., 2021). *Clostridium sensu stricto 1* was associated with elevating IBS symptoms in a recent study (Sun et al., 2018). *Alistipes* is an anaerobic bacterium that can be considered as a commensal which belongs to the Bacteroidota phylum. This genus has been associated with inflammation and bacteraemia in gastrointestinal disease (Parker et al., 2020). Moreover, a recent study reported a positive correlation between this genus and colorectal cancer (Moschen et al., 2016).

SCFA and BCFA analyses provided further insights into the metabolic activity of the gut microbiota in response to biscuit formulations. The SCFAs acetate, propionate, and butyrate are key metabolites produced through bacterial fermentation of dietary substrates in the colon (Silva et al., 2020). These SCFAs play diverse roles in gut health, including serving as energy sources for colonocytes, modulating immune function, and regulating intestinal motility (Rios-Covian et al., 2016). Butyrate, in particular, has garnered significant attention due to its role in maintaining colonic homeostasis and exerting anti-inflammatory effects (Rios-Covian et al., 2016). However, SCFAs have been implicated in the pathogenesis of IBS (Jiang et al., 2022). Butyrate can increase visceral hypersensitivity which is a pathophysiological mechanism in IBS (Bourdu et al., 2005, Xu et al., 2013). The noticeable decline in butyrate levels in samples fermented with LFP could potentially offer advantages in managing IBS symptoms.

In addition to SCFAs, the production of BCFAs is noteworthy in this context. Our results showed that isobutyrate levels were higher following fermentation in the presence of inulin and cellulose compared to BiFC, LFC, and LFP fermentates. Similarly, isovalerate levels were significantly elevated in BiFC fermentates compared to other formulations, indicating the influence of specific ingredients on BCFA production. BCFAs, such as isobutyrate and isovalerate, have been associated with various metabolic functions, including gut barrier integrity and immune modulation. BCFA levels showed negative correlation with fibre consumption (Francois et al., 2014, Rios-Covian et al., 2020), suggesting that dietary fibre may influence BCFA production in the gut.

It is essential to interpret these findings in the context of individual variability, type of disease and dietary tolerance. Although certain biscuit formulations may promote favourable alterations in the gut microbiota and SCFA and BCFA production, individual reactions to dietary interventions can vary widely due to the diverse dietary triggers for different types of IBS. The high relative abundance of *Escherichia-Shigella* and *Enterococcus* in some treatment groups, particularly in BiFC fermentates, may reflect a dysbiotic microbial profile associated with IBS. However, the exclusive use of biscuit fermentates in this model does not fully represent a typical human diet, warranting caution in the interpretation of these results. Variables such as baseline gut microbiota composition, host genetics, and gastrointestinal transit time can influence the effectiveness of dietary strategies in IBS management (Jeffery et al., 2020).

3.6 Conclusion

This study adds valuable insights to our understanding of the complex interactions between dietary components and the gut microbiota in terms of the pathophysiology of IBS. By examining the effects of specific biscuit formulations on microbial composition and fatty acid levels, we gained a deeper understanding of how dietary choices may impact gut health in individuals with IBS. The findings suggest that fortifying low FODMAP biscuit formulations with high fibre could potentially serve as a beneficial intervention for alleviating IBS symptoms. The study also demonstrates the efficacy of using *in vitro* colonic fermentation models to devise food formulations for gut microbiome-associated diseases. However, it is important to acknowledge the limitations inherent in *in vitro* studies. While these results are promising, the applicability of our findings to real-life scenarios may be constrained by the lack of direct human data. Therefore, further preclinical studies and dietary interventions are

necessary to fully elucidate the effects of these biscuit models on the human gut microbiome and to assess their efficacy in managing IBS symptoms in everyday dietary contexts.

3.7 Acknowledgements

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Chapter 4

Impact of Low FODMAP Sourdough Bread on Gut Microbiota Using an *in vitro* Colonic Fermentation Model

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4.1 Abstract

This study explores the development of whole-grain sourdough bread with reduced FODMAP (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) content to offer dietary solutions for individuals with irritable bowel syndrome (IBS). Three sourdough breads were prepared using different lactic acid bacteria (LAB) strains including *Lactiplantibacillus plantarum* FST1.7 (SD-FST1.7), *Lacticaseibacillus paracasei* R3 (SD-R3), and *Pediococcus pentosaceus* RYE106 (SD-RYE106). A control sourdough bread was prepared using baker's yeast (SD-control). *In vitro* digestion and *in vitro* colonic fermentation were employed on bread samples with cellulose (negative control) and inulin (positive control), followed by 16S rRNA gene sequencing and short-chain fatty acid (SCFA) analysis to evaluate the impact on gut microbiota and SCFA levels. Alpha and beta diversity did not reveal any significant differences within the groups following *in vitro* colonic fermentation (FDR > 0.05). Taxonomic analysis displayed Firmicutes as the predominant phylum across all faecal samples at the end of colonic fermentation. Actinobacteriota was significantly lower in cellulose fermented faecal samples compared to samples fermented with SD-Control (ANCOMBC, FDR = 0.02) and inulin (ANCOMBC, FDR = 0.0001). Faecal samples fermented with inulin had significantly higher Bacteroidota levels compared to those fermented with cellulose (ANCOMBC, FDR = 0.002). Acetate levels were higher in faecal samples fermented with SD-FST1.7 compared to those fermented with SD-R3 and SD-RYE106 (p = 0.03 for both). Positive correlations between butyrate and *Lachnospira*, *Agathobacter*,

and *Bifidobacterium* were observed, demonstrating the potential of sourdough fermentation to influence gut health and support IBS management.

4.2 Introduction

Cereal-based foods are pillars of nutrition, boasting a rich array of essential nutrients and are important components of a balanced diet (Mobley et al., 2013, Poutanen et al., 2022). Among these, whole-grain sourdough bread is distinguished by its heightened fibre content, biogenic compounds, vitamins, and minerals, contributing to a reduced risk of non-communicable diseases (Sakandar et al., 2019, Kissock et al., 2021, Lockyer and Spiro, 2020, Laskowski et al., 2019). However, individuals with irritable bowel syndrome (IBS) often face dietary restrictions due to the high concentrations of fermentable oligo-, di-, monosaccharides, and polyols (FODMAPs) in whole-grain products, including wheat bread (Whelan et al., 2011, Ispiryan et al., 2020, Koc et al., 2024). Consumption of FODMAPs can exacerbate gastrointestinal symptoms in IBS sufferers, influencing their quality of life and productivity (Corsetti, 2018, Bertin et al., 2024). The low-FODMAP diet has emerged as a cornerstone approach for managing IBS symptoms, characterized by the restriction of high-FODMAP foods and the promotion of low-FODMAP alternatives, effectively mitigating gastrointestinal distress among IBS sufferers (Gibson and Shepherd, 2010, Staudacher et al., 2011, Halmos et al., 2014, Bohn et al., 2015).

Despite the tangible relief offered by the low-FODMAP diet, it presents practical challenges, particularly in the realm of dietary diversity and accessibility (Staudacher et al., 2011, Bohn et al., 2015). Traditional bread varieties, including whole-grain sourdough bread, often contain high levels of FODMAPs, rendering them unsuitable for individuals with IBS. Furthermore, the limited availability of low-FODMAP bread options, primarily restricted to gluten-free variations, poses a barrier to dietary

adherence and enjoyment (Ispiryan et al., 2022, Moroni et al., 2009, El Khoury et al., 2018).

Sourdough fermentation, a natural leavening process driven by lactic acid bacteria (LAB) and wild yeast, offers immense potential for FODMAP reduction in bread (Borowska et al., 2023, Menezes et al., 2021, Boakye et al., 2022). The fermentation process, characterized by the gradual breakdown of complex carbohydrates and proteins by microbial enzymes, facilitates the conversion of fermentable carbohydrates into simpler, more digestible forms (Siddiqui et al., 2023). This enzymatic activity, coupled with the acidification of the dough, creates an environment conducive to the degradation of FODMAPs, thereby potentially reducing their presence in the final bread product (Loponen and Ganzle, 2018).

In light of these challenges, our study aims to explore innovative strategies to develop whole-grain sourdough bread with significantly reduced FODMAP content, thus offering a viable solution for individuals managing IBS symptoms. Reducing FODMAPs in breads has been studied in recent years (Pejcz et al., 2023, Shewry et al., 2022). However, those studies focused on chemical properties of bread. Leveraging the essential capacity of sourdough fermentation to modulate FODMAP levels, we aimed to harness this traditional bread-making technique to produce gut-friendly bread options that align with the principles of the low-FODMAP diet. Our previous study aimed to select FODMAP-reducing LAB for the production of sourdough (Borowska et al., 2023). The selected LAB strains included *L. plantarum* FST1.7 (FST1.7), *L. paracasei* R3 (R3), and *P. pentosaceus* RYE106 (RYE106). These LAB strains, chosen for their ability to thrive in sourdough environments and ferment

carbohydrates, served as the key drivers of FODMAP reduction in our bread formulation. In previous research by Borowska et al., these three strains were shown to reduce the FODMAPs including sorbitol, mannitol, fructans, oligosaccharides and polyols significantly in sourdough bread. The details of formulation and biochemical properties of each sourdough bread have been previously published by Borowska *et al.* (Borowska et al., 2023).

In this current study, through a comprehensive investigation employing *in vitro* digestion, *in vitro* colonic fermentation, 16S rRNA gene sequencing, and short-chain fatty acid (SCFA) analysis, we investigated the effects of these fortified breads on gut microbiota and metabolome changes. Our findings illuminated the potential mechanisms underlying the health-promoting effects of fortified sourdough breads, with significant implications for the development of functional foods aimed at enhancing gut health.

4.3 Materials and Methods

4.3.1 Sourdough bread preparation

Sourdough breads were prepared in four different recipes as previously described (Borowska et al., 2023). Three sourdough breads were prepared using different LAB strains: *L. plantarum* FST1.7 (SD-FST1.7), *L. paracasei* R3 (SD-R3), and *P. pentosaceus* RYE106 (SD-RYE106). A control sourdough bread was prepared using baker's yeast (SD-control).

4.3.2 *In vitro* digestion

Four types of sourdough bread using different LAB strains: *L. plantarum* FST1.7 (SD-FST1.7), *L. paracasei* R3 (SD-R3), and *P. pentosaceus* RYE106 (SD-RYE106), control sourdough bread with baker's yeast (SD-control), along with cellulose (negative control) and inulin (positive control), underwent *in vitro* digestion prior to *in vitro* colonic fermentation. The *in vitro* oral, gastric, and intestinal digestion process followed the INFOGEST 2.0 method to replicate the oral, gastric, and intestinal phases, including both negative and positive controls (Egger et al., 2016, Minekus et al., 2014, Brodkorb et al., 2019). Simulated salivary, gastric, and intestinal fluids were prepared at a stock concentration of 1.25x for each respective phase. During the oral phase, α-amylase (1,500 U mL^{-1}, Sigma, A0521) was added to the simulated salivary fluid electrolyte solution and incubated at 37°C with the food samples for 2 min. In the gastric phase, the liquid samples from the oral phase were mixed with simulated gastric fluid electrolyte solution and pepsin (25,000 U mL^{-1}, Sigma, P6887), adjusted to pH 3.0, and incubated in an incubating rocking platform shaker (VWR, Radnor, PA, United States) at 37°C for 2 h. The intestinal phase included the gastric chyme, simulated intestinal fluid electrolyte solution, pancreatin (800 U mL^{-1}, Sigma, P7545), and fresh bile (10 mM), which were adjusted to pH 7.0 and incubated in an incubating rocking platform shaker at 37°C for 2 h. Afterward, the post-intestinal phase liquids were transferred to dialysis tubes and immersed in water as the dialysate for 24 h. The water was refreshed at 1, 6, and 18 h, after which the retentate was removed from the dialysis tubes. Retentates were then transferred to sterile 110 mm × 110 mm petri

dishes and freeze dried for 24 h according to a previously described method (Koc et al., 2022).

4.3.3 *In vitro* colonic fermentation

Subsequent to *in vitro* digestion, *in vitro* colonic fermentation was conducted based on a previously established protocol (Koc et al., 2022). Participant recruitment and stool sample collection were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals [review reference numbers: ECM 4 (gg) 11/02/20 & ECM 3 (iiii) 22/02/2022]. Faecal samples were obtained from nine healthy individuals for use in a MicroMatrix™ *in vitro* benchtop fermentation system, adhering to a protocol outlined in previous literature (O'Donnell et al., 2016). For each MicroMatrix™ well, 0.25 g of pre-digested and freeze-dried sample was mixed with 4 mL of faecal fermentation media and 1 mL of faecal slurry. *In vitro* colonic fermentation was conducted in triplicate for the negative control (cellulose) and positive control (inulin), while pre-digested bread samples were evaluated in six replicates. Faecal fermentation was performed for 12 h and samples were collected at the baseline (T0) and at the end of fermentation (T12). These samples were collected into 2 mL sterile screw cap tubes. Subsequently, the tubes were centrifuged at 16,000 *g* for 10 min at 4°C and the pellet was utilized for DNA extraction to analyse microbial composition, while the supernatant was used for SCFA analysis.

4.3.4 DNA extraction and 16S rRNA gene library preparation

DNA extraction was performed using repeated bead beading protocol which was developed by Yu and Morrison (Yu and Morrison, 2004) and described in detail by

Koc *et al.* (Koc et al., 2022). Pellets were resuspended using 1 mL of lysis buffer composed of 500 mM NaCl, 50 mM Tris–HCl (pH 8.0), 50 mM EDTA, and 4% sodium dodecyl sulphate (SDS). The resulting suspension was transferred to 2 mL screwcap microtubes (Sarstedt, Nümbrecht, Germany) containing Zirconia/Silica beads (0.125 g each of 0.1 mm and 1.5 mm beads, along with a single 2.5 mm bead; Biospec, Oklahoma, United States). The mixture was subjected to mechanical disruption using a bead beater (Biospec, Oklahoma, United States) at 4,000 rpm for 3 min. Following this, the tubes were incubated at 70°C for 15 min. The samples were then centrifuged at 16,000 g for 5 min at 4°C. The supernatant was carefully transferred to a sterile 2 mL Eppendorf tube, while 300 µL of the lysis buffer was added to the pellet, and the homogenization, incubation, and centrifugation steps were repeated. The collected supernatants were combined in a 2 mL tube. Next, 260 µL of chilled 7.5 M ammonium acetate was added to the supernatant, and the mixture was incubated on ice for 5 min. The tubes were centrifuged again at 16,000 g for 10 min at 4°C, after which 650 µL of the supernatant was transferred into two separate 1.5 mL tubes. An equal volume (650 µL) of isopropanol was added to each tube, and the samples were incubated overnight at −20°C. After incubation, the samples were centrifuged at 16,000 g for 15 min at 4°C. The supernatant was discarded, and the pellet was washed with 200 µL of 70% ethanol. The pellet was then resuspended in 100 µL of Tris-EDTA buffer. To degrade RNA, 2 µL of RNase (10 mg/mL) was added, followed by incubation at 37°C for 15 min. Afterward, 15 µL of proteinase K (from the QIAamp DNA Stool Mini Kit) and 200 µL of AL buffer (from the same kit) were added to the samples, which were incubated at 70°C for 10 min. Subsequently, 200 µL of absolute ethanol was added to precipitate the DNA. The samples were then transferred to

QIAamp mini columns, and the wash and elution steps were carried out according to the QIAamp kit protocol. Finally, the extracted DNA was stored at −20°C until it was needed for 16S rRNA gene library preparation.

DNA samples were processed following the Illumina 16S Metagenomics Sequencing Library Preparation protocol. Amplification targeted the V3-V4 regions of the 16S rRNA gene using 16S Amplicon PCR Forward Primer = 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Amplicon PCR Reverse Primer = 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC. PCR reactions were carried out on a 2,720 thermal cycler (Life Technologies) using KAPA HiFi HotStart ReadyMix. The PCR conditions consisted of an initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final elongation step at 72°C for 5 min. For indexing, the Illumina Index primer set was applied following the manufacturer's instructions. The resulting PCR amplicons were visualized on a 1.5% agarose gel (Sigma-Merck, Germany) stained with SYBR Safe (Thermo Fisher, MA, United States) to confirm amplification. Subsequently, PCR products were purified using the AMPure XP bead system (Beckman Coulter, CA, United States) in accordance with the Illumina protocol. Prior to pooling, the concentration of the purified products was quantified using the Qubit dsDNA High-Sensitivity kit (Thermo Fisher, MA, United States) and the Qubit 3.0 Fluorometer (Thermo Fisher, MA, United States). Following library preparation, all subsequent libraries were precisely pooled to achieve a final concentration of 4 nM. Afterwards, sequencing was performed utilizing the NextSeq 2000 platform, leveraging the high-throughput capabilities of this technology.

Specifically, sequencing utilized the P1 600-cycle (2 × 300 bp) reagent kit sourced from Illumina (San Diego, United States). The sequencing procedures were conducted at the Teagasc Moorepark Research Centre Next Generation DNA Sequencing Facility.

4.3.5 Short chain fatty acid analysis

Samples were prepared for SCFA analysis according to a previously described protocol with minor adaptations as explained in the work by Lynch *et al.* (Lynch et al., 2021). The standards and samples underwent analysis via gas chromatography—flame ionization detection (GC-FID) employing an Agilent 8860 GC system equipped with a DB-FFAP column (30 m length, 0.32 mm internal diameter, 0.25 μM film thickness; Agilent, California, United States) coupled with a flame ionization detector. Automated sample loading via splitless injection was facilitated using an autosampler. Helium served as the carrier gas, maintaining a constant flow rate of 1.3 mL/min throughout the analysis. The initial temperature of the GC oven was set at 50°C and held for 0.5 min, followed by a programmed ramp to 140°C at a rate of 10°C/min, where it was maintained for an additional 0.5 min. Subsequently, the temperature was elevated to 240°C at a rate of 20°C/min and held for 5 min. The total run time encompassed 20 min. The detector and injection port temperatures were set at 300°C and 240°C, respectively.

Peak integration was accomplished using Agilent OpenLab (version 2.0) software. Concentrations of SCFAs were reported in milimolar (mM) units for each compound. To discriminate significant differences among experimental groups, a one-way ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc test was

employed. Statistical analyses were conducted utilizing the ‘ggpbur ()’ package, with visualization facilitated through the ‘ggplot2 ()’ package in R version 4.3.2, ensuring robust statistical assessment and clear representation of data trends via box plots.

4.3.6 Bioinformatics and statistical analysis

The raw sequencing data underwent processing via the DADA2 pipeline (version 1.16) as outlined by Callahan *et al*. (Callahan et al., 2016), incorporating specific parameters for optimal data refinement. These parameters included maxN=0, maxEE=c (2, 2), rm.phix=TRUE, truncQ=2, trimLeft=c (17, 21), truncLen=c (280,260), and maxN=0, ensuring stringent quality control and accurate sequence inference. Next, the processed data were utilized to generate an amplicon sequence variant (ASV) table and perform taxonomic assignment using SILVA v138.1 database (Quast et al., 2013). Integration of sample and group metadata, ASV table, and taxonomic information facilitated the creation of a phyloseq object, enabling comprehensive downstream analyses (McMurdie and Holmes, 2013).

Alpha diversity metrics, including Observed, Chao1, Shannon, and Simpson indices, were computed to characterize within-sample diversity. Beta diversity was assessed utilizing the Bray-Curtis distance matrix and visualized through principal coordinate analysis (PCoA) to elucidate between-sample dissimilarities.

Statistical analyses encompassed Dunn’s test to discern significant differences in alpha diversity measurements and taxonomic compositions at both phylum and genus levels. False discovery rate (FDR) was used to correct p values for multiple comparisons. FDR values were considered as significant if $q < 0.05$. Furthermore, to assess differential abundance of microbial taxa across the six experimental groups, we

employed the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC). This method accounts for compositional data characteristics and applies bias correction to identify significant differences in microbial abundance (Lin and Peddada, 2020). Cellulose (negative control) was used as the reference group for pairwise comparisons with the remaining groups. All statistical analyses were performed, and graphics were generated using R (version 4.3.2). The details of bioinformatics analysis have been reported in Supplementary Methods.

4.4 Results

4.4.1 Alpha and beta diversity

A total of 20,857,343 raw reads (2 × 300 bp) were obtained after sequencing with an average of reads per sample 651,791. After filtering and merging forward and reverse reads, we obtained a total of 13,159,481 reads with an average value of 411,233 reads/sample and a sequence length of 300 bp.

Alpha diversity was calculated (Figure 4.1) using four indices: Observed, Chao1, Shannon and Simpson following *in vitro* colonic fermentation of cellulose (negative control), inulin (positive control), sourdough bread fermented with baker's yeast (SD-control), sourdough bread fermented with *L. paracasei* R3 (SD-R3), sourdough bread fermented with *P. pentosaceus* RYE106 (SD-Rye106) and sourdough bread fermented with *L. plantarum* FST1.7 (SD-FST1.7). Shannon diversity was found to be higher in the presence of inulin compared to SD-Control ($p = 0.016$, FDR = 0.24) and SD-Rye106 ($p = 0.017$, FDR = 0.24) at the end of colonic fermentation. Similarly, inulin had greater diversity in Simpson index compared to SD-control ($p = 0.044$,

FDR = 0.66). Following false discovery rate (FDR) corrections, the comparisons were not significant ($q > 0.05$).

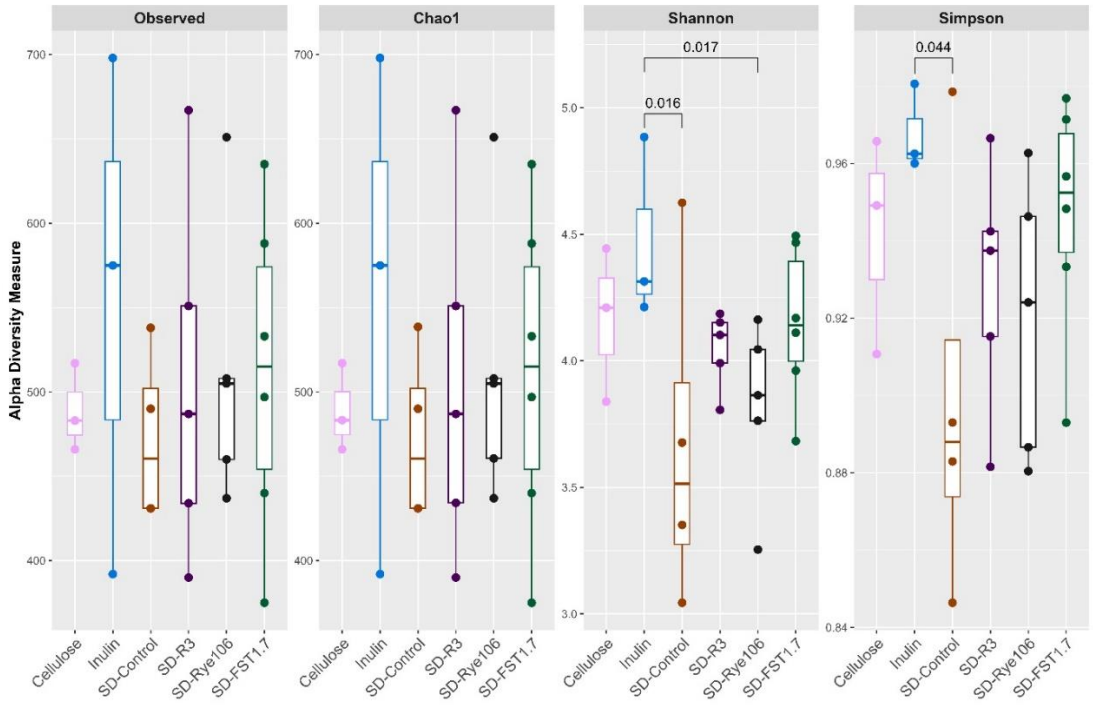

Figure 4.1 Alpha diversity assessed using Observed, Chao1, Shannon and Simpson indices following *in vitro* colonic fermentation of cellulose (negative control), inulin (positive control), sourdough bread fermented with baker's yeast (SD-control), *L. paracasei* R3 (SD-R3), *P. pentosaceus* RYE106 (SD-Rye106), and *L. plantarum* FST1.7 (SD-FST1.7). Significance was determined using the Dunn's test (FDR > 0.05 for all comparisons).

Beta diversity was measured using Bray-Curtis distance metric and principal coordinate analysis plot (Figure 4.2). Table 4.4.1 shows the Pairwise PERMANOVA results with FDR value indicating no significant separation between the groups.

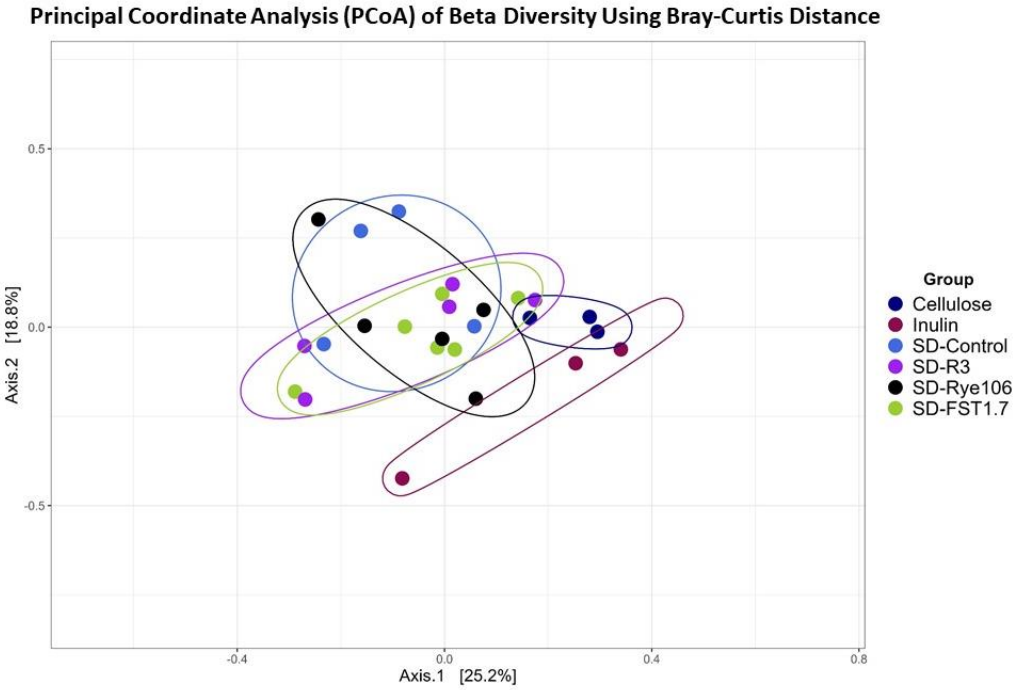

Figure 4.2 Principal Coordinate Analysis (PCoA) plot showing beta diversity of microbial communities following *in vitro* colonic fermentation of cellulose (negative control), inulin (positive control), SD-control, SD-R3, SD-Rye106, and SD-FST1.7.

Table 4.1 Pairwise PERMANOVA results of each fermentation group following *in vitro* colonic fermentation

Comparisons	**Sums of Squares**	**F-Model**	**R^2**	**p-value**	**FDR**
SD-Rye106 vs SD-FST1.7	0.0695	0.642266446	0.06660949	0.819	0.819
SD-Rye106 vs SD-R3	0.1181	0.983747924	0.109503064	0.47	0.542
SD-Rye106 vs Control	0.1729	1.328100476	0.159472197	0.222	0.302
SD-Rye106 vs Cellulose	0.4008	3.831855028	0.389738764	0.019	0.052
SD-Rye106 vs Inulin	0.3718	2.433193306	0.288525736	0.019	0.052
SD-FST1.7 vs SD-R3	0.0697	0.772130407	0.079013519	0.526	0.563
SD-FST1.7 vs Control	0.1829	1.916295024	0.193247077	0.075	0.125
SD-FST1.7 vs Cellulose	0.3583	5.227753322	0.427531795	0.019	0.052
SD-FST1.7 vs Inulin	0.3386	3.082511232	0.305728519	0.006	0.052
SD-R3 vs Control	0.1367	1.277842143	0.154368991	0.276	0.345
SD-R3 vs Cellulose	0.3248	4.187966012	0.411069884	0.017	0.052
SD-R3 vs Inulin	0.3138	2.49466764	0.293674543	0.057	0.106
Control vs Cellulose	0.3749	4.504268494	0.473920586	0.021	0.052
Control vs Inulin	0.3741	2.651294499	0.346515808	0.055	0.106
Cellulose vs Inulin	0.2539	2.408039482	0.375784121	0.1	0.15

4.4.2 Gut microbial composition

A total of nine phyla and 173 genera were identified, with Firmicutes being the dominant phylum across all samples, followed by Proteobacteria and Bacteroidota (Figure 4.3). Notably, no significant differences in Firmicutes abundance were

observed between the groups. Statistical differences determined by Dunn's test are provided in Supplementary Table 4.1. Moreover, the Proteobacteria levels exhibited significant variations, with higher relative abundances observed in faecal samples fermented with SD-R3 compared to faecal samples fermented with inulin ($p = 0.00296$, FDR = 0.044) following *in vitro* colonic fermentation. Actinobacteriota demonstrated a higher relative abundance in faecal samples containing inulin compared to those with SD-R3 and SD-Rye106 ($p = 0.017$ and $p = 0.004$) at the end of colonic fermentation, however after correcting *p* values, the differences were not significant (FDR = 0.13 and FDR = 0.06). Notably, faecal samples harbouring SD-Control exhibited elevated Actinobacteriota levels relative to those containing SD-Rye106 ($p = 0.033$, FDR = 0.14). However, at the phylum level, a discrepancy was noted in the relative abundance of Bacteroidota, with higher levels detected in faecal samples fermented with inulin compared to those fermented with SD-FST1.7 ($p = 0.033$, FDR = 0.17). Furthermore, Desulfobacterota levels were elevated in faecal samples fermented with cellulose at T12 compared to faecal samples fermented with SD-R3 ($p = 0.01$, FDR = 0.07), SD-Rye106 ($p = 0.01$, FDR = 0.07), and SD-FST1.7 ($p = 0.033$, FDR = 0.12).

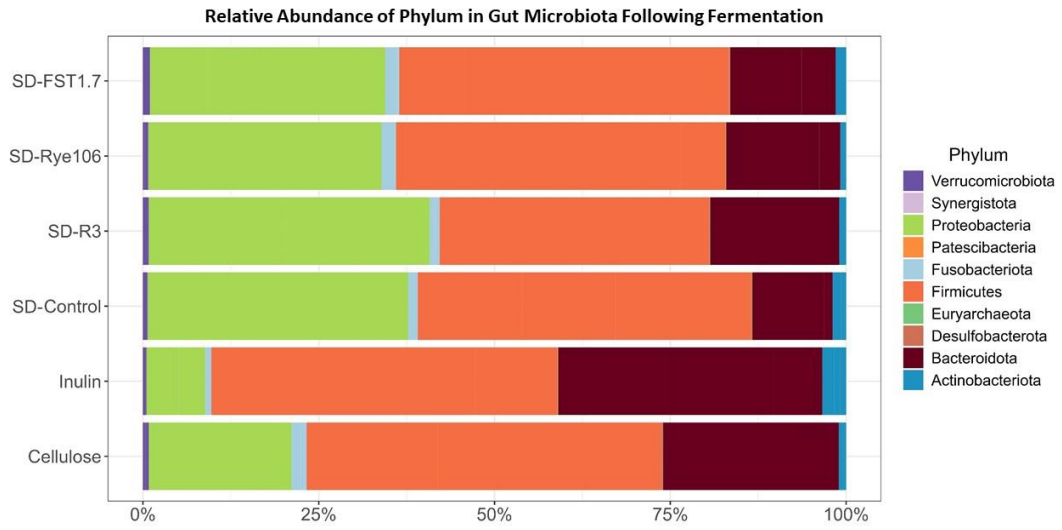

Figure 4.3 Bar charts showing taxonomic composition of gut microbiota at the phylum level (%) before (Baseline) and after *in vitro* colonic fermentation of cellulose (negative control), inulin (positive control), SD-control, SD-R3, SD-Rye106, and SD-FST1.7.

The top 25 genera were used to construct a bar chart (Figure 4.4), illustrating gut microbiota alterations across the faecal samples after *in vitro* colonic fermentation with various substrates. Statistical differences between the groups were assessed using Dunn's test, and the results are provided in Supplementary Table 4.3. *Agathobacter* exhibited higher levels in faecal samples fermented with SD-R3 and SD-Rye106 compared to samples fermented with cellulose ($p = 0.01$, FDR = 0.13 and $p = 0.04$, FDR = 0.18). Conversely, the relative abundance of *Bacteroides* was lower in the samples fermented with SD-FST1.7 compared to samples fermented with cellulose ($p = 0.03$, FDR = 0.089) and inulin ($p = 0.03$, FDR = 0.089). Similarly, faecal samples had lower levels of *Bacteroides* in the presence of SD-Rye106 compared to cellulose at the end of colonic fermentation ($p = 0.031$, FDR = 0.089). Relative

abundances of *Bifidobacterium* were significantly elevated in samples fermented with inulin compared to samples fermented with SD-Rye106 ($p = 0.002$, FDR = 0.04). In contrast, faecal samples exhibited significantly higher levels of C*itrobacter* in the presence of SD-Rye106 and SD-FST1.7 compared to those with inulin ($p = 0.002$, FDR = 0.035 and $p = 0.006$, FDR = 0.05). *Ligilactobacillus* levels were greater in faecal samples fermented with SD-Control compared to those fermented with cellulose ($p = 0.004$, FDR = 0.06). Faecal samples had lower *Veillonella* levels in the presence of SD-R3 and SD-Rye106 compared to cellulose ($p = 0.001$, FDR = 0.077 and $p = 0.006$, FDR = 0.077). *Clostridium sensu stricto 13* was found to be significantly higher in samples fermented with cellulose and inulin compared to those fermented with SD-Control ($p = 0.003$, FDR = 0.01 and $p = 0.003$, FDR = 0.01), SD-R3 ($p = 0.001$, FDR = 0.01 and $p = 0.002$, FDR = 0.01), SD-FST1.7 ($p = 0.01$, FDR = 0.03 and $p = 0.01$, FDR = 0.03). Similarly, *Clostridium sensu stricto 1* was significantly lower in samples fermented with SD-Control ($p = 0.003$, FDR = 0.04) and SD-Rye106 ($p = 0.008$, FDR = 0.05). The relative abundance of *Limosilactobacillus* was significantly greater in samples fermented with SD-FST1.7 and SD-control compared to those fermented with cellulose ($p = 0.0004$, FDR = 0.006, $p = 0.005$, FDR = 0.03). Similarly, SD-FST1.7 fermentates had higher levels of *Limosilactobacillus* compared to inulin fermentates ($p = 0.006$, FDR = 0.03).

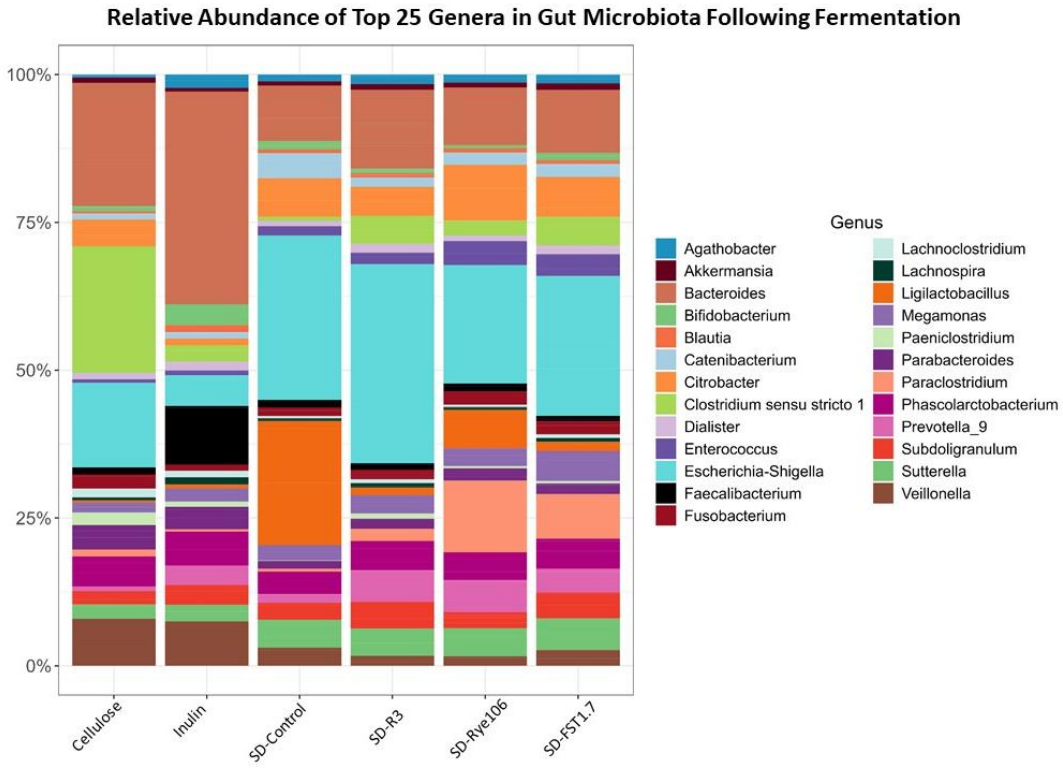

Figure 4.4 Bar charts depicting gut microbiota composition at the genus level across fermentation groups, including cellulose, inulin, SD-Control, SD-R3, SD-Rye106, and SD-FST1.7. The top 25 genera's relative abundances (%) are displayed and the remainders are categorized in other.

The relative abundances of *Lactobacillus*, *Megamonas*, *Parabacteroides*, and *Prevotella*_9 were found to be similar across all faecal samples at the end of fermentation ($q > 0.05$).

4.4.3 Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC)

We conducted an Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) to evaluate differential microbial abundance across the six experimental groups, using cellulose (negative control) as the reference group for

pairwise comparisons. The results, detailed in Supplementary Table 4, revealed significant log fold changes (lfc) in microbial taxa abundance at genus level with corresponding q values to indicate statistical significance ($q < 0.05$). All experimental groups demonstrated a significantly greater proportion of *Agathobacter* compared to cellulose ($q < 0.05$). In contrast, *Akkermansia* abundance was significantly lower in both the inulin and SD-R3 groups relative to cellulose ($q < 0.05$). *Bacteroides* levels were significantly reduced in SD-control, while *Catenibacterium* abundance was higher compared to SD-control ($q < 0.05$). In the SD-Rye106 group, *Citrobacter* abundance increased significantly compared to cellulose (lfc = 1.03, $q < 0.05$). *Clostridium sensu stricto 1* and *Clostridium sensu stricto 13* were reduced in all sourdough bread groups, with the exception of inulin ($q < 0.05$), while *Dialister* abundance increased 0.3-fold in SD-FST1.7 relative to cellulose ($q < 0.05$). *Enterococcus* levels were higher in SD-Rye106 and SD-FST1.7 compared to cellulose. The abundance of *Escherichia-Shigella* was significantly higher in SD-R3 compared to cellulose ($q < 0.05$), while *Faecalibacterium* levels were lower in cellulose than in SD-R3 ($q < 0.05$). The *Lachnospiraceae* NK4A136 group was more abundant in SD-FST1.7, SD-R3, and SD-Rye106 compared to cellulose ($q < 0.05$). Notably, *Lactobacillus* was present at significantly higher proportions in all bread groups except for inulin ($q < 0.05$). Furthermore, *Ligilactobacillus* was significantly more abundant in the SD-control and SD-FST1.7 groups compared to cellulose ($q < 0.05$), while *Megamonas* levels increased in SD-FST1.7 relative to cellulose. *Parabacteroides* was reduced in SD-control and SD-R3 groups compared to cellulose ($q < 0.05$). Additionally, *Paraclostridium* levels were significantly lower in SD-control compared to cellulose ($q < 0.05$). *Prevotella_9* showed significantly

higher fold changes in SD-FST1.7 compared to cellulose ($q < 0.05$), while SD-R3 and SD-Rye106 also displayed higher abundances close to significance (q values nearing 0.05). *Subdoligranulum* levels were elevated in SD-FST1.7 and SD-R3 relative to cellulose ($q < 0.05$), and *Sutterella* was more abundant in SD-control compared to cellulose. Finally, *UCG-002* showed higher abundance in SD-FST1.7, SD-R3, and SD-Rye106 compared to cellulose, while *Veillonella* levels were lower in all groups except inulin ($q < 0.05$).

4.4.4 Short-chain fatty acid analysis

Short-chain fatty acid analysis revealed a number of significant differences among groups after colonic fermentation (Figure 4.5). Acetate levels (Figure 4.5A) were found to be significantly higher in faecal samples fermented with SD-FST1.7, SD-R3 and SD-Rye106 following colonic fermentation compared to baseline ($p = 0.024$, $p = 0.036$, and $p = 0.036$). The concentration of acetate was also found to be significantly higher in faecal samples fermented with SD-FST1.7 compared to samples fermented with cellulose, SD-R3 and SD-Rye106 ($p = 0.024$, $p = 0.03$, and $p = 0.03$) at the end of *in vitro* colonic fermentation. Similarly, following *in vitro* colonic fermentation, faecal samples fermented with SD-Control had higher acetate levels compared to those fermented with SD-R3 ($p = 0.032$). In the presence of SD-FST1.7, faecal samples had significantly higher levels of butyrate at the end of the fermentation compared to baseline ($p = 0.048$) (Figure 4.5B). Butyrate levels were found to be higher in the faecal samples fermented with inulin compared to those fermented with SD-FST1.7, SD-R3 and SD-Rye106 ($p = 0.048$, $p = 0.36$, and $p = 0.036$).

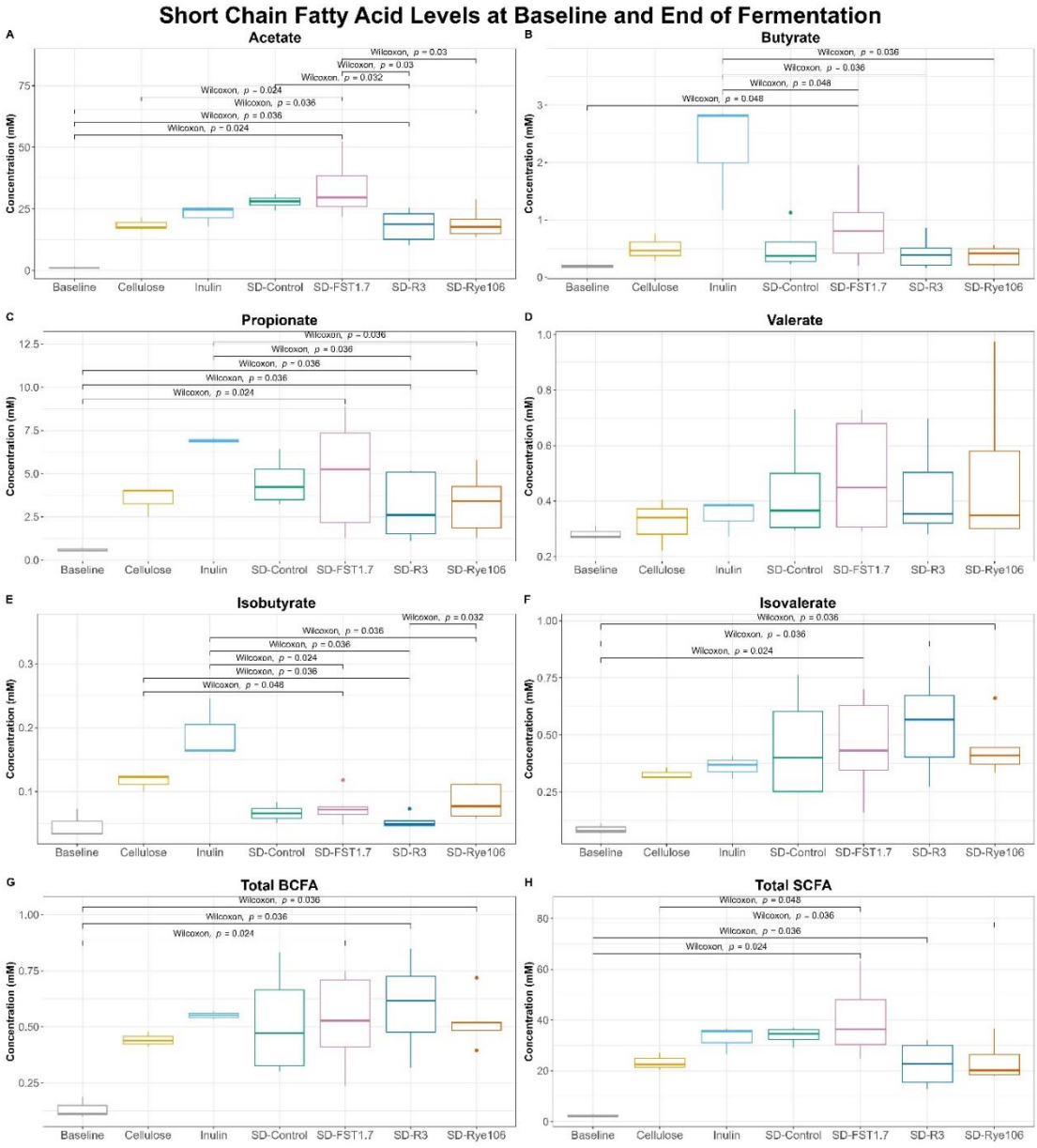

Figure 4.5 Box plots of short-chain fatty acid (SCFA) concentrations (mM) at baseline (T0) and after fermentation (T12) for cellulose, inulin, SD-Control, SD-R3, SD-Rye106, and SD-FST1.7. **(A)** Acetate, **(B)** butyrate, **(C)** propionate, **(D)** valerate, **(E)** isobutyrate, **(F)** isovalerate, **(G)** total BCFAs, and **(H)** total SCFAs. Wilcoxon rank test $p < 0.05$.

Faecal fermentation of all substrates including cellulose (negative control) and inulin (positive control) resulted in increased level of propionate (Figure 4.5C). Comparison

of baseline (T0) and T12 revealed significant differences in the samples fermented with SD-FST1.7, SD-R3, and SD-Rye106 ($p = 0.024$, $p = 0.36$, and $p = 0.036$). Faecal samples fermented with inulin displayed significantly higher levels of propionate than samples fermented with SD-R3 and SD-Rye106 ($p = 0.036$ and $p = 0.036$).

Isobutyrate levels (Figure 4.5E) were significantly higher in faecal samples fermented with cellulose, inulin and SD-Rye106 compared to samples fermented with SD-R3 ($p = 0.036$, $p = 0.036$, and $p = 0.032$). Faecal samples exhibited higher isobutyrate levels at T12 in the presence of inulin, compared to those with SD-FST1.7 and SD-Rye106 ($p = 0.024$ and $p = 0.036$). Similarly, faecal samples fermented with cellulose displayed higher concentration of isobutyrate compared to those fermented with SD-FST1.7 ($p = 0.048$). At the end of colonic fermentation, samples fermented with SD-FST1.7, SD-R3 and SD-Rye106 had significantly higher levels of isovalerate than baseline ($p = 0.034$, $p = 0.036$, and $p = 0.036$) (Figure 4.5F).

Total branched-chain fatty acid (BCFA) levels (Figure 4.5G) were found to be significantly higher in samples fermented with SD-FST1.7, SD-R3, and SD-Rye106 compared to baseline ($p = 0.024$, $p = 0.036$, and $p = 0.036$). Similarly, total SCFA levels (Figure 4.5H) were higher in all samples fermented with SD-FST1.7, SD-R3, and SD-Rye106 at T12 compared to baseline ($p = 0.024$, $p = 0.036$, and $p = 0.036$). Total SCFA levels were significantly higher in faecal samples fermented with SD-FST1.7 than those fermented with cellulose at the end of fermentation ($p = 0.048$).

4.4.5 Correlation between SCFAs and Top 25 Genera

Pearson correlation analysis was utilized to investigate the relationship between the top 25 genera identified in the microbiota analysis and SCFAs in the gut environment.

The analysis revealed several noteworthy correlations (Figure 4.6). Acetate exhibited a positive correlation with propionate, indicating a potential metabolic relationship between these SCFAs. Moreover, butyrate levels were positively correlated with *Lachnospira*, *Agathobacter*, *Blautia*, *Parabacteroides*, *Veillonella*, *Bacteroides*, *Bifidobacterium*, and *Faecalibacterium*, suggesting a potential role of these genera in butyrate production. Additionally, *Bifidobacterium* showed a positive correlation with propionate, indicating its involvement in propionate metabolism. Similarly, valerate displayed positive correlations with *Citrobacter*, *Enterococcus*, *Paraclostridium*, *Fusobacterium*, *Prevotella*_9, *Sutterella*, and *Blautia*, suggesting potential interactions between these genera and valerate production. In contrast, isovalerate showed negative correlations with all the top 25 genera. Finally, isobutyrate exhibited negative correlations with *Escherichia-Shigella* and *Citrobacter*, while showing positive correlations with *Bacteroides* and *Bifidobacterium*, suggesting differential effects on these genera.

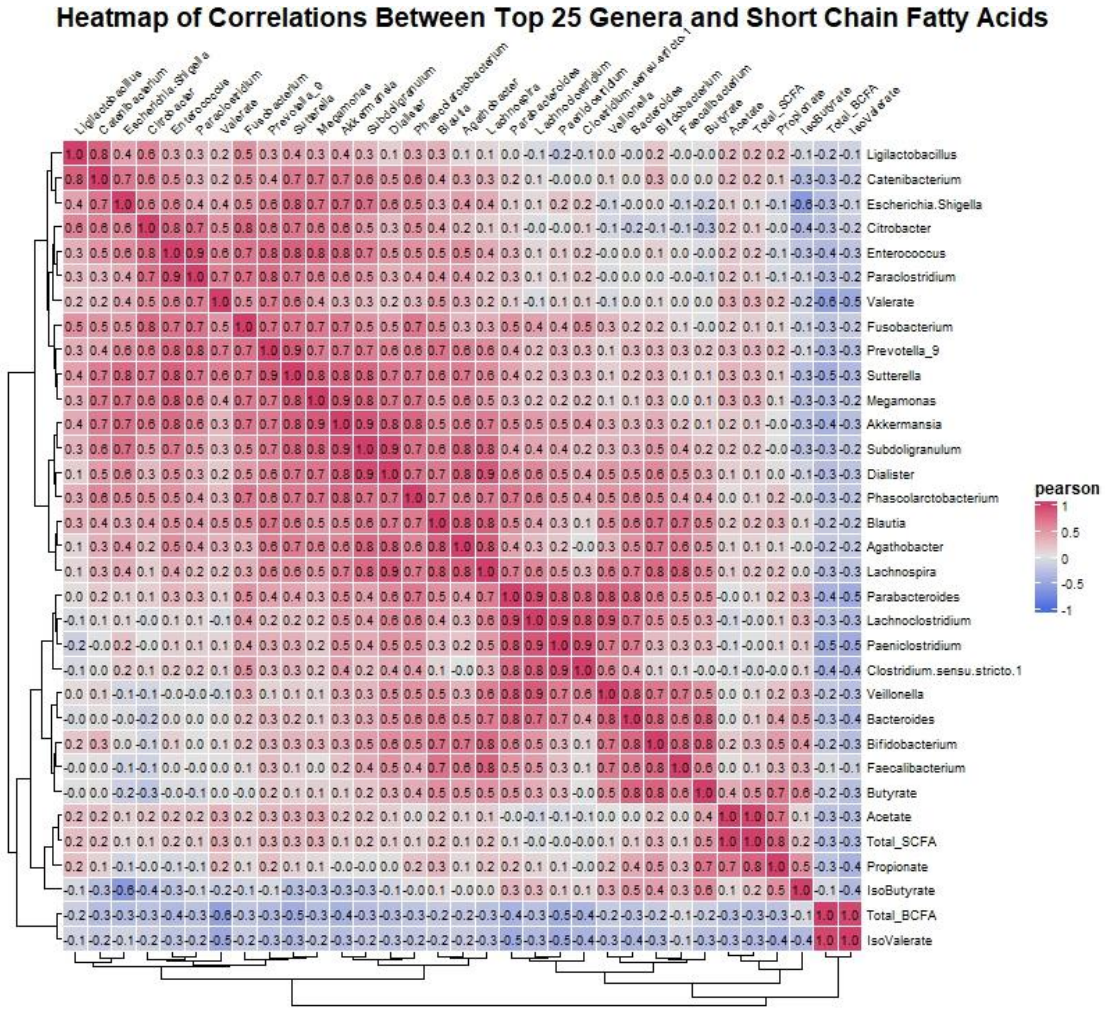

Figure 4.6 Heatmap displaying Pearson correlation coefficients between the top 25 genera's relative abundances and SCFA concentrations post-fermentation. Red indicates positive correlations, blue indicates negative, with intensity representing correlation strength.

4.5 Discussion

This study provides new insights into how three distinct sourdough breads fortified with selected LAB strains [*L. plantarum* FST1.7 (SD-FST1.7), *L. paracasei* R3 (SD-R3), and *P. pentosaceus* RYE106 (SD-RYE106)] modulate gut microbiome and offer a promising dietary approach for managing symptoms of IBS. Our results unveiled significant alterations in microbial composition and SCFA production following *in*

vitro colonic fermentation of various bread formulations, providing a mechanistic understanding of how fortified sourdough breads interact with the gut microbiome to promote gut health. Although a detailed chemical composition analysis post-digestion was not performed in our study, Borowska *et al.* (Borowska et al., 2023) provide valuable insights into LAB's robust utilization of FODMAPs. Their high-throughput FODMAP utilization assay supports our hypothesis that LAB strains, including *L. paracasei* R3 and *P. pentosaceus* RYE106, exhibited robust utilization of fructans, GOS, and other relevant carbohydrates, including fructose and sucrose, which are often implicated in IBS symptomatology. The results revealed that fructan levels decreased by up to 73% with RYE106 and 69% with R3, confirming our hypothesis that the beneficial effects of these sourdough breads are attributed to the substantial reduction of FODMAPs. The observed reductions in maltose, glucose, and fructose after 48 h of fermentation in sourdough bread samples suggest that these strains play a crucial role in mitigating FODMAP-related gut symptoms. Even though our study focuses on the traditional FODMAPs, it is also important to consider microbial-derived carbohydrates such as beta-glucan and dextran, which may have potential effects on IBS symptoms and require further investigation.

While alpha and beta diversity analyses did not reveal significant differences between the bread groups, investigation into relative abundances of taxonomic groups at the phylum and genus levels highlighted significant differences across different bread formulations. Interestingly, Bacteroidota levels were higher in inulin-treated faecal samples compared to those treated with specific sourdough bread formulations, suggesting distinct microbial modulation and highlighting the strain-specific effects of LAB fortification. Increased levels of Firmicutes and Bacteroidota are suggested for

healthy gut health (Ponnusamy et al., 2011) and intestinal inflammation is generally associated with reduced Firmicutes and Bacteroidota levels (Ghoshal et al., 2012). Furthermore, commensal species of Firmicutes and Bacteroidota were shown to induce T-regulatory cells and inhibit Th17-mediated inflammation in previous studies (Veiga et al., 2010, Mazmanian et al., 2008, Chow et al., 2010).

At the genus level, several noteworthy changes were observed. *Agathobacter* was elevated in faecal samples fermented with all breads and inulin compared to those fermented with cellulose (ANCOMBC, FDR < 0.05). *Agathobacter* is recognized for its role in butyrate production, which is crucial for maintaining intestinal homeostasis and mucosal integrity (Rios-Covian et al., 2016). The enhanced levels of *Agathobacter* in the presence of SD-R3 and SD-Rye106 breads indicated a synergistic effect between these strains and beneficial gut bacteria, which can facilitate SCFA production and contribute to improved gut health. In contrast, *Bacteroides* levels were lower in faecal samples fermented with SD-FST1.7 and SD-Rye106 compared to those fermented with cellulose, suggesting these specific two LAB strains may be effective in suppressing potentially pathogenic microbial populations associated with IBS (Scott et al., 2014). High levels of *Bacteroides* have been associated with gut dysbiosis and inflammation, while lower levels have been linked to improved gut barrier function and reduced risk of gastrointestinal disorders (Taketani et al., 2020). A recent study reported high levels of *Bacteroides* in severe IBS patients (Tap et al., 2017). Thus, the modulation of *Bacteroides* abundance through dietary interventions such as fortified sourdough breads could represent a novel approach to maintaining a balanced gut microbiota and effectively managing IBS symptoms by targeting dysbiosis. *Limosilactobacillus* (formerly *Lactobacillus*)

was the signature genus for samples fermented with SD-FST1.7. *Limosilactobacillus* belongs to *Lactobacillaceae* family and it includes probiotic strains such as *Limosilactobacillus reuteri* (Abuqwider et al., 2022, Ksiezarek et al., 2022). *L. reuteri* has been shown to reduce inflammation in intestine (Tang et al., 2019). Moreover, *Bifidobacterium* levels were the significantly elevated in inulin-treated samples, indicating a potential preference for growth in an inulin-rich environment. *Bifidobacterium*, known for its probiotic properties, plays a crucial role in fermenting dietary fibres and producing beneficial metabolites such as SCFA thereby promoting gut health and alleviating symptoms of IBS (Scott et al., 2014).

The findings also highlighted a reduction in *Veillonella* levels within faecal samples fermented with SD-R3 and SD-Rye106 compared to those fermented with cellulose at the end of *in vitro* colonic fermentation. Elevated *Veillonella* levels have been reported in individuals with IBS and are associated with inflammatory responses (Rigsbee et al., 2012, Tana et al., 2010). These bacteria have lipopolysaccharides which can contribute to gut inflammation and exacerbate IBS symptoms (Rocha et al., 2023). The ability of our sourdough formulations to decrease *Veillonella* offers a novel therapeutic avenue for mitigating IBS symptoms by targeting inflammation at its microbial source, thereby enhancing gut health.

Short-chain fatty acid analysis constituted a crucial aspect of our study, providing new insights into the metabolic interactions within the gut environment and their implications for gastrointestinal health, particularly in individuals with IBS. SCFAs, including acetate, propionate, and butyrate, serve as primary metabolites resulting from the fermentation of dietary fibres by gut microbiota in the colon (Koç et al., 2020,

Morrison and Preston, 2016). These SCFAs play multifaceted roles in maintaining gut homeostasis and exerting anti-inflammatory, immunomodulatory, and trophic effects on the colonic epithelium (Morrison and Preston, 2016, den Besten et al., 2013). Significant increases in acetate levels were observed following the fermentation of all sourdough formulations, with notable elevations observed in SD-FST1.7. Acetate, the most abundant SCFA in the colon, has been implicated in promoting gut barrier function, modulating immune responses, and attenuating inflammation (Morrison and Preston, 2016). Furthermore, butyrate, another critical SCFA, demonstrated notable variations across bread samples, with significantly higher levels detected post-fermentation in specific variants, in particular SD-FST1.7. Butyrate serves as a primary energy source for colonic epithelial cells and plays pivotal roles in epithelial integrity, mucosal immune regulation, and inflammation modulation (Rios-Covian et al., 2016). Propionate, the second major SCFA, exhibited intriguing dynamics across fermentation groups, with significant differences observed between baseline and post-fermentation levels in samples fermented with SD-FST1.7, SD-R3 and SD-Rye106. Propionate has garnered attention for its role in regulating appetite, glucose homeostasis, and lipid metabolism, in addition to exerting anti-inflammatory effects within the gut (Chambers et al., 2015). The observed alterations in acetate, butyrate and propionate levels underscore the potential of fortified sourdough breads to modulate metabolic processes and inflammatory pathways, highlighting their implicated in IBS pathophysiology.

The correlation analysis conducted in our study further elucidated the intricate relationship between microbial composition and SCFA production in the gut environment. Positive correlations were observed between specific bacterial genera

and SCFA levels, suggesting potential microbial contributors to SCFA production. For instance, butyrate levels were positively correlated with genera known for their butyrate-producing capabilities, such as *Lachnospira*, *Agathobacter*, *Blautia*, *Parabacteroides*, *Veillonella*, *Bacteroides*, , and *Faecalibacterium* (Rios-Covian et al., 2016). These findings underscore the potential of specific bacterial taxa to modulate SCFA production and contribute to gut health maintenance in individuals with IBS.

In summary, our study offers new insights and mechanistic understanding into how fortified sourdough breads influence SCFA production and microbial composition, highlighting their broader potential in dietary interventions for IBS symptom management. The observed alterations in SCFA profiles and their correlation with specific bacterial genera hold promising implications for the management of IBS symptoms and the promotion of gut health.

While this study utilized an *in vitro* digestion and fermentation model, which is useful for controlled experimentation, it does not fully capture the complexity of the human digestive system; thus, the results may differ in actual human physiology. Additionally, we used pooled faecal samples from healthy individuals to mimic the human gut microbiome. However, individual baseline microbiomes differ, potentially influencing fermentation outcomes in real-world conditions. Future studies should consider *in vivo* experiments or personalized models to validate these findings in clinical settings and explore the long-term effects of fortified sourdough bread consumption on gut microbiota composition and IBS symptom management.

4.6 Conclusion

In conclusion, our study demonstrates the potential of fortified sourdough breads as a dietary intervention for individuals managing symptoms of IBS. By harnessing the fermentative properties of sourdough and incorporating specific LAB strains, we have successfully developed bread variants with reduced FODMAP content and enhanced gut-friendly attributes. Our findings highlight the positive impact of these fortified breads on gut microbiota composition and SCFA production. Among the evaluated bread variants, SD-FST1.7 emerges as a promising candidate, exhibiting favourable outcomes in terms of microbial modulation and SCFA profiles. Moving forward, continued research including human clinical trials will be essential to validate the efficacy and long-term benefits of these gut-friendly bread options in improving the quality of life for individuals managing IBS.

4.7 Data Availability Statement

The raw sequencing data has been updated to NCBI SRA repositories. The accession number of BioProject is PRJNA1168565. https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1168565.

4.8 Acknowledgements

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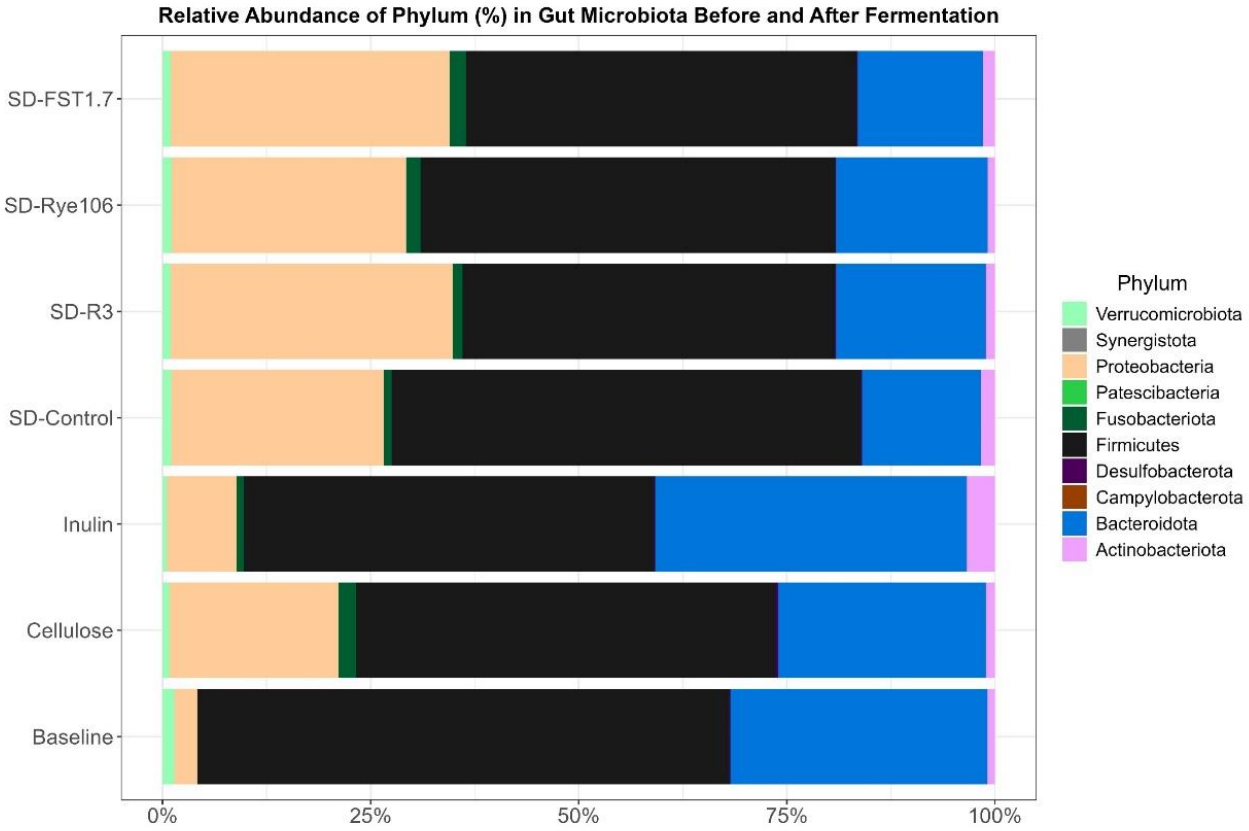
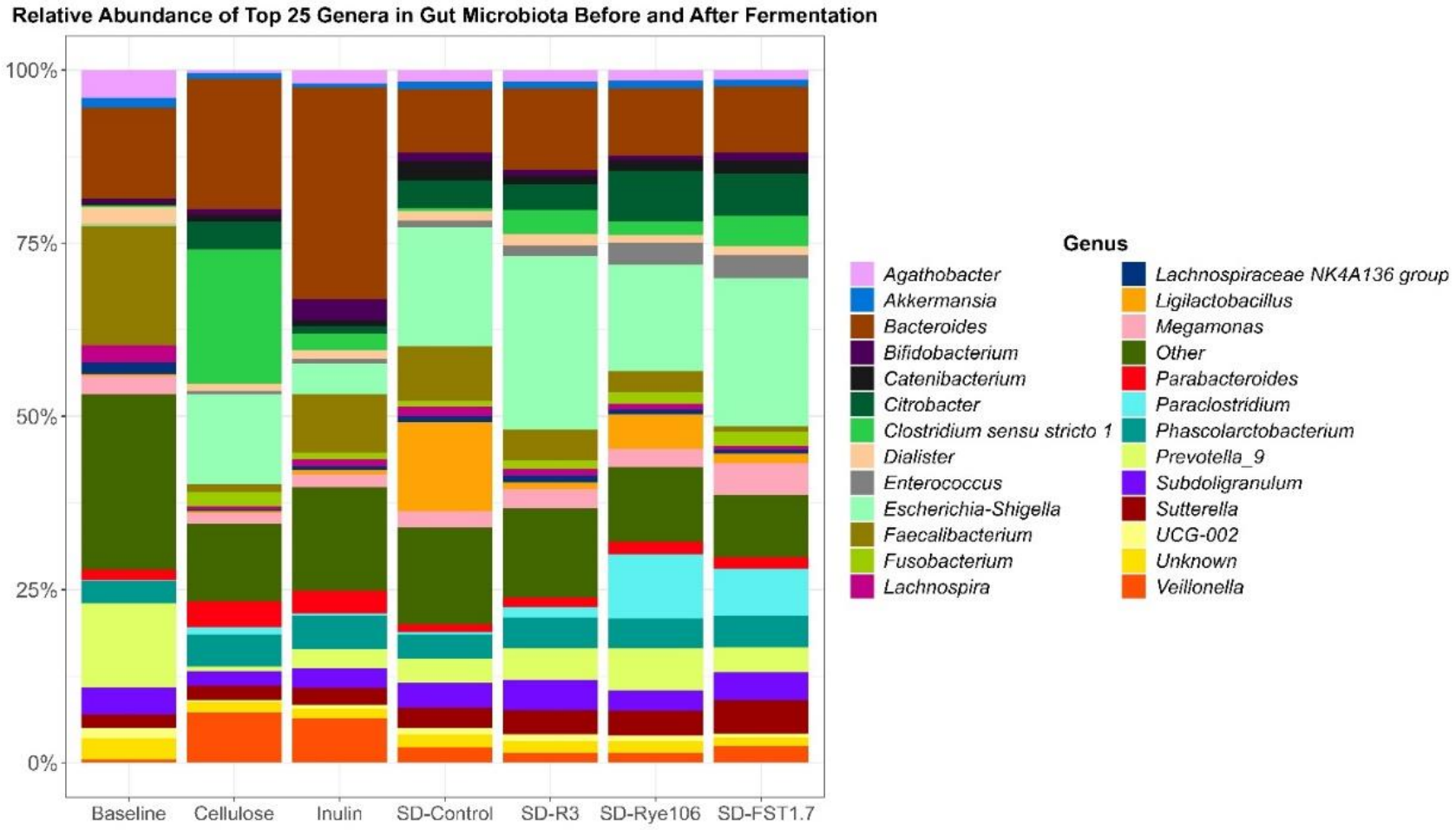
4.10 Supplementary Figures



Supplementary Figure 4.1 Relative abundance of phylum (%) in gut microbiota before and after fermentation



Supplementary Figure 4.2 Relative abundance of top 25 genera (%) in gut microbiota before and after fermentation

4.11 Supplementary Tables

Supplementary Table 4.1 Dunn's test results at Phylum level

Phylum	**group1**	**group2**	**p**	**p.adj**
Proteobacteria	Inulin	SD-R3	0.00296	0.044398
Actinobacteriota	Inulin	SD-Rye106	0.004022	0.060337
Desulfobacterota	Cellulose	SD-Control	0.00797	0.074502
Desulfobacterota	Cellulose	SD-R3	0.0149	0.074502
Desulfobacterota	Cellulose	SD-Rye106	0.012197	0.074502
Proteobacteria	Inulin	SD-Control	0.011571	0.08115
Proteobacteria	Inulin	SD-FST1.7	0.01623	0.08115
Proteobacteria	Inulin	SD-Rye106	0.021931	0.08224

Supplementary Table 4.2 Dunn's test results at Genus level

Genus	**group1**	**group2**	**p**	**p.adj**
Limosilactobacillus	Cellulose	SD-FST1.7	0.000443	0.00664363
Clostridium sensu stricto 13	Inulin	Control	0.003751	0.014065535
Clostridium sensu stricto 13	Inulin	SD-R3	0.002436	0.014065535
Clostridium sensu stricto 13	Cellulose	Control	0.003041	0.014065535
Clostridium sensu stricto 13	Cellulose	SD-R3	0.00194	0.014065535
Faecalitalea	Inulin	SD-R3	0.001884	0.014131263
Faecalitalea	Cellulose	SD-R3	0.001884	0.014131263

Barnesiella	Cellulose	SD-R3	0.001123	0.016847882
Sphingomonas	Inulin	SD-R3	0.001271	0.017538945
Sphingomonas	Cellulose	SD-R3	0.002339	0.017538945
Sellimonas	Inulin	SD-R3	0.003695	0.018472951
Sellimonas	Inulin	SD-Rye106	0.003695	0.018472951
Sellimonas	Inulin	SD-FST1.7	0.002715	0.018472951
Angelakisella	Cellulose	SD-R3	0.003695	0.018472951
Angelakisella	Cellulose	SD-Rye106	0.003695	0.018472951
Angelakisella	Cellulose	SD-FST1.7	0.002715	0.018472951
Barnesiella	Inulin	SD-R3	0.003127	0.02345478
Escherichia-Shigella	Inulin	SD-R3	0.001915	0.028719482
Faecalitalea	Inulin	SD-FST1.7	0.009867	0.029601126
Faecalitalea	Control	SD-R3	0.009672	0.029601126
Faecalitalea	Cellulose	SD-FST1.7	0.009867	0.029601126
Barnesiella	Cellulose	SD-FST1.7	0.005929	0.02964643
Clostridium sensu stricto 13	Cellulose	SD-FST1.7	0.010234	0.030702682
Clostridium sensu stricto 13	Inulin	SD-FST1.7	0.012509	0.031271449
Limosilactobacillus	Inulin	SD-FST1.7	0.006691	0.033452653
Limosilactobacillus	Cellulose	Control	0.005407	0.033452653
Citrobacter	Inulin	SD-Rye106	0.002339	0.03507789
Angelakisella	Cellulose	Inulin	0.009414	0.035303313
Sellimonas	Cellulose	Inulin	0.009414	0.035303313
Lachnospiraceae ND3007 group	Inulin	SD-Rye106	0.004852	0.036387771
Lachnospiraceae ND3007 group	Inulin	SD-FST1.7	0.004158	0.036387771
Lachnoclostridium	Cellulose	Control	0.003957	0.036387771
Lachnoclostridium	Cellulose	SD-Rye106	0.004852	0.036387771

Anaerostignum	Inulin	SD-R3	0.017539	0.037583796
Anaerostignum	Inulin	SD-Rye106	0.017539	0.037583796
Anaerostignum	Inulin	SD-FST1.7	0.014163	0.037583796
Anaerostignum	Cellulose	Control	0.014174	0.037583796
Anaerostignum	Cellulose	SD-R3	0.010311	0.037583796
Anaerostignum	Cellulose	SD-Rye106	0.010311	0.037583796
Anaerostignum	Cellulose	SD-FST1.7	0.008065	0.037583796
Microbacterium	Inulin	SD-R3	0.005072	0.038037594
Microbacterium	Cellulose	SD-R3	0.002863	0.038037594
Bifidobacterium	Inulin	SD-Rye106	0.002738	0.041065991
Faecalitalea	Inulin	SD-Rye106	0.01928	0.041314361
Faecalitalea	Cellulose	SD-Rye106	0.01928	0.041314361
Collinsella	Control	SD-R3	0.002774	0.041608539
Phocea	Cellulose	Control	0.011138	0.042143715
Phocea	Cellulose	SD-R3	0.003809	0.042143715
Phocea	Cellulose	SD-Rye106	0.011238	0.042143715
Phocea	Cellulose	SD-FST1.7	0.01121	0.042143715
Paeniclostridium	Cellulose	Control	0.002869	0.043031895
Anaerostignum	Inulin	Control	0.023139	0.043385557
Lachnospiraceae ND3007 group	Cellulose	SD-Rye106	0.011791	0.044216378
Lachnospiraceae ND3007 group	Cellulose	SD-FST1.7	0.010534	0.044216378
Butyricimonas	Cellulose	SD-R3	0.00296	0.044397825
Clostridium sensu stricto 1	Cellulose	Control	0.003148	0.047216398
UBA1819	Cellulose	SD-Rye106	0.006504	0.048778656
UBA1819	Cellulose	SD-FST1.7	0.005546	0.048778656
Candidatus Soleaferrea	Cellulose	SD-R3	0.00327	0.049056894

Collinsella	Control	SD-Rye106	0.00655	0.049127546
Citrobacter	Inulin	SD-FST1.7	0.006691	0.050178979
Haemophilus	Cellulose	SD-R3	0.004503	0.050178979
Haemophilus	Cellulose	SD-FST1.7	0.006691	0.050178979
Lachnospiraceae ND3007 group	Inulin	SD-R3	0.016984	0.050952602
Odoribacter	Cellulose	SD-Rye106	0.006989	0.052417801
Odoribacter	Cellulose	SD-FST1.7	0.005042	0.052417801
[Clostridium] innocuum group	Inulin	SD-R3	0.008954	0.054356735
[Clostridium] innocuum group	Inulin	SD-Rye106	0.018119	0.054356735
[Clostridium] innocuum group	Inulin	SD-FST1.7	0.008044	0.054356735
[Clostridium] innocuum group	Cellulose	SD-R3	0.017544	0.054356735
[Clostridium] innocuum group	Cellulose	SD-FST1.7	0.01623	0.054356735
Butyricimonas	Cellulose	SD-FST1.7	0.007339	0.055043506
Barnesiella	Inulin	SD-FST1.7	0.014729	0.05523337
Candidatus Soleaferrea	Cellulose	SD-FST1.7	0.00758	0.0568527
Collinsella	Cellulose	Control	0.011571	0.057854593
Alistipes	Cellulose	SD-R3	0.011791	0.058955171
Alistipes	Cellulose	SD-Rye106	0.011791	0.058955171
Alistipes	Cellulose	SD-FST1.7	0.005546	0.058955171
Clostridium sensu stricto 13	Cellulose	SD-Rye106	0.027794	0.059558442
Hungatella	Cellulose	Control	0.011991	0.059953138
Hungatella	Cellulose	SD-R3	0.004476	0.059953138
Hungatella	Cellulose	SD-Rye106	0.010592	0.059953138

Supplementary Table 4.3 ANCOM test results at genus level for groups compared to cellulose. lfc.taxon: taxonomic ID, lfc: log fold change, se: standard error.

lfc.taxon	lfc. SD-Control	se. SD-Control	W. SD-Control	p_val. SD-Control	q_val. SD-Control
Paeniclostridium	-3.549345491	0.301789414	-11.76100065	6.20E-32	1.20E-29
Limosilactobacillus	2.806940771	0.310617821	9.036637888	1.62E-19	1.56E-17
Clostridium sensu stricto 13	-5.93149399	0.678597154	-8.740817668	2.31E-18	1.49E-16
Lachnospiraceae ND3007 group	-1.571095411	0.196218612	-8.006862333	1.18E-15	4.54E-14
Lachnoclostridium	-1.530145539	0.194156529	-7.880989357	3.25E-15	1.04E-13
CAG-352	-3.086363951	0.4101127	-7.525648321	5.25E-14	1.45E-12
Hungatella	-4.372195841	0.614715047	-7.112557047	1.14E-12	2.75E-11
UBA1819	-1.511866216	0.225994714	-6.689829984	2.23E-11	4.79E-10
Adlercreutzia	1.088671497	0.168289365	6.469045136	9.86E-11	1.80E-09
Alistipes	-2.261630337	0.349918707	-6.463302164	1.02E-10	1.80E-09
Flavonifractor	-1.892562298	0.300143777	-6.305519029	2.87E-10	4.62E-09
Agathobacter	0.809447352	0.130246779	6.214720703	5.14E-10	7.63E-09
Candidatus Soleaferrea	-3.184249464	0.526295137	-6.050311393	1.45E-09	1.74E-08
Sphingomonas	2.164080361	0.357527306	6.052909312	1.42E-09	1.74E-08
Clostridium sensu stricto 1	-3.987511437	0.67642637	-5.894967452	3.75E-09	4.02E-08
Phocea	-3.465826034	0.600359579	-5.772917022	7.79E-09	7.91E-08
HT002	2.649948185	0.474459834	5.585189719	2.33E-08	2.25E-07
Methanobrevibacter	1.114033077	0.204966263	5.435202173	5.47E-08	5.03E-07
Butyricimonas	-2.542503994	0.470426173	-5.40468227	6.49E-08	5.70E-07
Acidaminococcus	-1.8282896	0.359161639	-5.090436726	3.57E-07	3.00E-06
Slackia	1.059589574	0.209070603	5.068094498	4.02E-07	3.23E-06
Anaerostipes	1.207608077	0.243678791	4.955737307	7.21E-07	5.35E-06

Incertae Sedis	-0.607975405	0.122564944	-4.96043473	7.03E-07	5.35E-06
Family XIII AD3011 group	1.065294237	0.222397994	4.790035277	1.67E-06	1.19E-05
Erysipelotrichaceae UCG-003	1.203007352	0.256216598	4.695274856	2.66E-06	1.84E-05
Barnesiella	-1.934719173	0.413376049	-4.680288513	2.86E-06	1.91E-05
Anaerofilum	-1.696659195	0.373676228	-4.54045259	5.61E-06	3.61E-05
Butyricicoccus	-1.008961404	0.231948146	-4.349943825	1.36E-05	8.21E-05
Collinsella	1.394939822	0.323898362	4.306720831	1.66E-05	9.69E-05
Parabacteroides	-1.204976153	0.301069077	-4.002324529	6.27E-05	0.000356046
Prevotella_7	-2.446327284	0.615984866	-3.971408095	7.14E-05	0.000393991
NK4A214 group	0.493158416	0.125808315	3.919919091	8.86E-05	0.00047488
Ligilactobacillus	3.324208756	0.873406359	3.806027654	0.000141217	0.000736616
Shuttleworthia	1.61847508	0.446746866	3.622801195	0.00029143	0.001480157
Bilophila	-2.110921707	0.588712195	-3.585659892	0.000336227	0.001663892
Allisonella	-1.239845429	0.353579515	-3.506553341	0.00045395	0.002190311
Alloprevotella	-1.372110039	0.402334834	-3.410368486	0.000648752	0.00305388
Odoribacter	-1.486838297	0.440908111	-3.372218068	0.000745654	0.003346772
Paludicola	-1.672294606	0.495199676	-3.377010706	0.000732782	0.003346772
Ochrobactrum	2.071365604	0.627454305	3.301221441	0.000962649	0.004222527
Haemophilus	-2.357679708	0.717924784	-3.284020499	0.001023375	0.004389141
Stenotrophomonas	1.653849971	0.50601255	3.268397142	0.001081585	0.004537954
Christensenellaceae R-7 group	-0.530371496	0.163619993	-3.241483418	0.001189094	0.004882873
[Eubacterium] ruminantium group	2.015929612	0.632640657	3.186531866	0.001439896	0.005789583
Colidextribacter	-0.915124039	0.288413784	-3.172955275	0.001508958	0.005943446
Lactobacillus	2.074290606	0.661870669	3.133981763	0.001724516	0.006656631
Paraclostridium	-0.930092591	0.300889472	-3.091143684	0.001993871	0.007545434
[Eubacterium] xylanophilum group	1.023555071	0.332673271	3.076757768	0.002092653	0.007652354

CAG-873	-2.09901762	0.682493998	-3.075510738	0.002101424	0.007652354
Bacteroides	-0.915536978	0.30461727	-3.005532082	0.002651166	0.009475465
Marvinbryantia	1.823085866	0.61500639	2.964336464	0.003033364	0.010644351
Fournierella	-2.41152054	0.836387761	-2.883256608	0.003935867	0.013316645
Negativibacillus	-2.734860369	0.950260087	-2.878012457	0.004001893	0.013316645
Succinatimonas	1.366999358	0.485933507	2.813140763	0.004906017	0.016048496
Faecalitalea	-1.216211219	0.444920655	-2.733546319	0.006265631	0.020154446
Catenibacterium	1.138111848	0.423900276	2.684857533	0.007256073	0.022957739
Howardella	-0.344840276	0.129482155	-2.663226265	0.007739535	0.024092424
Desulfovibrio	-1.228419593	0.463973321	-2.647608251	0.00810634	0.024833709
Cloacibacillus	-1.606787508	0.653425574	-2.45902146	0.013931629	0.041366221
Microbacterium	1.899236855	0.774864764	2.451055905	0.014243783	0.041652274
Veillonella	-0.999576712	0.411071234	-2.431638676	0.015030693	0.04329737
Anaerostignum	-1.87295674	0.772830247	-2.42350341	0.015371609	0.043628243
Streptococcus	0.702832601	0.29262941	2.401783881	0.016315344	0.045635673
Sutterella	0.752109526	0.314575833	2.390868739	0.016808561	0.046343604
[Eubacterium] siraeum group	-0.651563682	0.274729087	-2.371658892	0.017708431	0.048137002
Lachnospiraceae UCG-004	-0.54155441	0.229138068	-2.363441458	0.018106087	0.048534372
Acetanaerobacterium	-1.365481228	0.582805945	-2.342943204	0.019132295	0.050582644
Oxalobacter	-0.796669439	0.341049173	-2.335937169	0.019494516	0.050843806
GCA-900066575	1.286603452	0.558757249	2.302616124	0.021300451	0.054813161
Lachnospiraceae FCS020 group	1.11368571	0.488039073	2.281960136	0.022491694	0.057117065
[Ruminococcus] gnavus group	0.472384135	0.208283667	2.26798453	0.02333015	0.058476869
lfc.taxon	**lfc. SD-FST1.7**	**se. SD-FST1.7**	**W. SD-FST1.7**	**p_val. SD-FST1.7**	**q_val. SD-FST1.7**
Hungatella	-3.018728091	0.221365527	-13.63684818	2.42E-42	4.67E-40
Lachnospiraceae ND3007 group	-2.085033383	0.221262705	-9.423338572	4.37E-21	2.81E-19

Candidatus Soleaferrea	-3.100806417	0.328176584	-9.44859129	3.43E-21	2.81E-19
Limosilactobacillus	3.886180006	0.415564602	9.351566492	8.64E-21	4.17E-19
UBA1819	-1.220689308	0.130929846	-9.32323186	1.13E-20	4.36E-19
Sphingomonas	2.969073464	0.323395732	9.180929642	4.27E-20	1.37E-18
Anaerostipes	1.413795926	0.169401009	8.345853054	7.07E-17	1.95E-15
Proteus	2.646691191	0.33527885	7.89399986	2.93E-15	7.06E-14
Agathobacter	1.153675494	0.14876009	7.755275594	8.82E-15	1.89E-13
Microbacterium	3.433191496	0.46537929	7.377190115	1.62E-13	3.12E-12
Clostridium sensu stricto 13	-5.473438	0.752476118	-7.273902615	3.49E-13	6.13E-12
Odoribacter	-1.645121219	0.240399047	-6.84329344	7.74E-12	1.15E-10
Methanobrevibacter	1.244493412	0.187525706	6.63638835	3.21E-11	4.43E-10
Bilophila	-0.947526877	0.144508097	-6.556912026	5.49E-11	7.07E-10
[Clostridium] innocuum group	-1.126340176	0.17481852	-6.442911051	1.17E-10	1.38E-09
Dorea	0.637788635	0.099074729	6.437450177	1.21E-10	1.38E-09
Alistipes	-2.234996393	0.351650379	-6.355734344	2.07E-10	2.22E-09
Barnesiella	-2.781080279	0.465247279	-5.977638999	2.26E-09	2.30E-08
Butyricimonas	-2.557565329	0.431749603	-5.923723633	3.15E-09	2.89E-08
[Ruminococcus] gnavus group	1.318310258	0.228128446	5.778806975	7.52E-09	6.60E-08
Lachnospiraceae NK4A136 group	1.07624072	0.187100114	5.752218423	8.81E-09	7.39E-08
UC5-1-2E3	1.610926528	0.296120298	5.440108418	5.32E-08	4.11E-07
Intestinibacter	1.802507297	0.345137678	5.222574678	1.76E-07	1.31E-06
Subdoligranulum	0.727216992	0.141352318	5.144712193	2.68E-07	1.92E-06
Haemophilus	-2.194177804	0.438363144	-5.005388416	5.57E-07	3.84E-06
Fusicatenibacter	1.147827692	0.231377839	4.960836774	7.02E-07	4.67E-06
Adlercreutzia	0.762892488	0.15694071	4.861023566	1.17E-06	7.51E-06
Lachnoclostridium	-0.695824556	0.143880611	-4.836124547	1.32E-06	8.24E-06

UCG-007	1.637876175	0.340848355	4.80529289	1.55E-06	9.32E-06
Faecalitalea	-3.236442011	0.67566909	-4.789980866	1.67E-06	9.76E-06
Shuttleworthia	1.985653784	0.421238081	4.713851557	2.43E-06	1.38E-05
Megasphaera	1.111521964	0.236112959	4.707585594	2.51E-06	1.38E-05
Family XIII AD3011 group	1.134819229	0.255474933	4.441998337	8.91E-06	4.78E-05
Blautia	0.809352181	0.190880311	4.240103004	2.23E-05	0.000116539
Phocea	-2.961513753	0.705128016	-4.199966084	2.67E-05	0.000135585
CAG-352	-2.382267505	0.571489614	-4.168522834	3.07E-05	0.000151718
Lachnospiraceae AC2044 group	0.668313826	0.16240778	4.115035784	3.87E-05	0.000186785
Erysipelotrichaceae UCG-003	1.379574859	0.341044868	4.045141822	5.23E-05	0.000245172
UCG-002	1.004262866	0.248553492	4.040429514	5.34E-05	0.000245172
UCG-003	1.178715689	0.295494332	3.988962097	6.64E-05	0.000291092
Leuconostoc	0.777495701	0.194873956	3.989736326	6.61E-05	0.000291092
Paeniclostridium	-1.918530716	0.492601555	-3.894690744	9.83E-05	0.000421701
Enterococcus	1.637440797	0.422741405	3.87338637	0.000107333	0.000450334
Oscillibacter	1.126958279	0.296332089	3.803024782	0.00014294	0.000586967
Flavonifractor	-1.930491295	0.508536106	-3.796173509	0.000146947	0.000590848
Megamonas	1.328649586	0.379028258	3.505410373	0.000455904	0.001795703
HT002	2.064361713	0.597324906	3.456011449	0.000548232	0.002116176
[Eubacterium] brachy group	1.116749419	0.329677052	3.387404166	0.000705574	0.002670112
Streptococcus	0.594590059	0.177063951	3.358052588	0.000784937	0.002913323
Slackia	0.996838671	0.303787489	3.281368416	0.001033047	0.003761851
Monoglobus	0.827309787	0.254198316	3.254584057	0.001135585	0.003984873
Lactobacillus	2.101559371	0.679739072	3.091714832	0.001990039	0.006858528
[Eubacterium] eligens group	0.728975527	0.238592929	3.055310694	0.002248275	0.00761258
Fournierella	-1.41949992	0.476395396	-2.979667586	0.002885613	0.009602127

Negativibacillus	-2.544215006	0.857656506	-2.96647316	0.003012367	0.009854013
UCG-005	0.778553239	0.265621957	2.931057536	0.003378102	0.010866228
Paludicola	-1.39127939	0.480798461	-2.893685197	0.003807497	0.01204667
CAG-873	-1.867660331	0.656559614	-2.844616529	0.004446492	0.013841499
Frisingicoccus	0.609329013	0.215129313	2.832384882	0.00462022	0.013932851
Solobacterium	0.609329013	0.215129313	2.832384882	0.00462022	0.013932851
Lachnospiraceae UCG-001	1.607142786	0.571461447	2.812338077	0.004918278	0.014418902
Prevotella_9	2.172939733	0.794026554	2.736608395	0.006207614	0.017881634
DNF00809	1.071427133	0.392282379	2.731265004	0.006309172	0.017906914
CAG-56	1.230941213	0.467453799	2.633289568	0.008456222	0.02365291
[Eubacterium] fissicatena group	0.762993805	0.292377368	2.609619922	0.009064287	0.024991534
Clostridium sensu stricto 1	-1.845016496	0.716965337	-2.573369172	0.010071372	0.027377109
Prevotella_7	-1.823478885	0.712435207	-2.559501367	0.010482244	0.028098238
Marvinbryantia	1.919339278	0.763241989	2.514719193	0.011912722	0.03149528
Ligilactobacillus	1.338971525	0.54225348	2.469272349	0.013538813	0.03483988
Paraprevotella	1.496943071	0.605354157	2.472838507	0.01340447	0.03483988
Alloprevotella	-1.592922186	0.648921683	-2.454721776	0.014099366	0.03580497
Veillonella	-0.947804773	0.38947456	-2.433547326	0.014951681	0.037207207
Roseburia	0.899234033	0.369829292	2.431484069	0.01503711	0.037207207
Family XIII UCG-001	0.766224082	0.319417704	2.398815323	0.016448208	0.039442706
Peptococcus	0.848922237	0.354238055	2.396473853	0.016553674	0.039442706
[Eubacterium] ruminantium group	2.010900645	0.862407733	2.331728448	0.019714981	0.046402334
Dialister	0.350475379	0.150750797	2.324865846	0.020079135	0.046690036
Angelakisella	-1.042580274	0.456061769	-2.286050586	0.022251299	0.050523538
Anaerostignum	-1.781104846	0.782839281	-2.275185838	0.022894784	0.051380155
Pseudoflavonifractor	-1.765598174	0.787524771	-2.241958905	0.024964028	0.055379971

lfc.taxon	lfc.SD-R3	se.SD-R3	W. SD-R3	p_val. SD-R3	q_val. SD-R3
Bilophila	-1.52006212	0.08506623	-17.86916057	2.05E-71	3.96E-69
Candidatus Soleaferrea	-3.960566699	0.302851002	-13.07760802	4.42E-39	4.27E-37
Sphingomonas	3.744006781	0.354304242	10.56720845	4.23E-26	2.72E-24
Hungatella	-4.120736979	0.414573222	-9.939708508	2.80E-23	1.35E-21
Butyricimonas	-3.073811169	0.317511936	-9.680931075	3.63E-22	1.40E-20
Faecalitalea	-4.281550474	0.458462509	-9.338932608	9.73E-21	3.13E-19
Microbacterium	3.83362361	0.445905629	8.597387786	8.16E-18	2.25E-16
Clostridium sensu stricto 13	-6.042778867	0.70601406	-8.55900641	1.14E-17	2.75E-16
Lachnospiraceae ND3007 group	-2.218747622	0.264667839	-8.383140293	5.15E-17	1.11E-15
[Ruminococcus] gnavus group	1.16450087	0.143501868	8.114883002	4.86E-16	9.38E-15
Agathobacter	0.924430264	0.11425918	8.090643244	5.94E-16	1.04E-14
Barnesiella	-3.902995443	0.488261978	-7.993650164	1.31E-15	2.11E-14
Alistipes	-2.577917443	0.323434086	-7.970456909	1.58E-15	2.35E-14
Methanobrevibacter	1.21884679	0.173137967	7.039743007	1.93E-12	2.66E-11
Lachnoclostridium	-1.047565212	0.150977743	-6.938540667	3.96E-12	5.10E-11
Odoribacter	-1.739117824	0.252247651	-6.894485695	5.41E-12	6.52E-11
UBA1819	-1.542848714	0.230964447	-6.680026871	2.39E-11	2.56E-10
Dorea	0.422514262	0.063207913	6.684515312	2.32E-11	2.56E-10
Phocea	-3.843865991	0.589990085	-6.515136585	7.26E-11	7.01E-10
Flavonifractor	-1.448858769	0.227010344	-6.382346905	1.74E-10	1.60E-09
Subdoligranulum	0.391352664	0.070118134	5.581333091	2.39E-08	1.92E-07
Shuttleworthia	2.3949726	0.444714548	5.385415454	7.23E-08	5.58E-07
Haemophilus	-2.79034005	0.529827657	-5.266505082	1.39E-07	1.03E-06
Anaerostipes	1.303390118	0.253971559	5.132031804	2.87E-07	2.05E-06
Fusicatenibacter	1.100914708	0.215518064	5.10822474	3.25E-07	2.24E-06

[Clostridium] innocuum group	-1.693327153	0.337700108	-5.014292607	5.32E-07	3.54E-06
CAG-352	-2.311862243	0.516591424	-4.475223813	7.63E-06	4.91E-05
CAG-56	1.593009893	0.359391856	4.432515281	9.31E-06	5.80E-05
Veillonella	-1.761132994	0.405470181	-4.343434059	1.40E-05	8.46E-05
Lachnospiraceae NK4A136 group	0.949136154	0.222950814	4.257154918	2.07E-05	0.00012109
Eggerthella	-1.165783005	0.285518285	-4.083041495	4.45E-05	0.00025232
Acidaminococcus	-1.913933561	0.485134315	-3.945162196	7.97E-05	0.000439742
[Eubacterium] ruminantium group	2.938371145	0.797065852	3.686484798	0.000227373	0.001218972
UCG-002	0.945314812	0.263404273	3.588836284	0.000332157	0.001732604
Ochrobactrum	0.757334006	0.221287727	3.422394974	0.000620721	0.002994978
Colidextribacter	-0.868354257	0.253667707	-3.423195914	0.000618895	0.002994978
CAG-873	-2.176331572	0.63509029	-3.426806562	0.000610724	0.002994978
UCG-007	1.539237981	0.476264996	3.231893994	0.001229727	0.005650887
Marvinbryantia	1.995612979	0.622792467	3.204298518	0.00135392	0.005983471
UCG-003	1.015532436	0.317141807	3.202139904	0.001364107	0.005983471
Akkermansia	-0.285205903	0.0902354	-3.160687529	0.001573972	0.006750593
UCG-009	1.713134484	0.546584922	3.134251268	0.001722933	0.007228826
Negativibacillus	-2.538638577	0.825145611	-3.076594656	0.002093798	0.008444528
Parasutterella	-0.522599165	0.169913078	-3.075685348	0.002100193	0.008444528
Incertae Sedis	-0.603798865	0.200823776	-3.006610462	0.002641781	0.01040538
Romboutsia	-0.942308506	0.315398091	-2.987679802	0.002811039	0.01085061
Faecalibacterium	-0.44163143	0.148391351	-2.976126481	0.002919144	0.011046958
Megasphaera	0.706234933	0.237826977	2.969532476	0.002982533	0.011069785
Family XIII AD3011 group	0.938106828	0.325317022	2.883669661	0.003930708	0.014251848
Prevotella_7	-1.569983283	0.545295182	-2.879143877	0.003987564	0.014251848
Roseburia	1.023444224	0.357391818	2.863647609	0.004187936	0.014695849

Olsenella	-1.447469713	0.507577721	-2.851720342	0.004348334	0.014986222
Escherichia-Shigella	0.621539957	0.219355473	2.833482785	0.004604379	0.015590265
Monoglobus	0.646976009	0.229665032	2.817041862	0.004846821	0.015590607
Brevundimonas	1.382247035	0.489602355	2.823203405	0.00475464	0.015590607
Oxalobacter	-0.645469295	0.234378697	-2.753958884	0.005887916	0.018628982
Paraprevotella	1.614891202	0.592293946	2.726502968	0.006400938	0.0199255
Parabacteroides	-1.226729852	0.454860441	-2.696936776	0.006998056	0.021438488
Angelakisella	-1.245717045	0.47998265	-2.595337655	0.009449803	0.027633516
Caproiciproducens	0.561168155	0.215531725	2.603645261	0.009223817	0.027633516
Clostridium sensu stricto 1	-2.60801741	1.011698905	-2.577859278	0.009941447	0.028637304
Oscillibacter	0.876261955	0.343262859	2.552743273	0.010687824	0.03033456
Howardella	-0.424457107	0.16728505	-2.537328396	0.011170212	0.031244215
[Eubacterium] ventriosum group	1.044545222	0.412998686	2.529173231	0.011433158	0.031262746
Anaerostignum	-1.984241617	0.797012138	-2.489600249	0.012788685	0.03428078
[Eubacterium] fissicatena group	0.738628794	0.297880028	2.479618383	0.013152306	0.034772536
Paludicola	-1.474297288	0.598416269	-2.463665117	0.013752454	0.035867886
Lachnospiraceae UCG-003	0.780890613	0.318106526	2.454808533	0.014095964	0.036273615
Lactobacillus	1.65321008	0.67759535	2.439819102	0.014694618	0.037316596
Fournierella	-0.964250534	0.396119762	-2.434239908	0.0149231	0.037404654
Family XIII UCG-001	0.739334885	0.304935394	2.424562379	0.01532685	0.037924129
UC5-1-2E3	0.799084969	0.338589053	2.360043724	0.018272781	0.044641098
lfc.taxon	**lfc. SD-Rye106**	**se. SD-Rye106**	**W. SD-Rye106**	**p_val. SD-Rye106**	**q_val. SD-Rye106**
Hungatella	-3.064803951	0.154528151	-19.83330504	1.54E-87	2.96E-85
Candidatus Soleaferrea	-2.814485755	0.198005338	-14.21419133	7.48E-46	7.22E-44
Odoribacter	-1.499205054	0.170808112	-8.777130315	1.68E-18	1.08E-16
Agathobacter	1.299452144	0.14951323	8.691218429	3.59E-18	1.73E-16

Butyricimonas	-2.128218575	0.261981774	-8.12353677	4.53E-16	1.75E-14
Microbacterium	3.210799096	0.427919934	7.503270682	6.22E-14	1.72E-12
Haemophilus	-1.711531718	0.239623199	-7.142596064	9.16E-13	2.21E-11
Clostridium sensu stricto 13	-5.278770966	0.789161215	-6.689090729	2.25E-11	4.33E-10
Sphingomonas	3.061138877	0.471384659	6.493929781	8.36E-11	1.47E-09
UBA1819	-1.449332534	0.23828044	-6.082465405	1.18E-09	1.76E-08
Lachnospiraceae ND3007 group	-2.031674784	0.33493463	-6.065884502	1.31E-09	1.81E-08
Limosilactobacillus	2.308645968	0.38496949	5.996958272	2.01E-09	2.59E-08
Flavonifractor	-1.556747734	0.262873054	-5.922051381	3.18E-09	3.84E-08
Alistipes	-2.044420486	0.358606573	-5.701012306	1.19E-08	1.35E-07
Phocea	-3.299580547	0.606526381	-5.440127005	5.32E-08	5.71E-07
Faecalitalea	-2.989731107	0.563334686	-5.307202237	1.11E-07	1.13E-06
Anaerostipes	1.476348929	0.286438083	5.154164262	2.55E-07	2.46E-06
Shuttleworthia	2.142131079	0.424264507	5.049046164	4.44E-07	4.08E-06
CAG-352	-2.200141429	0.441471124	-4.983658744	6.24E-07	5.47E-06
Adlercreutzia	1.306111823	0.267745838	4.87817788	1.07E-06	8.98E-06
Methanobrevibacter	1.222752936	0.282640757	4.326173435	1.52E-05	0.00012201
HT002	2.346515836	0.550066845	4.265873967	1.99E-05	0.000153722
[Ruminococcus] gnavus group	1.040874349	0.248281158	4.192321147	2.76E-05	0.000204962
Lachnoclostridium	-0.873710307	0.211330138	-4.134338415	3.56E-05	0.000254459
[Eubacterium] ventriosum group	1.155398186	0.2845776	4.060046135	4.91E-05	0.000326523
Fournierella	-1.124238054	0.284823877	-3.947134157	7.91E-05	0.000497198
Citrobacter	1.036437603	0.262733984	3.944817446	7.99E-05	0.000497198
[Eubacterium] ruminantium group	2.38338897	0.609258584	3.911949759	9.16E-05	0.000552185
Enterococcus	1.776060816	0.460247756	3.858923356	0.000113888	0.00064648
[Clostridium] innocuum group	-0.95647956	0.247497597	-3.864601403	0.000111271	0.00064648

Paludicola	-1.806780478	0.477016864	-3.787665831	0.000152069	0.000838553
Roseburia	1.078078165	0.289200724	3.727785146	0.00019317	0.001007616
Marvinbryantia	2.237941216	0.611708266	3.658510668	0.000253685	0.001255416
Fusicatenibacter	1.016064509	0.277588474	3.660326716	0.000251894	0.001255416
Barnesiella	-2.587166467	0.711394117	-3.636755498	0.000276094	0.001332152
Blautia	0.958881	0.264968131	3.618854069	0.00029591	0.001392944
Veillonella	-1.361273534	0.381405043	-3.569102086	0.000358207	0.001646046
Erysipelotrichaceae UCG-003	1.171918426	0.332781313	3.521587241	0.000428971	0.001925384
Colidextribacter	-0.894752024	0.255526021	-3.501608246	0.000462459	0.002028514
Clostridium sensu stricto 1	-2.699429706	0.778252577	-3.468577922	0.000523221	0.002244036
Slackia	0.719867943	0.213344371	3.374206409	0.000740288	0.003039908
UCG-007	1.522232778	0.45071589	3.377366563	0.000731835	0.003039908
Stenotrophomonas	1.289282359	0.396385319	3.252598662	0.001143549	0.004598019
Monoglobus	0.731412588	0.226151542	3.234170248	0.001219967	0.004805176
Ochrobactrum	1.186546621	0.37270558	3.183603046	0.001454543	0.005614537
Bilophila	-1.243139509	0.397240103	-3.129441114	0.001751392	0.006627816
Negativibacillus	-2.585335189	0.829139458	-3.118094508	0.001820244	0.006755907
Family XIII AD3011 group	1.14372767	0.370653376	3.08570687	0.002030689	0.007257833
Megasphaera	0.839709802	0.279802703	3.001078237	0.002690254	0.009440347
GCA-900066575	1.602434929	0.53756093	2.980936373	0.002873685	0.00990395
[Eubacterium] brachy group	1.060824889	0.358467996	2.959329429	0.003083093	0.010439246
Eggerthella	-1.05444877	0.357278378	-2.951336644	0.003164019	0.010528545
Prevotella_7	-1.633791473	0.567652074	-2.878156438	0.004000067	0.012866883
Lactobacillus	1.965328593	0.715689053	2.746064908	0.006031483	0.019083217
Paeniclostridium	-2.500105828	0.91674319	-2.727160511	0.006388196	0.019885835
Lachnospiraceae NK4A136 group	0.844887594	0.315008345	2.682111781	0.007315901	0.022412205

Acidaminococcus	-1.156870755	0.444078488	-2.605104247	0.009184631	0.027697403
UCG-002	0.791390032	0.305988192	2.58634174	0.00970007	0.028801748
NK4A214 group	0.684973532	0.267762235	2.558140926	0.010523344	0.030772808
Oscillibacter	0.862655904	0.343710511	2.509832767	0.012078835	0.034282576
Angelakisella	-1.080855598	0.440050846	-2.456206161	0.014041258	0.038713754
Brevundimonas	0.76090028	0.31324793	2.429067225	0.015137725	0.041149028
lfc.taxon	**lfc.Inulin**	**se.Inulin**	**W. Inulin**	**p_val. Inulin**	**q_val. Inulin**
Agathobacter	1.41483318	0.146853209	9.634336142	5.73E-22	1.11E-19
Ochrobactrum	0.980700396	0.104822946	9.355779766	8.30E-21	8.01E-19
Rikenella	1.129462763	0.161720886	6.984025336	2.87E-12	1.85E-10
Alloscardovia	1.190236616	0.194467796	6.120481845	9.33E-10	4.50E-08
Sellimonas	1.071344968	0.176233521	6.079121389	1.21E-09	4.66E-08
Akkermansia	-0.787192693	0.148400177	-5.304526649	1.13E-07	3.63E-06
UC5-1-2E3	1.453055736	0.316298371	4.593939997	4.35E-06	0.000119923
Streptococcus	0.721020947	0.17089412	4.219109151	2.45E-05	0.000591713
[Ruminococcus] gnavus group	1.308290295	0.336400337	3.889087349	0.000100622	0.002157781
Holdemanella	1.820422689	0.495503152	3.673887205	0.000238888	0.004610543
Intestinibacter	2.145571005	0.593301499	3.616324935	0.000298815	0.005242849
Adlercreutzia	0.41794885	0.119958878	3.484101032	0.000493793	0.007426528
Bilophila	-0.998976002	0.287009954	-3.480631901	0.000500232	0.007426528
Coprococcus	0.893917202	0.268488212	3.329446738	0.000870187	0.01199615
Lachnospiraceae ND3007 group	0.654060889	0.198729565	3.291210788	0.000997571	0.01204987
Erysipelotrichaceae UCG-003	0.830998729	0.252520151	3.290821446	0.000998953	0.01204987
Anaerostipes	1.397725662	0.433136545	3.226986219	0.001251015	0.014202698
Peptococcus	1.048552535	0.349254274	3.002261137	0.002679822	0.028733646

Chapter 5

The Potential Prebiotic and Postbiotic Effects of Tarhana Produced from Different Flours on Human Gut Microbiome Using an *in vitro* Fermentation Model

Koç, F., Sabuncu M.,Yavuz G.G.,Duven G.,Abdo RMK., Bagci U., Sahan Y., Ozmen Togay S., Ross, R. P., & Stanton, C. Exploring Tarhanas Prebiotic and Postbiotic Potential Using Different Flours in an *in vitro* Fermentation Model. *Food and Function*, January 2025 (under revision).

5.1 Abstract

Tarhana, a traditional fermented food important to Turkish and Central Asian cuisines, is known for its unique composition of yoghurt, flour, vegetables, and herbs, and its potential health benefits through fermentation. This study evaluated the impact of tarhana on the human gut microbiome, focusing on prebiotic and postbiotic properties using an *in vitro* colonic fermentation model. To explore how variations in ingredients influence its functionality, we developed tarhana using four different flour types, including wheat, chickpea, einkorn, and purple potato and two fermentation methods, Baker's yeast (*Saccharomyces cerevisiae*) and sourdough fermentation. The aim of this study was to assess changes in gut microbial diversity, the growth of beneficial bacteria, as well as short-chain fatty acid (SCFA) and branched-chain fatty acid (BCFA) levels resulting from fermentation in the presence of flours associated with these variations.

Our findings demonstrate that both flour type and fermentation method significantly changed gut microbial composition. Sourdough-fermented tarhanas prepared with purple potato, chickpea and einkorn flour reduced *Veillonella* levels compared to the same flour fermented with baker's yeast. Moreover, purple potato sourdough fermented tarhana caused a reduction in *Escherichia-Shigella* levels compared to purple potato baker's yeast fermented faecal samples following *in vitro* colonic fermentation. Digested chickpea flour and purple potato flour tarhana soups caused increases in acetate and propionate levels in the faecal samples following *in vitro* colonic fermentation compared to baseline.

This study highlights that specific flour types paired with sourdough fermentation can enhance the functional properties of tarhana by promoting beneficial microbial shifts as well as acetate and propionate production. The results suggest that tarhana could be personalized as a functional food to support gut microbiota modulation and potentially alleviate symptoms in individuals with gut-related disorders. Future clinical trials are needed to validate these findings and further investigate the mechanistic roles of tarhana's bioactive compounds on gut health.

5.2 Introduction

Tarhana, a traditional fermented food, has been widely used in Turkish, Balkan, and Central Asian cuisines for centuries. Traditionally, tarhana is made by mixing together wheat flour, yoghurt, and vegetables such as peppers, onions, garlic, tomatoes, and a mixture of herbs (Degirmencioglu, 2005, Daglioglu, 2000). Tarhana undergoes a fermentation process that enhances its flavour as well as transforms its nutritional and bioactive properties (Temiz and Tarakci, 2017). This fermentation process increases the bioavailability of essential nutrients, including proteins, vitamins and minerals (O'Callaghan et al., 2019, Goncu and Celik, 2020). Traditionally prepared tarhana is dried and later rehydrated to make a nutrient-rich soup (Degirmencioglu, 2005). Given the rising interest in functional foods that promote gut health (Balasubramanian et al., 2024), tarhana's potential to modulate the human gut microbiome is of interest. The gut microbiome is a complex and dynamic ecosystem that plays a crucial role in digestion, immunity, and even neurological health (Koç et al., 2020, Ansari et al., 2020). The unique fermentation process used in tarhana results in the development of bioactive compounds that can support a healthy balance of gut bacteria, contributing to overall health and well-being. The fermentation increases the production of beneficial bioactive compounds, such as short-chain fatty acids (SCFAs), peptides, and various metabolites with antioxidant and antimicrobial properties (O'Callaghan et al., 2019). Fermentation is a critical step in the production of tarhana, as it not only preserves the food but also enhances its functional properties (Cankurtaran Kömürcü and Bilgiçli, 2022). Lactic acid fermentation, which is the traditional method used in tarhana production, relies on lactic acid bacteria (LAB), primarily from the

Lactobacillus genus (Temiz and Tarakci, 2017). This genus contains well-known probiotic species, which can confer health benefits by promoting a balanced gut microbiota, enhancing digestion, and boosting immune function (Bauerl et al., 2020, Abuqwider et al., 2022). LAB are particularly adept at converting sugars into lactic acid, lowering the pH of the gut environment, and inhibiting the growth of pathogens (Icer et al., 2023). Additionally, LAB fermentation produces postbiotics, such as SCFAs and bioactive peptides, which further promote gut health by reducing inflammation and supporting gut barrier function (Moradi et al., 2020). In contrast, yeast fermentation introduces a different microbial ecosystem. Yeast species, such as *Saccharomyces cerevisiae*, contribute to the fermentation process by producing ethanol and carbon dioxide (Maicas, 2020). While yeast fermentation has been less studied in relation to gut health, it may offer distinct benefits, such as promoting the growth of specific yeast species in the gut, which can contribute to the overall diversity of the gut microbiome (Siddik et al., 2022). Besides *Saccharomyces cerevisiae*, sourdough fermentation is another way to increase the health-promoting effects of cereal-based foods such as bread. Sourdough can be produced traditionally or industrially and is rich in bioactive peptides, amino acids and complex carbohydrates. Microbial metabolism can increase bioactive compounds in sourdough during fermentation (Gobbetti et al., 2014).

One key factor in determining tarhana's nutritional and functional properties is the type of flour used (Goncu and Celik, 2020). The flour component of tarhana serves not only as a source of carbohydrates and proteins but also as a prebiotic substrate for gut bacteria. Prebiotics are non-digestible compounds that promote the growth of beneficial gut bacteria, such as *Bifidobacterium* and *Lactobacillus* species (Gibson et

al., 2017). The different levels of fibre and resistant starch content in various flours can influence their prebiotic properties and by extension, their ability to support a healthy gut microbiome (Gondalia et al., 2022). Different flours used in tarhana production such as wheat, einkorn, emmer, and corn each contribute their own unique nutritional profiles (O'Callaghan et al., 2019, Daglioglu, 2000, Cankurtaran Kömürcü and Bilgiçli, 2022, Tarakçı et al., 2004). For example, einkorn flour is considered to be an ancient grain with high quality nutritional value with its high protein and antioxidant content (Hidalgo et al., 2006). It has been demonstrated that it stimulates the gut microbiota to produce SCFAs (Barone et al., 2018). Purple potato flour is rich in antimicrobial and antioxidant compounds such as phenolic acids, flavonoids and anthocyanins (Santiago et al., 2015, Gumul et al., 2017). Dietary purple potato has been shown to improve colitis symptoms in a mouse model as well as increase levels of *Lachnospiraceae-Ruminococcaceae*, major butyrate producer families in the gut microbiome (Zhu et al., 2024). Additionally, chickpea is one of the most abundant legumes in the world (Koul et al., 2022) and is rich in nutrients including carbohydrates, proteins, fibres, vitamins, minerals and bioactive compounds (Jukanti et al., 2012). Intake of cooked chickpea flour has been associated with enhanced colonic epithelial barrier and reduced disease symptoms such as metabolic syndrome (Monk et al., 2017, Han et al., 2021, Kang et al., 2023).

The composition of tarhana, however, can vary depending on the type of flour used and the fermentation process. These variables have the potential to significantly influence the nutritional profile and bioactivity of tarhana, and consequently, its impact on the gut microbiome. In this study, we aimed to evaluate the effects of tarhana formulations on the human gut microbiome by comparing four different flour types

including wheat, purple potato, chickpea, and einkorn and two fermentation methods: sourdough and Baker's yeast (*Saccharomyces cerevisiae*) fermentation. The primary objective was to investigate how these variations in ingredients and fermentation processes modulate the gut microbiome, specifically focusing on changes in microbial diversity, the growth of beneficial bacteria, and the production of postbiotic compounds.

5.3 Materials and Methods

5.3.1 Preparation of sourdough

Three hundred grams of chickpeas were soaked in warm water with the addition of 5 grams of salt for 12 hours and then ground in a food processor. Then, 50 g of wheat flour and 10 g of sugar were added and fermented at room temperature for 24 hours. After gas formation in the dough, more flour was added to continue the dough fermentation. The resulting sour chickpea starter was stored at 4^{o}C until used for tarhana production.

5.3.2 Production of tarhana

Tarhana was produced according to a previously described protocol (Degirmencioglu, 2005) with slight adjustments. For each tarhana prototype, different flours including wheat, einkorn, chickpea and purple potato flour were used. Briefly, flour (100%), yoghurt (40%), salt (1%), onion powder (0.05%), and garlic powder (0.05%) were mixed for 30 seconds. The dough was then split into two equal pieces for fermentation. Baker's yeast (2%) or chickpea sourdough (2%) were used for fermentation. A dough kneader was used to mix tarhana dough (Electrolux, Sweden). Then, tarhana doughs

were fermented at 30ºC for 96 hours. Tarhana dough was stirred twice a day using a wooden spoon. Then, the doughs were divided into small pieces and placed on drying cloths, then placed into a hot air drying unit (at 45°C, 7% relative humidity) for 24 hours. Afterwards, the dried tarhana pieces were ground and then sieved (1 mm) into powder form. The produced tarhana samples were stored in airtight glass jars under refrigerated conditions (4°C) until further analyses were performed. Tarhana samples are labelled as CFB (Chickpea Flour Baker's yeast fermented tarhana), CFS (Chickpea Flour Sourdough fermented tarhana), EFB (Einkorn Flour Baker's yeast fermented tarhana), EFS (Einkorn Flour Sourdough fermented tarhana), PPB (Purple Potato flour Baker's yeast fermented tarhana), PPS (Purple Potato flour Sourdough fermented tarhana), WFB (Wheat Flour Baker's yeast fermented tarhana), and WFS (Wheat Flour Sourdough fermented tarhana).

5.3.3 Preparation of tarhana soup prior to *in vitro* digestion

Tarhana samples were prepared in a soup format to represent human consumption. Soup names were abbreviated as CFB_S (Chickpea Flour Baker's yeast fermented Soup), CFS_S (Chickpea Flour Sourdough fermented Soup), EFB_S (Einkorn Flour Baker's yeast fermented Soup), EFS_S (Einkorn Flour Sourdough fermented Soup), PPB_S (Purple Potato flour Baker's yeast fermented Soup), PPS_S (Purple Potato flour Sourdough fermented Soup), WFB_S (Wheat Flour Baker's yeast fermented Soup), WFS_S (Wheat Flour Sourdough fermented Soup). Thirty grams of Tarhana granules were mixed with 2 grams of NaCl and 500 mL distilled water and the mixture was incubated at room temperature for 30 minutes. Following incubation, soups were

boiled for 12 minutes at 100^{o} C. Samples then were left to cool down to room temperature prior to preparation for the *in vitro* digestion.

5.3.4 *In vitro* digestion and *in vitro* colonic fermentation

In vitro digestion was performed according to INFOGEST 2.0 method which was described previously (Brodkorb et al., 2019). Ten mL of boiled tarhana soup was used as the starting material. Following simulated salivary, gastric and intestinal digestion, samples were used in triplicate for the *in vitro* colonic fermentation experiment. Cellulose was used as negative control and inulin was used as positive control for *in vitro* digestion and *in vitro* colonic fermentation in triplicate. Digested soups were abbreviated as CFB_SD (Chickpea Flour Baker's yeast fermented Soup Digested), CFS_SD (Chickpea Flour Sourdough fermented Soup Digested), EFB_SD (Einkorn Flour Baker's yeast fermented Soup Digested), EFS_SD (Einkorn Flour Sourdough fermented Soup Digested), PPB_SD (Purple Potato flour Baker's yeast fermented Soup Digested), PPS_SD (Purple Potato flour Sourdough fermented Soup Digested), WFB_SD (Wheat Flour Baker's yeast fermented Soup Digested), WFS_SD (Wheat Flour Sourdough fermented Soup Digested).

Subsequent to *in vitro* digestion, *in vitro* colonic fermentation was conducted based on a previously established protocol (Koc et al., 2022). Participant recruitment and stool sample collection were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (review reference numbers: ECM 4 (gg) 11/02/20 & ECM 3 (iiii) 22/02/2022). Faecal samples were obtained from nine healthy individuals and pooled in equal amounts for use in a MicroMatrix^{TM} *in vitro* benchtop fermentation system, adhering to a protocol outlined previously (O'Donnell et al., 2016). Faecal

fermentation was performed for 12 hours and samples were collected at baseline (T0) and at the end of fermentation (T12). All samples were collected in 2 mL sterile screw cap tubes. Subsequently, the tubes containing the samples were centrifuged at 16,000 g for 10 minutes at 4°C. Following centrifugation, the pellet was used for DNA extraction to analyse the microbial composition and the supernatant was processed for short-chain fatty acid (SCFA) and branched-chain fatty acid (BCFA) analysis.

5.3.5 DNA extraction and 16S rRNA gene library preparation

DNA extraction and subsequent 16S rRNA gene library preparation adhered to a previously established protocol (Koc et al., 2024a). Following library preparation, all subsequent libraries were precisely pooled to achieve a final concentration of 4 nanomolar (nM). Afterwards, sequencing was performed utilizing the NextSeq 2000 platform, leveraging the high-throughput capabilities of this technology. Specifically, sequencing utilized the P1 600-cycle (2x300 bp) reagent kit sourced from Illumina (San Diego, USA). The sequencing procedures were conducted at the Teagasc Moorepark Research Centre Next Generation DNA Sequencing Facility.

5.3.6 Short-chain fatty acid and branched chain fatty acid analyses

Samples were prepared for SCFA and BCFA analysis according to a previously described protocol (Koc et al., 2024b). Briefly, the standards and samples underwent analysis via gas chromatography - flame ionization detection (GC-FID) employing an Agilent 8860 GC system equipped with a DB-FFAP column (30 m length, 0.32 mm internal diameter, 0.25 µM film thickness; Agilent, California, United States) coupled with a flame ionization detector. Automated sample loading via splitless injection was

facilitated using an autosampler. Helium served as the carrier gas, maintaining a constant flow rate of 1.3 mL/min throughout the analysis. The initial oven temperature was set at 50°C and held for 0.5 minutes, followed by a programmed ramp to 140°C at a rate of 10°C/min, where it was maintained for an additional 0.5 minutes. Subsequently, the temperature was elevated to 240°C at a rate of 20°C/min and held for 5 minutes. The total run time encompassed 20 minutes. The detector and injection port temperatures were set at 300°C and 240°C, respectively. Peak integration was accomplished using Agilent OpenLab version 2.0 software (Agilent, California, United States). Concentrations of SCFAs and BCFAs were reported in milimolar (mM) units for each compound. To discriminate significant differences among experimental groups, Dunn's test was employed using false discovery rate (FDR) correction for multiple group comparisons. Statistical tests were performed graphics were generated on R version 4.3.2.

5.3.7 Bioinformatics and statistical analysis

The raw sequencing data underwent processing via the DADA2 pipeline (version 1.16) as outlined by Callahan et al. (Callahan et al., 2016a), incorporating specific parameters for optimal data refinement. These parameters included maxN=0, maxEE=c (2, 2), rm.phix=TRUE, truncQ=2, trimLeft=c (17, 21), truncLen=c (290,260), and maxN=0, ensuring stringent quality control and accurate sequence inference.

Next, the processed data were utilized to generate an amplicon sequence variant (ASV) table and perform taxonomic assignment, following a rigorously validated protocol detailed in the previously published paper (Koc et al., 2024b). Integration of sample

and group metadata, ASV table, and taxonomic information facilitated the creation of a phyloseq object, enabling comprehensive downstream analyses (Callahan et al., 2016b).

Alpha diversity metrics, including Observed, Chao1, Shannon, and Simpson indices were computed to characterize within-sample diversity. Beta diversity was assessed utilizing the Bray-Curtis distance matrix and visualized through principal coordinate analysis (PCoA) to elucidate between-sample dissimilarities.

Statistical analyses encompassed the Dunn test to detect significant differences in alpha diversity measurements and taxonomic compositions at both phylum and genus levels. FDR was used to correct p values. $q<0.05$ was considered statistically significant.

To assess differential microbial abundance across the experimental groups, we employed ANCOM-BC (Analysis of Compositions of Microbiomes with Bias Correction), which accounts for compositional data and applies bias correction to identify significant differences (Lin and Peddada, 2020). Wheat flour tarhana (either Baker's yeast fermented or sourdough fermented) was used as the reference group for pairwise comparisons. Groups were sorted in the phyloseq object, with "Wheat flour" as the reference. The following parameters were used for ANCOM-BC analysis: p_adj_method = "fdr", formula = "Group", group = "Group", neg_lb = TRUE, tol = 1e-5, max_iter = 100, conserve = TRUE, alpha = 0.05, global = TRUE.

Following ANCOM-BC analysis, differential gene expression analysis (DESeq2) was employed (Love et al., 2014) on the same flour using a different fermentation to see if the fermentation affects the gut microbiome at genus level. Benjamini Hochberg p

adjustment method was used and $q<0.05$ was considered if the fold change was significant.

5.4 Results

Our initial analysis started with extracting DNA from tarhana samples (fermented tarhana granules, tarhana soup and digested tarhana soup) to show their microbiome profiles at phylum and genus level. Then, we performed *in vitro* colonic fermentation using MicroMatrix^{TM} to see how digested tarhana soups produced with different flour and fermentation types modulate the human gut microbiome *in vitro*. Group names are listed in Table 5.1 with the abbreviations used throughout the manuscript.

Table 5.1 Abbreviations used to identify samples throughout the manuscript

Group Name	Abbreviation
Wheat flour baker's yeast tarhana	WFB
Chickpea flour baker's yeast tarhana	CFB
Einkorn flour baker's yeast tarhana	EFB
Purple potato flour baker's yeast tarhana	PPB
Wheat flour sourdough tarhana	WFS
Chickpea flour sourdough tarhana	CFS
Einkorn flour sourdough tarhana	EFS
Purple potato flour sourdough tarhana	PPS
Wheat flour baker's yeast tarhana soup	WFB_S
Chickpea flour baker's yeast tarhana soup	CFB_S
Einkorn flour baker's yeast tarhana soup	EFB_S
Purple potato flour baker's yeast tarhana soup	PPB_S
Wheat flour sourdough tarhana soup	WFS_S
Chickpea flour sourdough tarhana soup	CFS_S
Einkorn flour sourdough tarhana soup	EFS_S
Purple potato flour sourdough tarhana soup	PPS_S
Digested wheat flour baker's yeast digested tarhana soup	WFB_SD
Digested chickpea flour baker's yeast digested tarhana soup	CFB_SD
Digested einkorn flour baker's yeast digested tarhana soup	EFB_SD
Digested purple potato flour baker's yeast digested tarhana soup	PPB_SD
Digested wheat flour sourdough digested tarhana soup	WFS_SD
Digested chickpea flour sourdough digested tarhana soup	CFS_SD
Digested einkorn flour sourdough digested tarhana soup	EFS_SD
Digested purple potato flour sourdough digested tarhana soup	PPS_SD
Baseline (faecal samples collected from time point 0)	Baseline
Faecal samples fermented with digested cellulose	Cellulose_DF
Faecal samples fermented with digested inulin	Inulin_DF
Faecal samples fermented with digested wheat flour baker's yeast soup	WFB_SDF
Faecal samples fermented with digested purple potato baker's yeast soup	PPB_SDF
Faecal samples fermented with digested einkorn flour baker's yeast soup	EFB_SDF
Faecal samples fermented with digested chickpea flour baker's yeast soup	CFB_SDF
Faecal samples fermented with digested wheat flour sourdough soup	WFS_SDF
Faecal samples fermented with digested purple potato sourdough soup	PPS_SDF
Faecal samples fermented with digested einkorn flour sourdough soup	EFS_SDF
Faecal samples fermented with digested chickpea flour sourdough soup	CFS_SDF

5.4.1 Tarhana samples microbiome profiles

DNA was extracted from tarhana (granules), tarhana soup and *in vitro* digested tarhana soup. Relative abundances were calculated at phylum (Figure 5.1) and genus level (Figure 5.2).

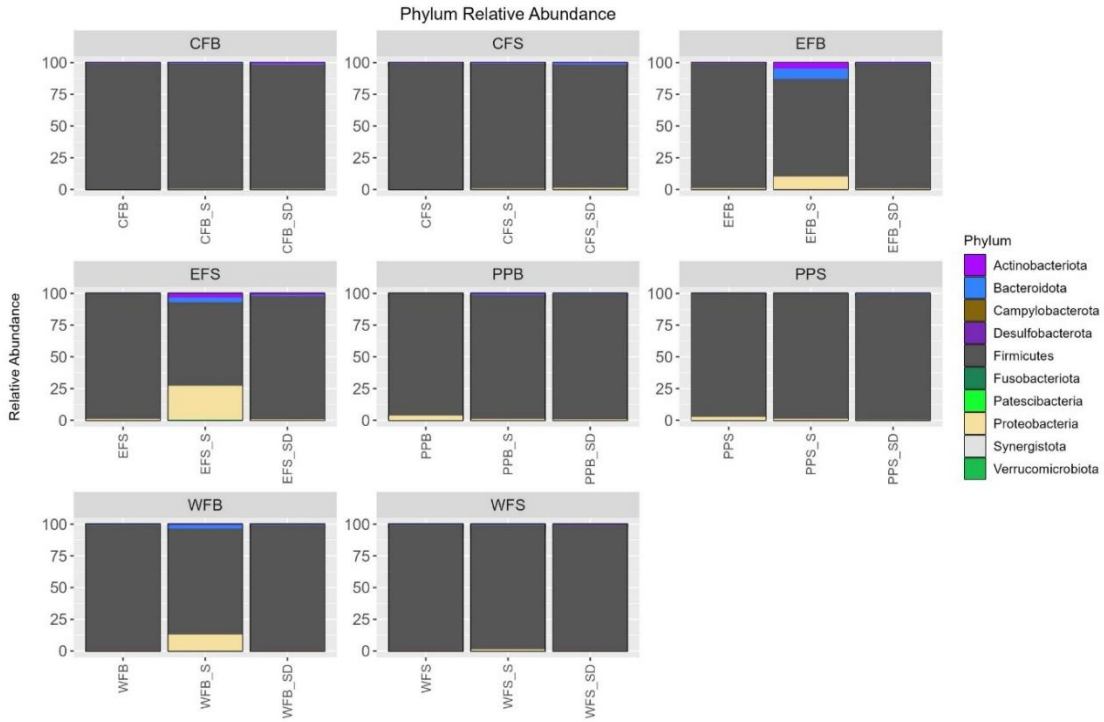


Figure 5.1 Phylum relative abundance for each tarhana sample. **CFB** (chickpea flour baker's yeast fermented tarhana), **CFB_S** (chickpea flour baker's yeast fermented soup), **CFB_SD** (chickpea flour baker's yeast fermented tarhana soup digested), **CFS** (chickpea flour sourdough fermented tarhana), **CFS_S** (chickpea flour sourdough fermented soup), **CFS_SD** (chickpea flour sourdough fermented tarhana soup digested), **EFB** (einkorn flour baker's yeast fermented tarhana), **EFB_S** (einkorn flour baker's yeast fermented soup), **EFB_SD** (einkorn flour baker's yeast fermented tarhana soup digested), **EFS** (einkorn flour sourdough fermented tarhana), **EFS_S** (einkorn flour sourdough fermented soup), **EFS_SD** (einkorn flour sourdough fermented tarhana soup digested), **PPB** (purple potato flour baker's yeast fermented tarhana), **PPB_S** (purple potato flour baker's yeast fermented soup), **PPB_SD** (purple

potato flour baker's yeast fermented tarhana soup digested), **PPS** (purple potato flour sourdough fermented tarhana), **PPS_S** (purple potato flour sourdough fermented soup), **PPS_SD** (purple potato flour sourdough fermented tarhana soup digested), **WFB** (wheat flour baker's yeast fermented tarhana), **WFB_S** (wheat flour baker's yeast fermented soup), **WFB_SD** (wheat flour baker's yeast fermented tarhana soup digested), **WFS** (wheat flour sourdough fermented tarhana), **WFS_S** (wheat flour sourdough fermented soup), **WFS_SD** (wheat flour sourdough fermented tarhana soup digested).

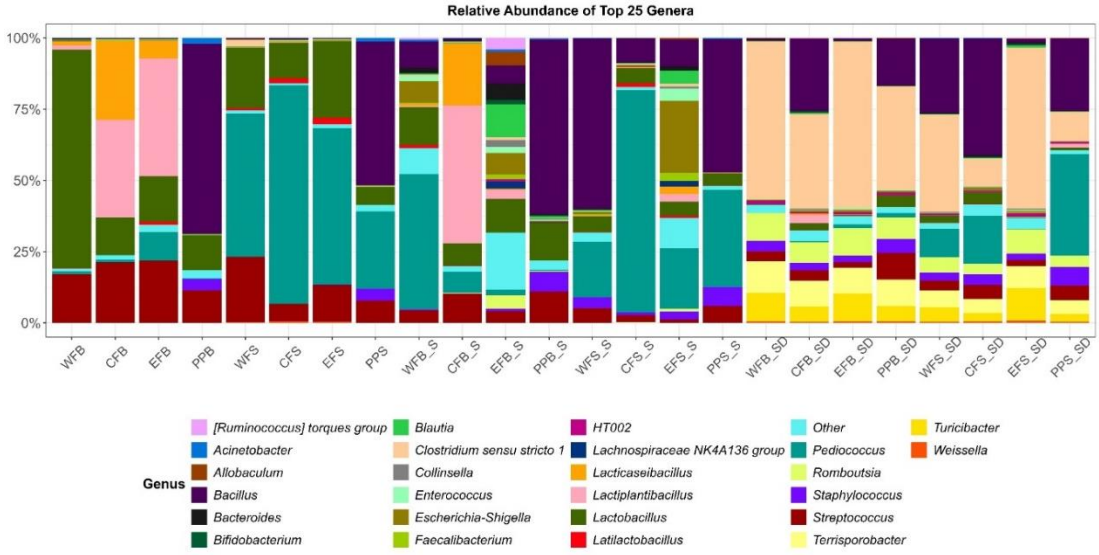


Figure 5.2 Top 25 genera for each tarhana, tarhana soup and digested tarhana soup **WFB** (wheat flour baker's yeast fermented tarhana), **CFB** (chickpea flour baker's yeast fermented tarhana), EFB (einkorn flour baker's yeast fermented tarhana), **PPB** (purple potato flour baker's yeast fermented tarhana), **WFS** (wheat flour sourdough fermented tarhana), **CFS** (chickpea flour sourdough fermented tarhana), **EFS** (einkorn flour sourdough fermented tarhana), **PPS** (purple potato flour sourdough fermented tarhana), **WFB_S** (wheat flour baker's yeast fermented soup), **CFB_S** (chickpea flour baker's yeast fermented soup), **EFB_S** (einkorn flour baker's yeast fermented soup), **PPB_S** (purple potato flour baker's yeast fermented soup), **WFS_S** (wheat flour sourdough fermented soup), **CFS_S** (chickpea flour sourdough fermented soup), **EFS_S** (einkorn flour sourdough fermented soup), **PPS_S** (purple potato flour sourdough fermented soup), **WFB_SD** (wheat flour baker's yeast fermented tarhana

soup digested), **CFB_SD** (chickpea flour baker's yeast fermented tarhana soup digested), **EFB_SD** (einkorn flour baker's yeast fermented tarhana soup digested), **PPB_SD** (purple potato flour baker's yeast fermented tarhana soup digested), **WFS_SD** (wheat flour sourdough fermented tarhana soup digested), **CFS_SD** (chickpea flour sourdough fermented tarhana soup digested), **EFS_SD** (einkorn flour sourdough fermented tarhana soup digested), **PPS_SD** (purple potato flour sourdough fermented tarhana soup digested).

The top 25 genera were created for each sample to determine their microbial composition before using them for the *in vitro* colonic fermentation experiment. Following *in vitro* digestion of all tarhana soups, the relative abundances of *Terrisporobacter*, *Clostridium sensu stricto 1* and *Turicibacter* were found to be higher in all digested samples, whereas the relative abundances of *Lactobacillus* and *Streptococcus* were lower than tarhana granules. Sourdough fermented tarhana granules including wheat flour sourdough fermented tarhana (WFS), chickpea flour sourdough fermented tarhana (CFS), einkorn flour sourdough fermented tarhana (EFS), and purple potato sourdough fermented tarhana (PPS) have higher levels of *Pediococcus* than their baker's yeast fermented counterparts.

5.4.2 Microbiota analysis of faecal samples following *in vitro* colonic fermentation of digested tarhana soup

Eight tarhana soups, as well as negative (cellulose) and positive (inulin) controls, were subjected to *in vitro* oral, gastric and intestinal digestion, then the digested samples were subjected to *in vitro* colonic fermentation using the MicroMatrix^{TM} for 12 hours. Faecal samples were collected from each MicroMatrix^{TM} cassette well at the baseline

(T0) and the end of fermentation (T12) to perform 16S rRNA gene sequencing analysis, SCFA and BCFA analyses.

Following initial filtering steps to remove less than 5000 reads, a phyloseq object was generated from the taxonomic table, ASV table and metadata to perform downstream analyses. Alpha diversity was calculated at the baseline and following *in vitro* colonic fermentation of each digested tarhana soup to see their diversity changes. Observed, Chao1, Shannon and Simpson indices (Figure 5.3) revealed that the diversity of faecal samples significantly reduced following *in vitro* colonic fermentation of digested cellulose and inulin compared to baseline faecal samples. Shannon and Simpson indices also showed lower diversity in faecal samples fermented with digested chickpea flour sourdough fermented tarhana soup (CFS_SDF) compared to baseline. Similarly, Chao1 index showed a significant reduction in faecal samples fermented with digested chickpea flour baker's yeast fermented tarhana soup (CFB_SDF) compared to baseline.

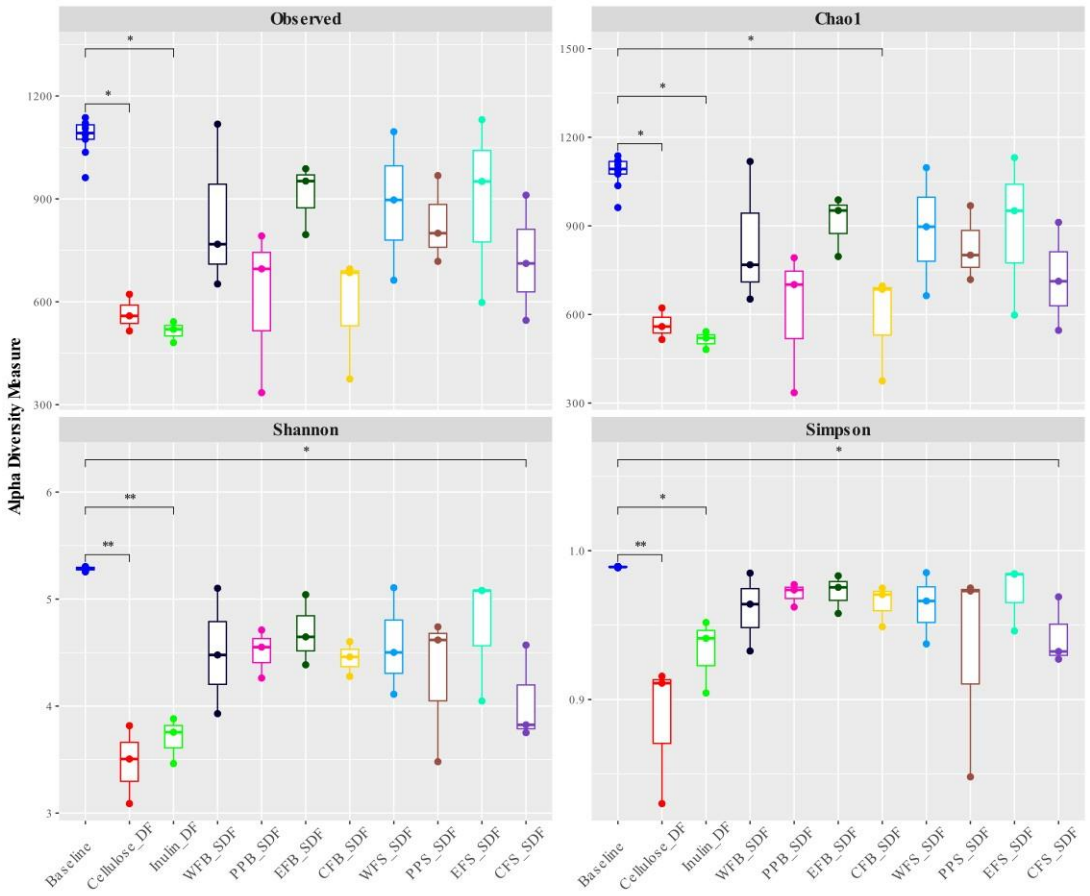


Figure 5.3 Alpha diversity measurement using four indices including Observed, Chao1, Shannon, and Simpson at the baseline and end of *in vitro* fermentation. Faecal samples fermented with different substrates and labelled with the substrate name; **Baseline** (Faecal samples collected from time point 0 without any substrate), **Cellulose_DF** (digested cellulose), **Inulin_DF** (digested inulin), **WFB_SDF** (digested wheat flour baker's yeast soup), **PPB_SDF** (digested purple potato baker's yeast soup), **EFB_SDF** (digested einkorn flour baker's yeast soup), **CFB_SDF** (digested chickpea flour baker's yeast soup), **WFS_SDF** (digested wheat flour sourdough soup), **PPS_SDF** (digested purple potato flour sourdough soup), **EFS_SDF** (digested einkorn flour sourdough soup), **CFS_SDF** (digested chickpea flour sourdough soup). The Dunn test was performed to determine significant differences. False discovery rate (FDR) was used as p adjustment method (q). * q<0.05, ** q<0.01, *** q<0.001.

Beta diversity analysis did not reveal any significant separation within the groups (Figure 5.4 and Supplementary Table 5.1).

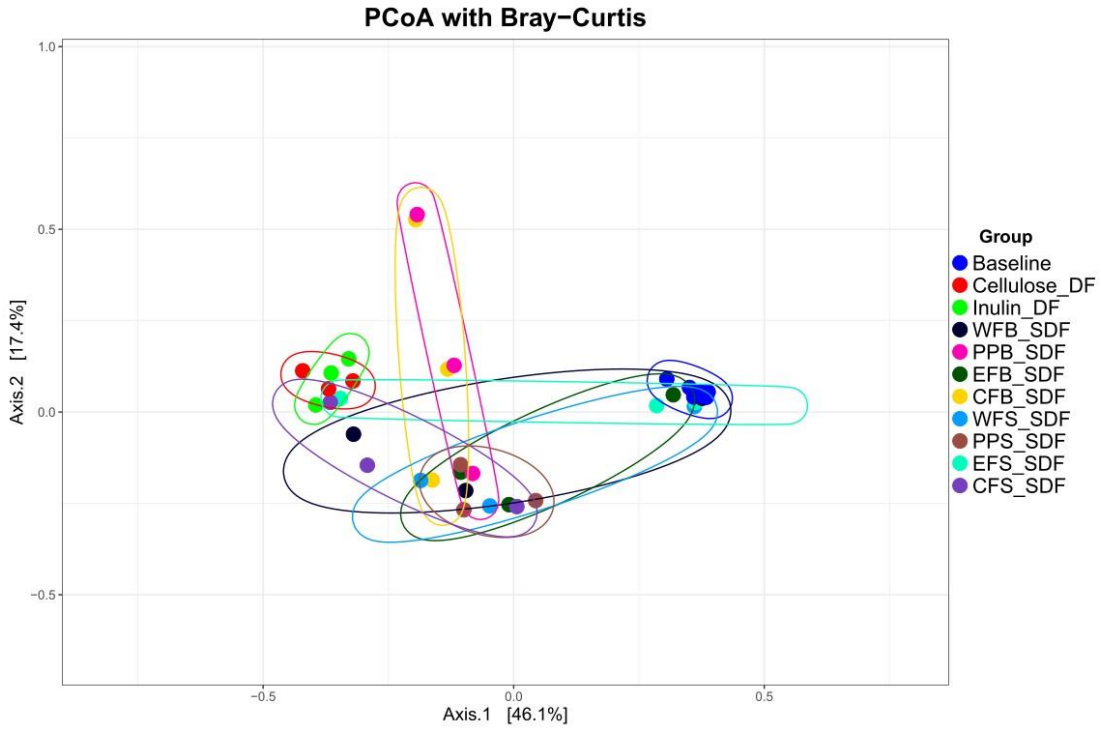


Figure 5.4 Beta diversity analysis was performed using the Bray-Curtis distance matrix, and Principal Coordinate Analysis (PCoA) was generated to assess significant separation between groups. Faecal samples were fermented with different substrates and labelled with the substrate name: **Baseline** (faecal samples collected from time point 0 without any substrate), **Cellulose_DF** (digested cellulose), **Inulin_DF** (digested inulin), **WFB_SDF** (digested wheat flour baker's yeast soup), **PPB_SDF** (digested purple potato baker's yeast soup), **EFB_SDF** (digested einkorn flour baker's yeast soup), **CFB_SDF** (digested chickpea flour baker's yeast soup), **WFS_SDF** (digested wheat flour sourdough soup), **PPS_SDF** (digested purple potato sourdough soup), **EFS_SDF** (digested einkorn flour sourdough soup), and **CFS_SDF** (digested chickpea flour sourdough soup).

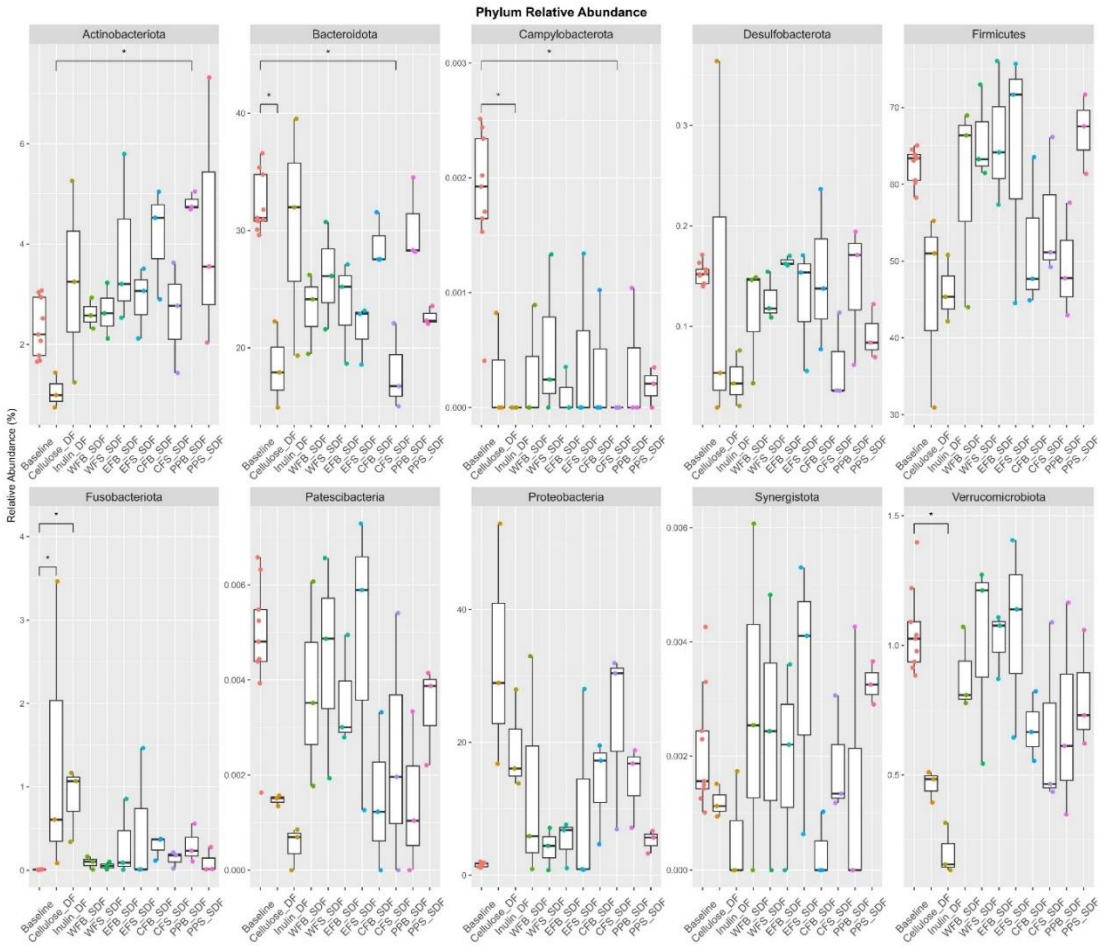


Figure 5.5 Box plots showing the relative abundance of each phylum. Data points represent faecal samples collected before (**Baseline**: faecal samples collected from time point 0 without substrate) and after *in vitro* colonic fermentation with the following substrates: **Cellulose_DF** (digested cellulose), **Inulin_DF** (digested inulin), **WFB_SDF** (digested wheat flour baker's yeast soup), **WFS_SDF** (digested wheat flour sourdough soup), **EFB_SDF** (digested einkorn flour baker's yeast soup), **EFS_SDF** (digested einkorn flour sourdough soup), **CFB_SDF** (digested chickpea flour baker's yeast soup), **CFS_SDF** (digested chickpea flour sourdough soup), **PPB_SDF** (digested purple potato baker's yeast soup), and **PPS_SDF** (digested purple potato sourdough soup). Statistical significance was assessed using the Dunn test with FDR correction ($q < 0.05$).

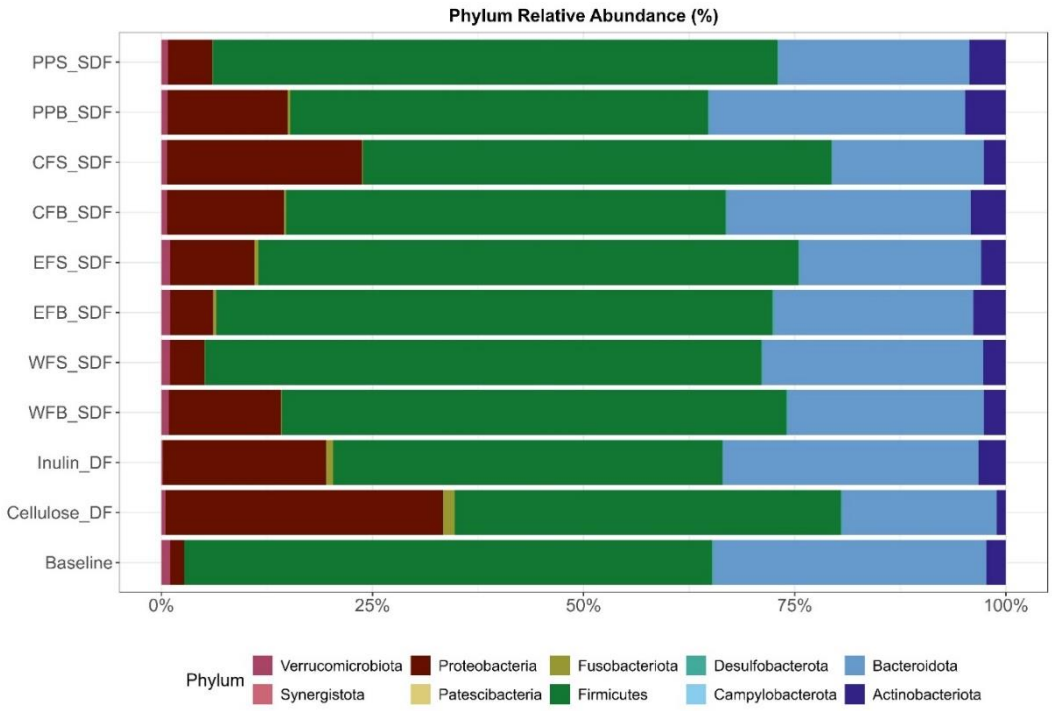


Figure 5.6 Bar plot illustrating the relative abundance of phyla at baseline and after *in vitro* colonic fermentation with different treatments. The **Y-axis** lists the treatments: **Baseline** (faecal samples collected from time point 0 without substrate), **Cellulose_DF** (digested cellulose), **Inulin_DF** (digested inulin), **WFB_SDF** (digested wheat flour baker's yeast soup), **WFS_SDF** (digested wheat flour sourdough soup), **EFB_SDF** (digested einkorn flour baker's yeast soup), **EFS_SDF** (digested einkorn flour sourdough soup), **CFB_SDF** (digested chickpea flour baker's yeast soup), **CFS_SDF** (digested chickpea flour sourdough soup), **PPB_SDF** (digested purple potato baker's yeast soup), and **PPS_SDF** (digested purple potato sourdough soup). The **X-axis** indicates the relative abundance of each phylum as a percentage (%).

A bar plot was generated to illustrate the relative abundances of phyla following *in vitro* colonic fermentation of different digested soup formulations (Figure 5.6). Firmicutes emerged as the dominant phylum in all faecal samples from baseline and following *in vitro* colonic fermentation. Dunn's test was employed with the FDR to determine if the difference was statistically significant. The relative abundance of Actinobacteriota (Figure 5.5) was greater in PPB_SDF samples compared to those

treated with digested cellulose (FDR=0.036). Bacteroidota levels were found to be reduced in the CFS_SDF samples and digested cellulose samples compared to baseline (FDR=0.015 and FDR=0.015). Likewise, Campylobacterota (Figure 5.5) relative abundances were also found to be low after *in vitro* colonic fermentation in the faecal samples treated with digested inulin (FDR=0.02) and CFS_SDF samples (FDR=0.02). Relative abundance of Verrucomicrobiota was found to be reduced in faecal samples fermented with digested inulin (FDR=0.04) whereas Fusobacteriota levels were higher in samples fermented with digested inulin (FDR=0.015) and cellulose (FDR=0.04) compared to baseline.

5.4.2.1 The effect of different flour types fermented with baker's yeast on human gut microbiota composition

At the genus level, the relative abundances of the genera were calculated to see differences within the digested tarhana soup fermented with baker's yeast. Figure 5.7 summarizes the top 25 genera at baseline and in the presence of different substrates. Dunn's test was performed on all samples at the genus level to determine if any of the differences were significant or not. Digested cellulose fermented faecal samples had lower relative abundance of *Erysipelatoclostridium* compared to PPB_SDF and EFB_SDF samples significantly (q=0.017 and q=0.021, Supplementary Table 5.2). The relative abundance of *Holdemania* was found to be higher in EFB_SDF samples compared to cellulose_DF samples (q=0.029), while it was significantly lower than inulin_DF (q=0.029, Supplementary Table 5.2). *Megamonas* was found to be reduced in inulin_DF compared to CFB_SDF samples (q=0.032) and PPB_SDF samples (q=0.032).

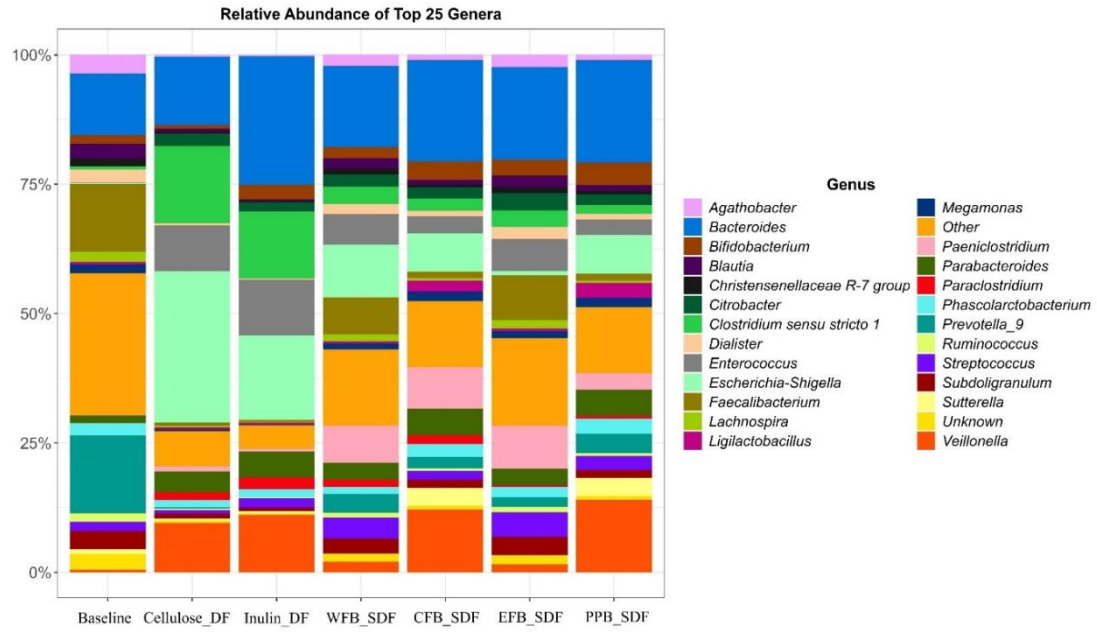


Figure 5.7 Bar plot showing the relative abundance of the top 25 genera in faecal samples fermented with tarhana prepared with different flour and baker's yeast as well as **Baseline**, negative (cellulose) and positive (inulin) control. Treatments include **Cellulose_DF** (digested cellulose), **Inulin_DF** (digested inulin), **WFB_SDF** (digested wheat flour baker's yeast soup), **CFB_SDF** (digested chickpea flour baker's yeast soup), **EFB_SDF** (digested einkorn flour baker's yeast soup), and **PPB_SDF** (digested purple potato baker's yeast soup).

The relative abundance of *Bifidobacterium* was found to be increased in PPB_SDF samples compared to cellulose_DF samples (q=0.035). Following *in vitro* colonic fermentation, *Haemophilus* levels were found to be reduced in EFB_SDF and WFB_SDF samples compared to cellulose_DF samples (q=0.035 and q=0.041). The relative abundances of *Streptococcus* were lower in cellulose_DF samples compared to EFB_SDF and WFB_SDF samples (q=0.042 and q=0.049). *Parasutterella* relative abundance was higher in PPB_SDF samples compared to cellulose_DF samples (q=0.0448) and inulin_DF samples (q=0.0448). At the end of colonic fermentation, the

relative abundance of *Akkermansia* was significantly greater in the EFB_SDF samples compared to inulin_DF samples (q=0.049).

5.4.2.2 Effects of different flour types using sourdough fermentation on human gut microbiota composition

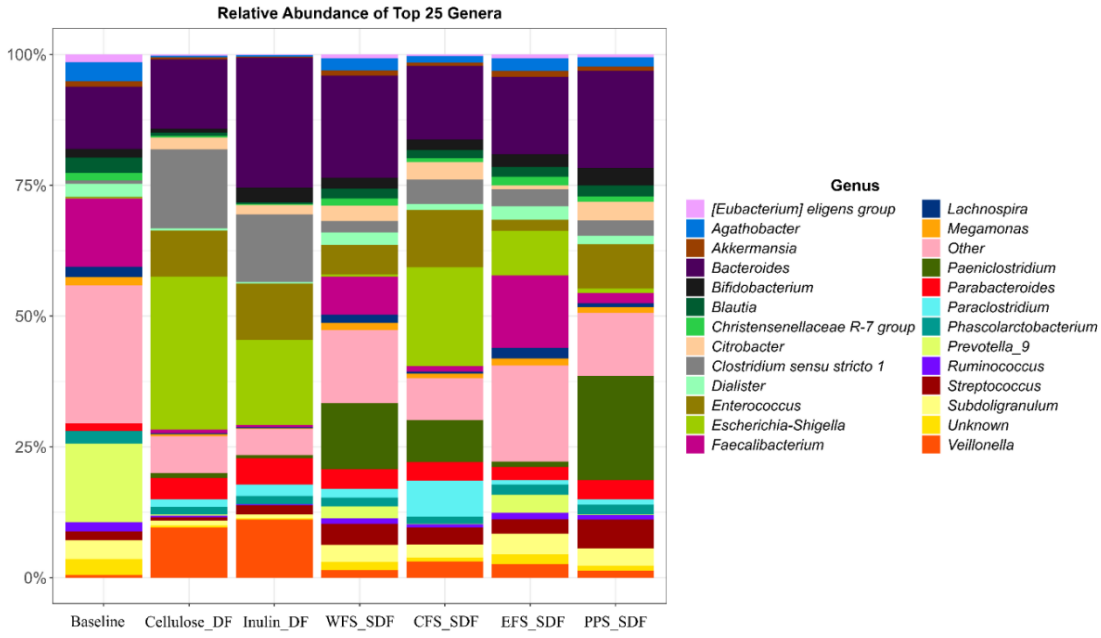


Figure 5.8 Bar plot depicting the relative abundance of the top 25 genera in faecal samples fermented with different digested substrates. Treatments include **Cellulose_DF** (digested cellulose), **Inulin_DF** (digested inulin), **WFS_SDF** (digested wheat flour sourdough), **CFS_SDF** (digested chickpea flour sourdough), **EFS_SDF** (digested einkorn flour sourdough), and **PPS_SDF** (digested purple potato flour sourdough).

Sourdough fermented tarhana soups using four different flours were further evaluated to see how sourdough fermentation affects gut microbiome at genus levels. First, we calculated the top 25 genera, presented in Figure 5.8, from faecal samples fermented with digested tarhana soups. Then, we performed Dunn's test to see if the differences at genus level were statistically significant or not. The significant results of Dunn's

test have been presented in Supplementary Table 5.3. The relative abundance of *Haemophilus* reduced in CFS_SDF, PPS_SDF and EFS_SDF samples compared to cellulose_DF samples following the colonic fermentation (q=0.014, q=0.019, and q=0.02). Similarly, inulin_DF samples had higher relative abundance of *Haemophilus* compared to CFS_SDF, PPS_SDF and EFS_SDF samples (q=0.019, q=0.03, and q=0.04).

The relative abundances of *Streptococcus* were greater in CFS_SDF faecal samples, PPS_SDF faecal samples, WFS_SDF faecal samples and EFS_SDF faecal samples compared to cellulose_DF faecal samples (q=0.019, q=0.019, q=0.019 and q=0.049). Similarly, *Erysipelatoclostridium* levels were found to be lower in faecal samples fermented with digested cellulose compared to CFS_SDF, PPS_SDF, EFS_SDF, and WFS_SDF samples (q=0.014, q=0.014, q=0.035 and q=0.035). Relative abundance of *Terrisporobacter* was increased in PPS_SDF and WFS_SDF samples compared to cellulose_DF (q=0.025 and q=0.039) and inulin_DF (q=0.025 and q=0.039) samples. Likewise, the relative abundance of *Turicibacter* was greater in PPS_SDF and CFS_SDF samples compared to cellulose_DF (q=0.025 and q=0.038) and inulin_DF (q=0.025 and q=0.045) faecal samples. Following *in vitro* fermentation, EFS_SDF faecal samples had higher relative abundance of *Dialister* compared to inulin_DF samples (q=0.044). *Lachnospiraceae NK4A136 group* was also greater in faecal samples of EFS_SDF compared to inulin_DF samples (q=0.032). Similarly, PPS_SDF and WFS_SDF samples had higher levels of *UBA1819* compared to inulin_DF samples (q=0.035 and q=0.049). *Lachnospiraceae UCG-004* and *UCG_003* were also lower in inulin_DF samples compared to EFS_SDF samples (q=0.039 and q=0.044). Whereas the relative abundance of *Akkermansia* was greater in WFS_SDF and

EFS_SDF samples compared to inulin_DF, however, after p-value adjustment, the differences were not significant (q=0.052).

5.4.2.3 The effects of tarhana produced with different flour types on human gut microbiota compared to wheat flour using baker's yeast fermentation

To evaluate the impact of tarhana soups made with different flour types on the gut microbiome, we applied the ANCOM-BC test to faecal samples fermented with digested baker's yeast-based tarhana soups (Supplementary Table 5.5). Faecal samples fermented with digested wheat flour with baker's yeast tarhana soup (WFB_SDF) served as the reference group. Faecal samples fermented with digested einkorn flour baker's yeast tarhana soup (EFB_SDF) showed a significant increase in log-fold change of *Candidatus Soleaferrea* (q=0.00069), *TM7x* (q=0.00139), *Caproiciproducens* (q=0.00511), and *Pediococcus* (q=0.04120). Similarly, chickpea flour baker's yeast tarhana samples (CFB_SDF) showed significantly higher log-fold changes in genera such as *Megamonas* (q=0.01315), *Gordonibacter* (q=0.01315), *Phascolarctobacterium* (q=0.02179), *HT002* (q=0.04361), *Leuconostoc* (q=0.04361), *Coprobacter* (q=0.04361). *Senegalimassilia* was 2.3-logs lower (q=2.91E-13) in faecal samples fermented with digested purple potato flour baker's yeast tarhana soup (PPB_SDF), and *Rothia* (q=1.87E-08), *Parasutterella* (q=1.69E-05), *Acinetobacter (q*=3.50E-05), *Phascolarctobacterium* (q=4.55E-05*), Candidatus Soleaferrea* (q=6.40E-05), *Gordonibacter* (q=0.000638571), *Bifidobacterium* (q=0.001791646), *Megamonas* (q=0.002211872) were greater significantly.

5.4.2.4 The effects of different flour types on human gut microbiota compared to wheat flour using sourdough fermentation

In this section, we used the ANCOM-BC test to assess the differential abundance of bacterial genera across faecal samples treated with digested sourdough-fermented tarhana soups (Supplementary Table 5.6). Faecal samples fermented with digested wheat flour with sourdough tarhana (WFS_SDF) were used as the reference group. Significant differences were observed in the log fold changes of several key bacterial genera. *Catenibacillus* increased by 2.9 log in faecal samples fermented with digested einkorn flour sourdough tarhana soup (EFS_SDF) (q=1.71E-10). Similarly, faecal samples fermented with digested chickpea flour sourdough soup (CFS_SDF) displayed positive log-fold changes of *Catenibacillus* (q=4.39E-13), *Olsenella* (q=0.00255199), *Senegalimassilia* (q=0.00255199), *Hungatella* (q=0.004987057), *Enterorhabdus* (q=0.013087146), *Clostridium sensu stricto 13* (q=0.018418127), *Adlercreutzia* (q=0.041262572), *Granulicatella* (q=0.045713277) and negative fold changes of *Eubacterium eligens group* (q=2.09E-14), *Alistipes* (q=4.39E-13), *Paraprevotella* (q=8.54E-05), *Acidaminococcus* (q=0.000228032), *Desulfovibrio* (q=0.00255199), *Fusicatenibacter* (q=0.005215846), *UCG-003* (q=0.011785648), *Solobacterium* (q=0.013087146), *Fournierella* (q=0.013087146), *Merdibacter* (q=0.015819957), *Eubacterium xylanophilum group* (q=0.015819957), *Eubacterium ventriosum group* (q=0.016655534), *Coprococcus* (q=0.018418127), *CAG-56* (q=0.018648596), *Oscillospira* (q=0.022175381), *Oscillibacter* (q=0.022175381), *CAG-352* (q=0.023436436), *Dialister* (q=0.029543846), *Lachnospiraceae NK4A136 group* (q=0.038106552), *Oxalobacter* (q=0.040640716), *NK4A214 group* (q=0.040640716), *Sellimonas* (q=0.040640716), *DTU089* (q=0.040640716),

Agathobacter (q=0.040640716), *Roseburia* (q=0.041262572) and *Colidextribacter* (q=0.047176628). Lastly, faecal samples treated with purple potato flour sourdough fermented tarhana soup (PPS_SDF) resulted in increased log-fold change of *Catenibacillus* (q=2.25E-09) and *Olsenella* (q=3.66E-09), *CHKCI002* (q=0.00453109), *Mogibacterium* (q=0.005545546), *Collinsella* (q=0.034868758), *Libanicoccus* (q=0.034868758) and negative fold changes of *Solobacterium* (q=6.46E-05), *Eubacterium eligens group* (q=0.003474266), *Prevotella_7* (q=0.010502181), *Acetanaerobacterium* (q=0.010502181) following *in vitro* colonic fermentation.

5.4.2.5 Comparison of fermentation types for each flour

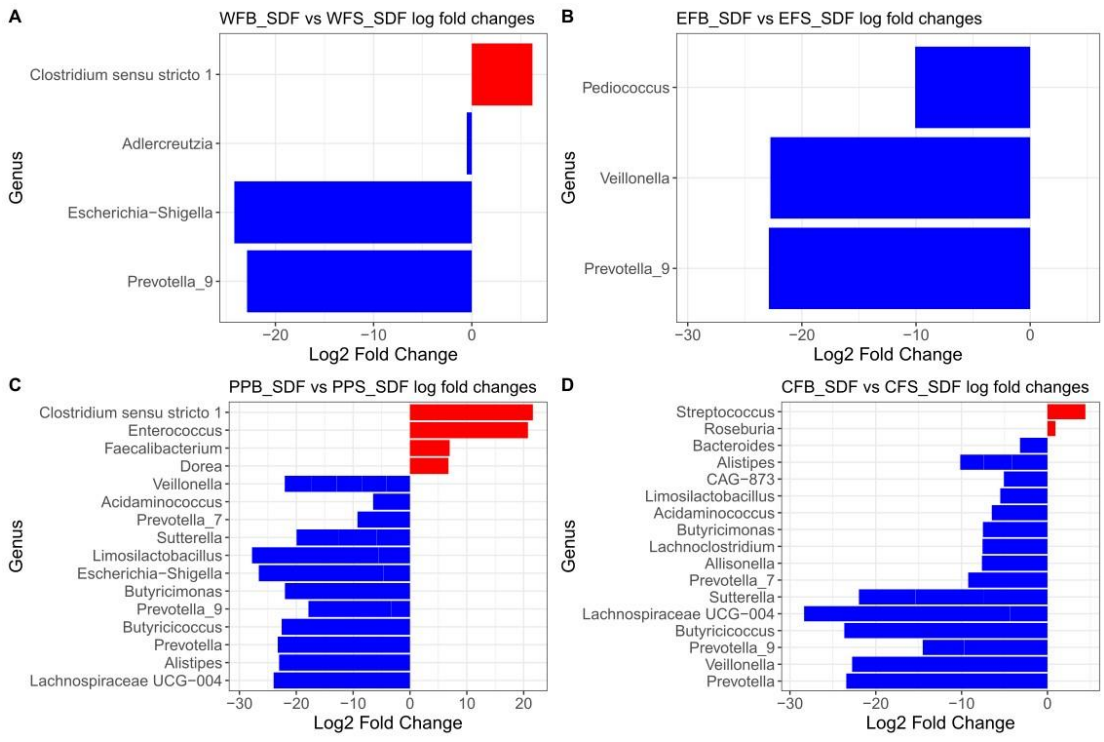


Figure 5.9 DESeq2 results in waterfall plot to show log2 fold changes between fermentation types for each flour.

We then employed DESeq2 to each fermentation paired with the same flour type to evaluate the differential abundant genera (Figure 5.9). We agglomerated taxonomy to

genus level and ran DESeq2 fold change analysis using the Benjamini Hochberg p adjustment method. Adjusted $p<0.05$ was considered statistically significant. Faecal samples fermented with digested wheat flour sourdough tarhana soup (WFS_SDF) caused an increase in *Clostridium sensu stricto 1* and a decrease in *Adlercreutzia*, *Escherichia-Shigella* and *Prevotella* compared to faecal samples of digested wheat flour baker's yeast tarhana soup (WFB_SDF) (Figure 5.9A). Faecal samples fermented with digested einkorn flour sourdough tarhana soup (EFS_SDF) had a reduction in *Pediococcus, Veillonella* and *Prevetolla_9* compared to einkorn flour baker's yeast (EFB_SDF) (Figure 5.9B). Faecal samples fermented with digested purple potato flour sourdough tarhana soup (PPS_SDF) (Figure 5.9C) had positive log2 fold changes in *Clostridium sensu stricto 1, Enterococcus, Faecalibacterium* and *Dorea* and negative fold changes for *Veillonella*, *Acidaminococcus*, *Prevotella_7*, *Lachnospiraceae UCG−004*, *Alistipes*, *Prevotella*, *Butyricicoccus*, *Prevotella_9*, *Butyricimonas*, *Escherichia−Shigella*, *Limosilactobacillus* and *Sutterella*. Lastly, Faecal samples fermented with digested chickpea flour sourdough tarhana soup (CFS_SDF) (Figure 5.9D) had positive log fold changes in *Streptococcus* and *Roseburia*, and negative fold changes in *Bacteroides, Prevotella, Veillonella, Prevotella_9, Butyricicoccus, Lachnospiraceae UCG−004, Sutterella, Prevotella_7, Allisonella, Lachnoclostridium, Butyricimonas, Acidaminococcus, Limosilactobacillus, CAG−873,* and *Alistipes*.

5.4.2.6 Short-chain and branched-chain fatty acid analyses

SCFA and BCFA analyses were performed on faecal samples fermented with digested tarhana soup as well as negative (cellulose) and positive (inulin) controls before and

after colonic fermentation to determine acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate levels. Several significant differences were observed (Figure 5.10). Acetate levels (Figure 5.10A) were found to be increased significantly in CFB_SDF, CFS_SDF, PPB_SDF and inulin_DF samples following the *in vitro* colonic fermentation compared to baseline. Similarly, in the presence of inulin, acetate levels were higher compared to EFB_SDF, EFS_SDF and PPS_SDF following *in vitro* colonic fermentation. Likewise, CFB_SDF samples had higher levels of acetate compared to EFS_SDF samples. Faecal butyrate levels (Figure 5.10B) were significantly higher in the presence of inulin compared to WFB_SDF following colonic fermentation. Propionate concentrations (Figure 5.10C) were greater in inulin_DF, CFB_SDF, and PPB_SDF samples compared to baseline; likewise, inulin_DF samples had also higher levels of propionate compared to EFB_SDF. EFB_SDF and EFS_SDF samples had lower levels of propionate compared to CFB_SDF, and PPB_SDF samples. Isobutyrate (Figure 5.10E) levels increased in inulin_DF faecal samples compared to baseline. Isovalerate, valerate and total BCFA levels were similar following the fermentation of substrates (Figure 5.10D, 5.10F and 5.10G). Total SCFA levels (Figure 5.10H) were higher in inulin_DF faecal samples compared to baseline, EFB_SDF, EFS_SDF and PPB_SDF samples. CFB_SDF and PPB_SDF faecal samples had higher total SCFA levels compared to baseline.

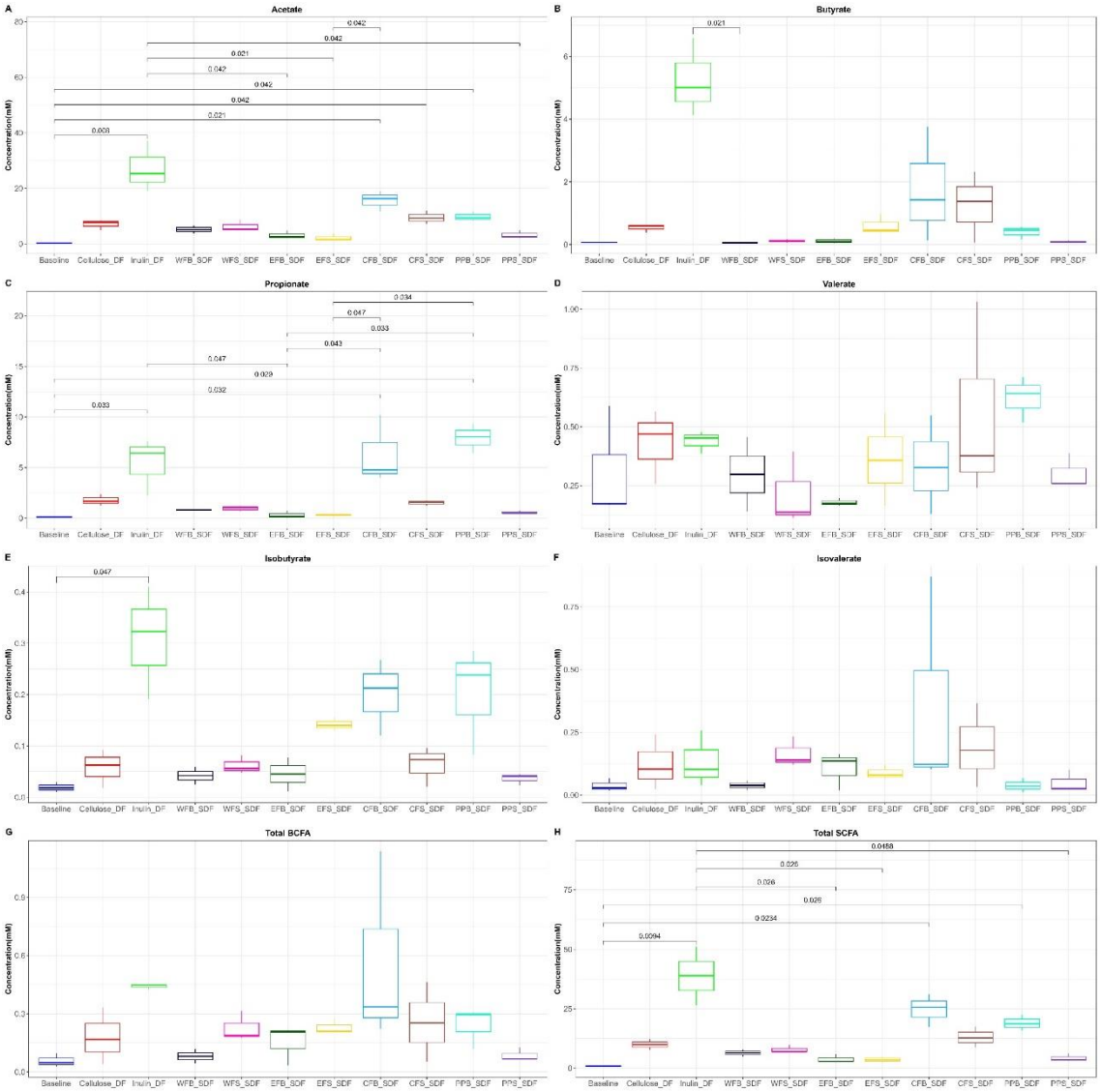


Figure 5.10 Short-chain and branched-chain fatty acid levels in millimolar concentrations before and after fermentation. Dunn's test was employed with false discovery rate p-value correction to determine significant changes within the groups.

5.5 Discussion

The impact of functional foods on gut microbiota composition is gaining increasing interest, with complex, whole-food interventions such as tarhana soup offering novel insights. In this study, the effects of tarhana soup, prepared with four different flours (chickpea, purple potato, wheat, and einkorn) and fermented by baker's yeast or

sourdough, were assessed for their influence on gut microbiota diversity, community composition, as well as SCFA and BCFA levels for gut health. This study provides a comparative evaluation of gut microbial communities following *in vitro* colonic fermentation of different digested tarhana soups, highlighting significant shifts at both phylum and genus levels as well as SCFA and BCFA concentrations, suggesting tarhana's potential role in modulating gut microbiota in ways beneficial for gastrointestinal health. Our investigation started with evaluating the effects of four flour types using baker's yeast, and then the same flours were fermented with chickpea sourdough to increase the health benefits of tarhana. After, we explored which fermentation type had better effects on the human gut microbiota by comparing the fermentation types for each flour.

Microorganisms in tarhana with potential probiotic attributes help maintain the balance of the gut microbiome by inhibiting the growth of pathogens and supporting digestive health. Meanwhile, the prebiotic fibres from different flour types provide a substrate for these beneficial bacteria to thrive, enhancing their colonization and activity in the gut (Han et al., 2021, Barone et al., 2018, Santiago et al., 2015). Postbiotics produced during fermentation, including SCFAs like butyrate and acetate, further promote gut health by reducing inflammation, supporting the gut barrier, and promoting overall metabolic health (Prajapati et al., 2023). Dietary complexity has been shown to drive modulations in microbial composition, as varied substrates support a broader range of microbial niches, particularly those involved in the fermentation of carbohydrates and dietary fibres (Cronin et al., 2021).

At the phylum level, the dominance of Firmicutes across all digested tarhana soup-treated faecal samples aligns with their established role in fibre and carbohydrate breakdown, which can lead to beneficial SCFA production critical for colon health and systemic immunity (Sun et al., 2023, Koh et al., 2016). Recent meta-analyses underscore the role of these taxa in reducing inflammatory markers and enhancing gut health outcomes, suggesting that tarhana could serve as a functional food in the dietary management of gut-related conditions (Sun et al., 2023). *Firmicutes* also include several probiotic genera, known to interact with gut epithelial cells to reinforce the gut barrier (Rastogi and Singh, 2022) such as *Faecalibacterium*, *Roseburia*, and *Lactobacillus. Faecalibacterium* levels were found to be increased in PPS_SDF faecal samples compared to PPB_SDF, indicating that sourdough fermentation resulted in positive modulation of the gut microbiome. *Faecalibacterium* species have been shown to improve immune health whereas low levels of *Faecalibacterium* have been associated with inflammation (Parsaei et al., 2021, Leylabadlo et al., 2020, Martin et al., 2023). Furthermore, the enrichment of *Roseburia* in faecal samples of CFS_SDF may indicate the health-promoting effects of chickpea and sourdough fermentation of tarhana on the gut microbiome. CFS_SDF faecal samples led to a significant increase of acetate compared to baseline however it did not cause an increase of butyrate or propionate levels significantly. *Roseburia* have been shown to modulate the immune system and produce SCFAs such as acetate, propionate, and butyrate (Tamanai-Shacoori et al., 2017, Nie et al., 2021). Previous studies have found that *R. intestinalis* and *F. prausnitzii* are the most abundant butyrate-producing bacteria in human faeces (Hold et al., 2003, Duncan et al., 2004). Thus, these suggest that sourdough fermentation of tarhana may favour specific microbial taxa associated with beneficial

SCFA profiles, aligning with recent findings on sourdough fermentation's role in producing beneficial metabolites during fermentation (Salminen et al., 2022).

Increased relative abundance of Actinobacteriota in PPB_SDF faecal fermentates compared to cellulose_DF highlights the ability of purple potato in tarhana samples to support beneficial bacterial populations which can contribute to saccharolytic activity and SCFA production (Kumar et al., 2020). The elevated abundance of Actinobacteriota in PPB_SDF faecal samples suggests potential benefits, as these bacteria are associated with the production of SCFA. PPB_SDF faecal samples also showed significant increases in acetate, propionate and total SCFA levels compared to baseline. Acetate has anti-inflammatory properties, can inhibit pathogen growth, and plays a crucial role in maintaining the gut barrier (Tedelind et al., 2007). *Actinobacteriota* includes *Bifidobacterium*, which is often used as a probiotic for its role in enhancing gut health and immunity (Sun et al., 2024). This observation aligns with studies suggesting that specific dietary fibres selectively enhance *Bifidobacterium* spp., thereby contributing to gut health (Riviere et al., 2016). *Bifidobacterium* enrichment in PPB_SDF samples is of interest as this genus plays a central role in carbohydrate fermentation and has established health benefits, including immunomodulation and production of SCFAs (Dong et al., 2022, Gavzy et al., 2023). The prebiotic potential of purple potato flour, suggested by *Bifidobacterium* abundance, aligns with research indicating that anthocyanin-rich foods, such as purple potatoes, support *Bifidobacterium* spp. through selective carbohydrate metabolism (Boto-Ordonez et al., 2014). This is especially relevant in the context of gut health, as increased *Bifidobacterium* is associated with positive outcomes in gastrointestinal health, including reduced symptoms of irritable bowel syndrome (IBS) and decreased

inflammation (Staudacher et al., 2017). *Bifidobacterium* has been shown to produce acetate to reduce inflammation as well as suppress non-alcoholic fatty liver disease-associated hepatocellular carcinoma (Song et al., 2023, Fukuda et al., 2012). *Adlercreutzia* is another genus that belongs to the Actinobacteriota phylum that has anti-inflammatory properties (Onate et al., 2023). Its abundance increased in WFS_SDF samples compared to WFB_SDF which shows the beneficial effect of sourdough fermentation.

Additionally, the increased relative abundance of *Akkermansia* (phylum Verrucomicrobiota) in WFS_SDF and EFS_SDF samples, near the threshold for significance (q=0.052), points to potential gut barrier support. Notably, *Akkermansia* levels increased in EFB_SDF samples significantly compared to inulin-fermented faecal samples (q=0.049), which is intriguing due to its documented role in mucus layer maintenance and anti-inflammatory effects (Liu et al., 2022). *Akkermansia* has gained attention for its potential to improve metabolic and immune health, making it relevant to probiotic interventions (Neef and Sanz, 2013, Rodrigues et al., 2022). *Akkermansia muciniphila*, specifically, is known for its mucin-degrading capabilities, which have been linked to improved intestinal integrity and protection against metabolic diseases (Cani, 2016). The increase in *Akkermansia* in wheat and einkorn flour sourdough samples may reflect the influence of these specific flours and fermentation methods on taxa that thrive in low-pH, carbohydrate-rich environments, offering possible protective effects on gut mucosal integrity.

Streptococcus was significantly enriched in EFB_SDF, WFB_SDF, WFS_SDF, EFS_SDF, PPS_SDF and CFS_SDF samples compared to cellulose_DF. Following

ANCOMBC analysis, *Streptococcus* levels did not significantly change in each fermentation group. However, DESeq2 analysis revealed a log2 fold increase of *Streptococcus* in CFS_SDF samples compared to CFB_SDF samples. Increased *Streptococcus* levels in samples fermented with tarhana might reflect the sample microbial composition as yoghurt is one of the ingredients of tarhana and it has *Streptococcus* (Demirci et al., 2022).

Veillonella levels were found to be reduced in sourdough fermented flour types except wheat flour. *Veillonella* is an anaerobic and opportunistic pathogenic bacterium that can colonize the oral cavity and colon (Giacomini et al., 2023, Zhan et al., 2022). Even though increased levels of *Veillonella* were observed in the elite athlete gut microbiome following exercise (Scheiman et al., 2019), it has been associated with diseases such as autism spectrum disorder and autoimmune diseases (Zhan et al., 2022, Liu et al., 2019).

Interestingly, there was a notable reduction in relative abundance of Campylobacterota following *in vitro* colonic fermentation with inulin and chickpea flour sourdough samples (CFS_SDF). Campylobacterota phylum includes pathogenic bacterial genera such as *Helicobacter* and *Campylobacter* spp. (van der Stel and Wosten, 2019). Previous research has highlighted that such reductions are often indicative of dietary patterns that select against pathogenic or dysbiotic genera, promoting beneficial taxa instead (Desai et al., 2016). Differential genus-level responses provide critical insights into tarhana's microbial community dynamics, with genera associated with beneficial metabolites differing across flour types and fermentation methods. The suppression of *Escherichia-Shigella* (Proteobacteria) in wheat flour and purple potato flour

sourdough fermentates (WFS_SDF and PPS_SDF) compared to their baker's yeast fermented counterparts further supports sourdough's antimicrobial properties. *Escherichia* and *Shigella* are well-studied pathogenic bacteria, which can cause diarrhoea and gastrointestinal disorders (Kaberdin and Arana, 2021, Devanga Ragupathi et al., 2018, Wang et al., 2012). Acidic byproducts of sourdough fermentation, such as lactic acid, create inhospitable environments for potential pathogens like *Escherichia-Shigella*, a common gut pathogen associated with gastrointestinal inflammation (Wang et al., 2015, Zare Mirzaei et al., 2018). This observation is consistent with literature suggesting that sourdough fermentation can reduce pathogenic load, thereby potentially contributing to gut health and reducing inflammation risk (Perez-Alvarado et al., 2022).

Given the distinctive microbial profiles elicited by the different flour types and fermentation processes, sourdough fermentation of tarhana particularly modulated the microbiome positively. The gut microbiome following *in vitro* digestion revealed the purple potato and chickpea flours had lower pathogenic bacteria and higher potential probiotic bacteria. Thus, a combination of sourdough fermentation with purple potato flour and chickpea flour might increase the potential health benefits of tarhana. Future research could focus on human studies assessing dietary intervention with sourdough-fermented tarhana's effects on gut health markers, inflammation, and gastrointestinal symptom management. Additionally, further exploration of molecular mechanisms underlying the selective microbial enrichment in tarhana could reveal novel insights into functional food design, specifically targeting prebiotic effects.

5.6 Limitations

Limitations of this study include the use of *in vitro* fermentation models, which may not fully capture the complexities of human gut dynamics. Variability in individual gut microbiota profiles and dietary habits should also be considered when translating these findings to clinical settings.

5.7 Conclusion

Results of this study provide a deeper understanding of how tarhana can be optimized as a functional food for gut health, with specific emphasis on how different flours and fermentation methods influence its probiotic, prebiotic, and postbiotic properties. These results underscore tarhana's potential as a functional food for gut health, supporting beneficial taxa and SCFA production. The selective microbial responses observed indicate that tarhana could be tailored for dietary interventions targeting microbial modulation. Future studies could expand these findings by exploring tarhana’s gut microbiome promoting effects to validate its potential health benefits *in vivo*.

5.8 References

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5.9 Supplementary Tables

Supplementary Table 5.1 Pairwise adonis to compare groups. False discovery rate (FDR) did not reveal significant differences between the groups.

Comparisions	SumsOfSqs	F.Model	R2	p.value	FDR
EFB_SDF vs Baseline	0.345289	8.547079	0.460832	0.005	0.062857
PPB_SDF vs Baseline	0.708333	9.550177	0.488496	0.003	0.062857
CFB_SDF vs Baseline	0.772377	10.39725	0.509738	0.007	0.062857
WFS_SDF vs Baseline	0.394589	9.218727	0.479674	0.008	0.062857
Baseline vs PPS_SDF	0.663179	22.58587	0.693119	0.008	0.062857
Baseline vs Cellulose_DF	1.299687	38.76525	0.794936	0.006	0.062857
Baseline vs Inulin_DF	1.314739	45.5416	0.819955	0.008	0.062857
Baseline vs CFS_SDF	0.898878	24.54735	0.710542	0.01	0.06875
EFS_SDF vs Baseline	0.215941	3.910034	0.281094	0.013	0.079444
WFB_SDF vs Baseline	0.405516	7.821507	0.43888	0.02	0.11

Supplementary Table 5.2 Table shows Dunn’s Test Results at Phylum Level

Phylum	group1	group2	p	p.adj (FDR)
Bacteroidota	Baseline	Cellulose	0.000561	0.01543
Bacteroidota	Baseline	CFS_L	0.000477	0.01543
Fusobacteriota	Baseline	Inulin	0.000289	0.015882
Campylobacterota	Baseline	Inulin	0.00075	0.020631

Campylobacterota	Baseline	CFS_L	0.00075	0.020631
Actinobacteriota	Cellulose	PPB_L	0.00067	0.036853
Fusobacteriota	Baseline	Cellulose	0.001673	0.04602
Verrucomicrobiota	Baseline	Inulin	0.00086	0.047284

Supplementary Table 5.3 Table shows Dunn test results at Genus level of baker's yeast samples

Genus	**group1**	**group2**	**p**	**p.adj (FDR)**
Erysipelatoclostridium	Cellulose	PPB_L	0.000828	0.017389
Erysipelatoclostridium	Cellulose	EFB_L	0.002028	0.021296
Holdemania	Cellulose	EFB_L	0.002347	0.029383
Holdemania	EFB_L	Inulin	0.002798	0.029383
Megamonas	CFB_L	Inulin	0.004671	0.032696
Megamonas	Inulin	PPB_L	0.003973	0.032696
Bifidobacterium	Cellulose	PPB_L	0.001704	0.035783
Haemophilus	Cellulose	EFB_L	0.001704	0.035783
Haemophilus	Cellulose	WFB_L	0.003973	0.041712
Streptococcus	Cellulose	EFB_L	0.002028	0.042593
UBA1819	EFB_L	Inulin	0.002028	0.042593
Parasutterella	Cellulose	PPB_L	0.005479	0.044871
Parasutterella	Inulin	PPB_L	0.00641	0.044871
Streptococcus	Cellulose	WFB_L	0.004671	0.049045
Akkermansia	EFB_L	Inulin	0.004671	0.049045

Supplementary Table 5.4 Table shows Dunn test results at Genus level of faecal samples fermented with pre-digested tarhana from sourdough fermentation

Genus	**group1**	**group2**	**p**	**p.adj (FDR)**
Haemophilus	Cellulose	CFS_L	0.000687	0.014429
Erysipelatoclostridium	Cellulose	CFS_L	0.001194	0.014994
Erysipelatoclostridium	Cellulose	PPS_L	0.001428	0.014994
Streptococcus	Cellulose	CFS_L	0.002853	0.019968
Streptococcus	Cellulose	PPS_L	0.001194	0.019968
Streptococcus	Cellulose	WFS_L	0.002028	0.019968
Haemophilus	Cellulose	PPS_L	0.002028	0.019968
Haemophilus	CFS_L	Inulin	0.002853	0.019968
Paludicola	EFS_L	Inulin	0.001635	0.020576
Haemophilus	Cellulose	EFS_L	0.004671	0.024522
Turicibacter	Cellulose	PPS_L	0.001428	0.025287
Terrisporobacter	Cellulose	PPS_L	0.002408	0.025287
Turicibacter	Inulin	PPS_L	0.002408	0.025287
Terrisporobacter	Inulin	PPS_L	0.002408	0.025287
Haemophilus	Inulin	PPS_L	0.007482	0.031425
Lachnospiraceae NK4A136 group	EFS_L	Inulin	0.004671	0.032696
Erysipelatoclostridium	Cellulose	EFS_L	0.008712	0.035418
Erysipelatoclostridium	Cellulose	WFS_L	0.010119	0.035418
UBA1819	Inulin	PPS_L	0.001704	0.035783
Turicibacter	Cellulose	CFS_L	0.005479	0.03835
Lachnospiraceae UCG-004	EFS_L	Inulin	0.005479	0.03903

Terrisporobacter	Cellulose	WFS_L	0.007482	0.039281
Terrisporobacter	Inulin	WFS_L	0.007482	0.039281
Dialister	EFS_L	Inulin	0.00641	0.044871
UCG-003	EFS_L	Inulin	0.00641	0.044871
Turicibacter	CFS_L	Inulin	0.008712	0.045738
Haemophilus	EFS_L	Inulin	0.015631	0.046893
UBA1819	Inulin	WFS_L	0.004671	0.049045
Streptococcus	Cellulose	EFS_L	0.011726	0.049249

Supplementary Table 5.5 ANCOMBC Results of faecal samples fermented with Baker's yeast produced tarhana. Wheat flour baker's yeast tarhana soup used as a reference.

lfc.taxon	**lfc.EFB_SDF**	**se. EFB_SDF**	**W. EFB_SDF**	**p_val. EFB_SDF**	**q_val. EFB_SDF**
Candidatus Soleaferrea	0.398571833	0.085987065	4.635253376	3.57E-06	0.000691611
TM7x	-0.142055005	0.032739456	-4.338954317	1.43E-05	0.001388674
Caproiciproducens	-0.590011243	0.149464378	-3.947504077	7.90E-05	0.005106738
Pediococcus	-1.852972067	0.555425872	-3.336128476	0.000849539	0.041202638
lfc.taxon	**lfc.CFB_SDF**	**se.CFB_SDF**	**W.CFB_SDF**	**p_val.CFB_SDF**	**q_val.CFB_SDF**
Megamonas	1.162701104	0.304682325	3.816109465	0.000135572	0.013150521
Gordonibacter	1.64266587	0.42885072	3.830390838	0.00012794	0.013150521
Phascolarctobacterium	1.45526617	0.40592679	3.585045886	0.000337019	0.021793892
HT002	2.489811817	0.763209285	3.262292358	0.001105151	0.043608317
Leuconostoc	1.954102283	0.609626665	3.205408152	0.001348711	0.043608317
Coprobacter	2.185151343	0.6762565	3.231246345	0.001232517	0.043608317
lfc.taxon	**lfc.PPB_SDF**	**se.PPB_SDF**	**W.PPB_SDF**	**p_val.PPB_SDF**	**q_val.PPB_SDF**
Senegalimassilia	-2.317131625	0.290476849	-7.976992428	1.50E-15	2.91E-13
Rothia	0.880408257	0.138274217	6.367118027	1.93E-10	1.87E-08
Parasutterella	1.827860911	0.354974054	5.149280318	2.61E-07	1.69E-05
Acinetobacter	2.537169589	0.511971992	4.955680447	7.21E-07	3.50E-05
Phascolarctobacterium	1.605837779	0.330394802	4.8603603	1.17E-06	4.55E-05
Candidatus Soleaferrea	0.903483875	0.189980772	4.755659554	1.98E-06	6.40E-05
Gordonibacter	1.588270691	0.375195854	4.233177613	2.30E-05	0.000638571
Bifidobacterium	1.406020546	0.354748938	3.963424252	7.39E-05	0.001791646
Megamonas	1.139370677	0.293324921	3.884329617	0.000102613	0.002211872
Solobacterium	1.554855553	0.52553169	2.958633293	0.003090066	0.059947275

Supplementary Table 5.6 ANCOMBC Results of faecal samples fermented with sourdough produced tarhana. Wheat flour sourdough tarhana soup used as a reference.

lfc.taxon	**lfc.EFS_SDF**	**se.EFS_SDF**	**W.EFS_SDF**	**p_val. EFS_SDF**	**q_val. EFS_SDF**
Catenibacillus	2.94134613	0.41133115	7.150798405	8.63E-13	1.71E-10
lfc.taxon	**lfc.CFS_SDF**	**se.CFS_SDF**	**W. CFS_SDF**	**p_val. CFS_SDF**	**q_val.CFS_SDF**
[Eubacterium] eligens group	-0.403405759	0.048611981	-8.298484208	1.05E-16	2.09E-14
Alistipes	-0.948161056	0.121009621	-7.835418791	4.67E-15	4.39E-13
Catenibacillus	2.856068806	0.366584808	7.791017916	6.65E-15	4.39E-13
Paraprevotella	-3.247945395	0.679043018	-4.783121698	1.73E-06	8.54E-05
Acidaminococcus	-1.238252607	0.273039222	-4.535072276	5.76E-06	0.000228032
Senegalimassilia	0.601447916	0.153802091	3.910531475	9.21E-05	0.00255199
Desulfovibrio	-0.415335755	0.106762843	-3.890265014	0.000100135	0.00255199
Olsenella	0.693693895	0.178641925	3.883152826	0.000103111	0.00255199
Hungatella	0.531320466	0.144096403	3.687256978	0.000226684	0.004987057
Fusicatenibacter	-0.405499795	0.111131137	-3.648840509	0.000263427	0.005215846
UCG-003	-0.795836097	0.233529959	-3.407854394	0.000654758	0.011785648
Fournierella	-0.422492416	0.127552944	-3.312290581	0.000925354	0.013087146
Enterorhabdus	0.335059587	0.100557137	3.332031903	0.000862144	0.013087146
Solobacterium	-0.408163076	0.122332464	-3.336506627	0.000848384	0.013087146
[Eubacterium] xylanophilum group	-1.892370518	0.586696271	-3.225468803	0.001257665	0.015819957
Merdibacter	-1.728653503	0.536717328	-3.220789439	0.00127838	0.015819957
[Eubacterium] ventriosum group	-0.452613432	0.141950851	-3.188522132	0.001430021	0.016655534
Clostridium sensu stricto 13	2.447258634	0.778987684	3.141588352	0.001680341	0.018418127
Coprococcus	-0.644941759	0.206264696	-3.126767559	0.001767396	0.018418127
CAG-56	-1.302720224	0.419153104	-3.107981811	0.001883697	0.018648596

Oscillibacter	-0.546434659	0.180476314	-3.027736136	0.002463931	0.022175381
Oscillospira	-0.70701598	0.233476723	-3.028207575	0.00246009	0.022175381
CAG-352	-1.960746998	0.654136618	-2.997457938	0.002722414	0.023436436
Dialister	-0.388931355	0.133521044	-2.912884325	0.003581072	0.029543846
Lachnospiraceae NK4A136 group	-0.685606028	0.243174912	-2.819394577	0.004811433	0.038106552
Agathobacter	-0.303792706	0.109980416	-2.76224365	0.005740562	0.040640716
NK4A214 group	-0.75503877	0.274050355	-2.755109625	0.005867247	0.040640716
Oxalobacter	-0.681962501	0.248958267	-2.739264336	0.006157684	0.040640716
DTU089	-0.462350582	0.166073491	-2.784011938	0.005369106	0.040640716
Sellimonas	-1.260655524	0.460158018	-2.739614384	0.006151131	0.040640716
Roseburia	-0.95084763	0.349209069	-2.722860642	0.006471935	0.041262572
Adlercreutzia	0.538669782	0.198554931	2.712950909	0.006668699	0.041262572
Granulicatella	0.389507485	0.145964478	2.668508745	0.00761888	0.045713277
Colidextribacter	-1.064889461	0.402174483	-2.647829501	0.008101037	0.047176628
lfc.taxon	**lfc.PPS_SDF**	**se.PPS_SDF**	**W.PPS_SDF**	**p_val.PPS_SDF**	**q_val.PPS_SDF**
Catenibacillus	2.86828237	0.42254977	6.788034387	1.14E-11	2.25E-09
Olsenella	1.395308366	0.210909875	6.615661609	3.70E-11	3.66E-09
Solobacterium	-0.453339241	0.092597165	-4.895822036	9.79E-07	6.46E-05
[Eubacterium] eligens group	-0.186572711	0.046928854	-3.975650303	7.02E-05	0.003474266
CHKCI002	0.831416969	0.215516935	3.857780227	0.000114421	0.00453109
Mogibacterium	1.818321385	0.483241086	3.762762393	0.000168047	0.005545546
Prevotella_7	-2.383384343	0.675730416	-3.527123075	0.000420101	0.010502181
Acetanaerobacterium	-1.174060776	0.333116954	-3.524470198	0.000424331	0.010502181
Collinsella	0.835331582	0.267064656	3.127825275	0.001761048	0.034868758
Libanicoccus	0.945152389	0.300233577	3.148056919	0.001643597	0.034868758

Chapter 6

Shaping Metabolic Health: The Role of *Lactobacillus mucosae* DPC6426 and Dietary Fibre in a Porcine Model of Metabolic Syndrome

6.1 Abstract

Metabolic syndrome (MetS) is a complex disorder characterized by a cluster of conditions, including insulin resistance, obesity, hypertension, and dyslipidaemia, all of which significantly increase the risk of cardiovascular diseases. This study investigated the effects of *Lactobacillus mucosae* DPC6426 and dietary fibre, individually and in combination (synbiotic), on metabolic profiles in a porcine model of MetS. We set up two pig trials; in Trial 1, we developed a MetS pig model using a high fat, sugar, and salt diet (HFD) and compared their faecal metabolome with control animals. In Trial 2, MetS pigs (HFD) were administered *Lb mucosae* DPC6426 (HFD_Lb), fibre (HFD_fibre) or their combination (HFD_Lb_fibre). Utilizing the TMIC (The Metabolomic Innovation Centre) Mega Assay, we analysed faecal samples from week 6 and week 12 from both Trial 1 and 2 as well as plasma metabolites at week 2, week 6, and week 12 from Trial 2. Our findings revealed significant alterations in lipid metabolism, amino acid metabolism, and neurotransmitter activity in MetS pigs at week 6 and 12. At week 6, histamine (p=1.21E-05) was significantly elevated, alongside with other metabolites such as C18:1, kynurenic acid, and C18, whereas 3-methyladipic acid (p=2.77E-07), arginine (p=0.0079149), and pyruvic acid (p=0.00011268) were significantly reduced in MetS pigs faecal samples. By week 12, N2-acetyl-ornithine (p=2.77E-07) was significantly higher, along with C18:1, orotic acid, and serotonin, while arginine and pyruvic acid levels remained reduced. Notably, the synbiotic combination of *Lb. mucosae* DPC6426 and fibre generated more favourable metabolite profiles than either treatment alone. Key metabolites including spermidine (p=0.046143) and 3-deoxyglucosone (p=9.21E-05) were elevated in the

faecal metabolome of the synbiotic group (HFD_Lb_fibre), while serum levels of N-acetyl-glycine ($p=0.0093112$), and serine ($p=0.014102$) were significantly higher in week 6. The synbiotic intervention also led to increased (variable importance projection>1.5) serum lysophospholipids and reduced levels of N2-acetyl-ornithine, putrescine, and uric acid, suggesting improved lipid metabolism, enhanced fatty acid oxidation, and insulin sensitivity. These results underscore the potential of combining probiotics and fibre as a synergistic strategy to modulate metabolic pathways, thereby aiding in the management of metabolic syndrome.

6.2 Introduction

Metabolic syndrome (MetS) is a multifaceted disorder characterized by insulin resistance, obesity, systemic hypertension, and dyslipidaemia, significantly elevating the risk for cardiovascular diseases (McCracken et al., 2018, Watanabe et al., 2008). The pathophysiological mechanisms underlying MetS involve visceral adiposity, low-grade inflammation, and oxidative stress (Roberts et al., 2013, Eckel et al., 2005, Reaven, 1988). Key lifestyle factors such as smoking, high-fat diet, high fructose consumption, low dietary fibre intake, and physical inactivity are known to worsen MetS (Gantenbein and Kanaka-Gantenbein, 2021, Martinez-Hernandez et al., 2024, Taskinen et al., 2019, Balhara, 2012, Deehan et al., 2024). Research has increasingly highlighted the importance of diet in both the development and management of MetS (Abutair et al., 2018, Gantenbein and Kanaka-Gantenbein, 2021, Chavez et al., 2023, Bulsiewicz, 2023); however, the specific mechanisms underpinning these dietary effects remain unclear, necessitating further investigation. A high fat, sugar and salt diet is particularly implicated in the exacerbation of MetS through alterations in gut microbiota and associated metabolic changes (O'Donovan et al., 2020). The gut microbiota, a complex community of trillions of microorganisms, plays a critical role in metabolizing dietary components, such as fibres, to produce metabolites like short-chain fatty acids (SCFAs), which promote health (Hills et al., 2019). SCFAs; including acetate, propionate, and butyrate are crucial for colonic health and metabolic regulation, providing 60-70% of the energy required by colonocytes and enhancing insulin sensitivity through activation of key receptors that stimulate the secretion of glucagon-like peptide 1 (GLP-1) (Roediger, 1982, Green et al., 2020).

Emerging studies have demonstrated differences in gut microbiota composition between MetS patients and healthy individuals, reinforcing the notion that gut microbial health is pivotal in managing MetS (Hills et al., 2019, O'Donovan et al., 2020). Specific metabolites associated with metabolic dysfunction have received significant attention; elevated levels of certain fatty acids have been linked to increased insulin resistance and inflammation. Stearic acid has been shown to lower low-density lipoprotein which is a risk factor for coronary heart diseases (van Rooijen and Mensink, 2020). Increased tryptophan and tyrosine levels were observed in obese subjects compared to healthy controls (Reddy et al., 2018, Chen et al., 2016). Phosphatidylcholine and lysophosphatidylcholine have been shown to have an anti-inflammatory effect on *in vitro* cell culture experiments (Treede et al., 2007).

Probiotics and prebiotics have shown promise in modulating gut microbiota and ameliorating symptoms of MetS (He and Shi, 2017). Probiotic intake has been associated with improvements in body mass index, blood pressure, glucose metabolism, and lipid profiles (Tenorio-Jimenez et al., 2020). Similarly, high dietary fibre intake has been linked to increased abundance of beneficial gut bacteria, such as *Bifidobacterium* and *Lactobacillus*, and the production of health-promoting metabolites like SCFAs and bile acids (Abutair et al., 2018). Combining prebiotics and probiotics to form synbiotics can further enhance these benefits by synergistically improving metabolic health more effectively than either component alone (Jiang et al., 2022).

There are several animal models including rodents, rabbits, and pigs to study the effects of administration of probiotics, prebiotics and synbiotics on gut microbiome

and metabolome (Martin et al., 2010, Smith et al., 2021a). Pigs are excellent animal models for studying cardiovascular and metabolic diseases due to their physiological and anatomical similarities to humans, including comparable heart structure, lipid profiles, and lipoprotein metabolism (Zhang and Lerman, 2016, Jia et al., 2024). A previous study from our research group demonstrated significant gut microbiome changes in a pig model of MetS, underscoring the relevance of this model for studying the gut microbiota-metabolic syndrome axis (O'Donovan et al., 2020).

In this current study, we aimed to investigate the effects of administering probiotics, prebiotics, and a synbiotic on the blood and faecal metabolome in a pig model of MetS. Two pig trials were thus conducted. In Trial 1, a MetS pig model was developed using a high fat, sugar, and salt diet (HFD). In Trial 2, MetS pigs were administered the same HFD diet supplemented with *Lactobacillus mucosae* DPC6426 (HFD_Lb), fibre (HFD_fibre) or their combination as a synbiotic (HFD_Lb_fibre). Utilizing the comprehensive TMIC Mega Assay from the Metabolomics Innovation Centre at the University of Alberta, we profiled metabolic changes at week 2 (T2), week 6 (T6), and week 12 (T12) from plasma samples of Trial 2 and at week 6 and week 12 from faecal samples to elucidate the metabolic shifts associated with Trial 1 and Trial 2. By understanding these metabolic changes, we hope to shed light on the underlying mechanisms linking diet, gut microbiota, and MetS while determining the potential impacts of *Lb. mucosae* DPC6426 and dietary fibre, individually and combined.

6.3 Materials and Methods

6.3.1 Metabolic syndrome development of porcine model (Trial 1) and sample collection

All experimental procedures used during the study were reviewed and approved by the University College Cork Ethics Committee and Animal Welfare Body (AWB), under a license issued by the Health Products Regulatory Authority (HPRA; reference no. AE19130-P041 and AE19130-P097) in exercise of the powers conferred on it under the European Union (protection of animals used for scientific purposes) Regulations 2012 (S.I. No. 543 of 2012) as amended and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purpose.

Effect size was calculated on G Power (version 3.1.9.2, University Kiel, Germany), and a sample-size estimation was computed. For both trials, three-month-old, 25 kg Landrace female piglets were acquired from Blarney pigs (Company number: 580987) and housed in individual stalls in the Biological Service Unit at University College Cork, Cork, Ireland. After a week of acclimatization, animals were separated into two groups including the control and metabolic syndrome model (MetS). The control group (n=10) was fed 1kg/day Connolly's Redmills normal chow (20% protein, 3.5% fibre, 6% oil, 20% Hi-Density Weaner Pellets), and the MetS group (n=10) was fed 0.5 kg/day Connolly's Redmills normal chow diet supplemented with 1 kg/day 4% salt, 4% cholesterol, 25% crude fat, 0.5% cholate, and 26% sugar [high fat, salt, and sugar diet (HFD), R8620 version 0011, SAFE, Augy, France]. Water was provided to the animals ad libitum. In addition to the HFD, the MetS group received a subcutaneous

implant of deoxycorticosterone acetate (DOCA) pellets (200 mg/kg, 90-day release, Innovative Research of America) in the inguinal region prior to the start of the trial to promote sodium and water retention, leading to hypertension. The detailed study protocol was published by O'Donovan et al., 2020 (O'Donovan et al., 2020).

Faecal samples were collected from Trial 1 pigs at baseline (T0) to the end of the study (T12) every two weeks. For faecal metabolomics analysis, week 6 (T6) and week 12 (T12) samples were processed.

6.3.2 Oral administration of *Lb. mucosae* DPC6426 and dietary fibre (Trial 2)

In Trial 2, in order to determine the effects of *Lb. mucosae* DPC6426 and dietary fibre singly and in combination (synbiotic), three-month-old Landrace female piglets were used. The starting weight of piglets was 20-25 kg. Animals were separated into 4 groups: HFD (n=6) were fed 0.5 kg/day Connolly's Redmills normal chow (20% Hi-Density Weaner Pellets; 20% protein, 3.5% fibre, 6% oil) supplemented with 1 kg/day of HFD; HFD + *Lb. mucosae* DPC 6426 (n = 6) fed with 0.5kg/day Connolly's Redmills normal chow supplemented with 1kg/day of HFD and 1g of *Lb. mucosae* DPC 6426 (HFD_Lb) at 10^{10} CFU/day; HFD + PROMITOR 70® Soluble Fiber (Tate & Lyle LLC) (HFD_fibre) (n = 6) fed with 0.5kg/day Connolly's Redmills normal chow supplemented with 1kg/day of HFD and 43g of fibre; and HFD + *Lb. mucosae* DPC 6426 + PROMITOR 70® Soluble Fiber (Tate & Lyle LLC) (HFD_Lb_fibre) (n = 6) fed with 0.5kg/day Connolly's Redmills normal chow supplemented with 1kg/day of HFD, 1g of *Lb. mucosae* DPC 6426 at 10^{10} CFU/g and 43g of fibre. All animals had free access to water. In conjunction with the diet, animals underwent surgical implantation of a subcutaneous DOCA depot similar to the method described in

Methods 2.1. All four groups were fed the diets over a 12-week period. Faecal samples were collected every two weeks for a total of seven-time points from baseline (T0) to week 12 (T12). Faecal samples from week 6 (T6) and week 12 (T12) were used for metabolomics analysis. Blood samples were also collected at three time points: T2, T6 and T12. Metabolomics analyses using liquid chromatography-tandem mass spectrophotometry LC-MS/MS were performed on faecal and blood samples.

6.3.3 Metabolomics analysis

TMIC (The Metabolomic Innovation Centre) Mega Kit (TMIC, Edmonton, Alberta, Canada) was used to analyse blood and faecal samples. The details of standards, calibration curves, isotopes and methods have been described in a previously published paper (Zhang et al., 2024). Formic acid, water, methanol, and acetonitrile, all of LC/MS grade (Optima™), as well as ammonium acetate, were obtained from Fisher Scientific (Ottawa, ON, Canada). Other materials purchased from Fisher Scientific (Ottawa, ON, Canada) included Optima™ LC/MS grade phosphate-buffered saline (PBS). Additional chemicals, including potassium phosphate monobasic and dibasic, D_2O (99.9%), HPLC grade water, pyridine, ethanol, chloroform, PITC (phenylisothiocyanate), 3-NPH (3-nitrophenylhydrazines), EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), and BHT (butylated hydroxytoluene) were procured from Sigma-Aldrich (Oakville, ON, Canada). Similarly, Nunc® 96 DeepWell™ plates and Multiscreen “solvinert” hydrophobic filter plates (PTFE, 0.45 μm) were also purchased from Sigma-Aldrich (Oakville, ON, Canada). The 2-chloropyrimidine-5-carboxylic acid (98%) was sourced from ArkPharm (Libertyville, IL, USA), and the Amicon 1.5 mL 3 kDa molecular weight cut-off (MWCO) filtration

units were acquired from Millipore Sigma (St. Louis, MO, USA). Chemical standards and isotope-labelled internal standards (ISTDs) were obtained from Sigma-Aldrich, Cayman Chemical (Ann Arbor, MI, USA), Toronto Research Chemicals (North York, ON, Canada), Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA), IsoSciences (Ambler, PA, USA), and C/D/N Isotopes Inc. (Pointe-Claire, QC, Canada). Notably, 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) was synthesized by the University of Alberta (Khaniani et al., 2018).

6.3.3.1 Standard preparation

To ensure accurate metabolite quantification, isotope-labelled ISTDs and derivatization reagents were utilized. Chemicals were precisely weighed using a Sartorius CPA225D semi-micro electronic balance (Mississauga, ON, Canada) with a precision of 0.0001 g. Stock solutions for analytes were prepared by dissolving accurately measured amounts of each chemical in appropriate solvents. Seven point calibration standards (Cal1 to Cal7), spanning various concentration ranges to reflect physiological or pathological levels in human serum and plasma were prepared. The details of standards have been reported in a previously published protocol (Zhang et al., 2024). Amino acid derivatives, biogenic amines, nucleotides/nucleosides, and amino acids were dissolved in double-distilled water to prepare stock, calibration, QC (quality control) mixtures, and working ISTD solutions. The working ISTD solution combined 48 isotope-labelled ISTDs at specific concentrations (Zhang et al., 2024). Lipid stock solutions were dissolved in chloroform, while methanol was used for acylcarnitines and hexose. Organic acid stock solutions were prepared in 75% methanol. Calibration and ISTD solutions for PITC and 3-NPH panels were dissolved

in water or 75% methanol, respectively. All calibration solutions were stored at −86 °C (Thermo Scientific, Waltham, MA, USA).

6.3.3.2 Sample Preparation for Liquid Chromatography–Mass Spectrometry (LC-MS)

Faecal samples were prepared using two extraction buffers, designated Buffer A and Buffer B prepared by mixing ethanol (Sigma-Aldrich, Oakville, ON, Canada) and 20 mM phosphate buffer in specific proportions. For Buffer A, 340 mL of ethanol was combined with 60 mL of 20 mM phosphate buffer to achieve a total volume of 400 mL. Buffer B was prepared by mixing 80 mL of ethanol with 320 mL of 20 mM phosphate buffer, also to a final volume of 400 mL. The 20 mM phosphate buffer was prepared by dissolving four PBS (phosphate buffer saline) tablets (Fisher Scientific, Ottawa, ON, Canada) in 400 mL of Optimater (Fisher Scientific, Ottawa, ON, Canada).

Each faecal sample was weighed with minimum masses of 50 mg for Buffer B and 20 mg for Buffer A. Three times the sample weight of each extraction buffer was added to the respective sample. Samples were then briefly vortexed, followed by shaking at 1500 rpm for 15 minutes. Subsequently, samples were sonicated for 15 minutes and briefly vortexed again. The samples were then centrifuged at 10,000 x g for 10 minutes at 4^{o}C. The supernatant was carefully transferred to a 96-well collection plate and stored at -80°C until further processing.

TMIC Mega Kit (TMIC, Edmonton, Alberta, Canada) was used to analyse blood and faecal samples. Serum samples and faecal extracts were stored at −80 °C until analysed. Before analysis, samples were thawed on ice, vortexed for 15 seconds on a

Fisherbrand™ analog multitube vortexer (Ottawa, CA, USA), and centrifuged at 13,000 × g for 10 minutes. A 96-well plate was utilized for automated, high-throughput analysis, with 40 μL of serum or faecal extract required per sample. For PITC derivatization, 10 μL of serum, faecal extract or QC standard, 30 μL of ISTD mixture, and 10 μL of various standards were pipetted into filter plate wells. After drying the plate under steam nitrogen for 30 min, 50 μL of 5% PITC solution (300 uL of PITC derivatization reagent was added to a mixture of ethanol, water and pyridine, each 1900 uL) was added to each well, followed by incubation at room temperature for 20 min. Then, the plate was kept under gentle nitrogen steam for 90 minutes. Targeted analytes were extracted with addition of 300 μL methanol containing 5 mM ammonium acetate. The plate was covered and shaken at 330 rpm using a Thermo Scientific Thermal Mixer (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at room temperature. Then the plate was centrifuged at 50 × g for 5 min to collect the extracts from the upper filter plate to the bottom collection plate. Extracts (50 uL from each well) were transferred to a new 96-deep-well plate, diluted with 450 uL of water, and subjected to LC–MS/MS for quantifying amino acids, amino acid derivatives, biogenic amines, and nucleotides/nucleosides. A 10 μL aliquot of the remaining extracts were transferred to a separate 96-deep-well plate. Each aliquot was diluted with 490 μL of direct flow injection (DFI) buffer, composed of 60 μL of formic acid, 10 mL of water, and 290 mL of methanol. The prepared samples were then subjected to DFI–tandem mass spectrometry (DFI–MS/MS) analysis to quantify lipids, acylcarnitines, and glucose/hexose.

In order to quantify organic acids, the 3-NPH derivatization method was employed, 90 μL of ice-cold methanol was combined with 30 μL of each blank sample, calibration

standard, QC standard, and serum sample in a 600 µL microfuge tube to precipitate proteins. The mixture was vortexed thoroughly for 30 seconds and then stored at −20 °C overnight to facilitate protein precipitation. The following morning, the samples were centrifuged at 13,000× g for 20 minutes at 4°C. For faecal extracts, overnight methanol extraction was not performed. From each sample (blank sample, calibration standard, QC standard, and serum), 50 µL of supernatant was transferred to a designated well of a 96-deep-well plate as well as 10 uL of faecal extract mixed with 40 uL of methanol. Then, 75 µL of derivatization reagent was added to each well. This reagent consisted of 25 µL of 250 mM 3-NPH in 50% aqueous methanol, 25 µL of 150 mM EDC in methanol, and 25 µL of 7.5% pyridine in 75% aqueous methanol. A working internal standard (ISTD) solution was prepared in a 1.5 mL microfuge tube by mixing 50 µL of the ISTD mixture solution with 75 µL of an isotope-labelled derivatization reagent. The isotope-labelled reagent contained 25 µL of 250 mM 13C6-3-NPH in 50% aqueous methanol, 25 µL of 150 mM EDC in methanol, and 25 µL of 7.5% pyridine in 75% aqueous methanol. The 96-deep-well plate was shaken at 500 rpm for 2 hours at room temperature using a Thermo Scientific 13687721 Thermal Mixer. Afterwards, 325 µL of water and 50 µL of BHT (2 mg/mL in methanol) were added to each well. The working ISTD solution, prepared under identical conditions, was diluted with 1125 µL of water. Subsequently, 10 µL of this diluted ISTD solution was added to each well of a new 96-deep-well plate, except for the double blank sample position. From the derivatized samples, 25 µL was transferred to the corresponding wells in the new 96-well plate. Finally, 215 µL of water was added to each well in the new plate to prepare the samples for LC–MS/MS analysis.

6.3.3.3 LC/DFI–MS/MS Analysis

Derivatized samples were analysed using an Agilent 1260 series ultra-high performance liquid chromatography (UHPLC) system (Agilent Technologies, Palo Alto, CA, USA) with an Agilent reversed-phase Zorbax Eclipse XDB C18 column (3.0 mm × 100 mm, 3.5 μm particle size, 80 Å pore size) and a Phenomenex (Torrance, CA, USA) SecurityGuard C18 guard column (4.0 mm × 3.0 mm). Mass spectrometric detection was conducted using an ABSciex 5500 QTrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA).

For the UHPLC separation of the PITC panel in LC–MS/MS and DFI–MS/MS analyses, a gradient method was employed using solvent A (0.2% (v/v) formic acid in water) and solvent B (0.2% (v/v) formic acid in acetonitrile). The gradient was programmed as follows: at minute 0, 0% solvent B was used, which remained constant until t=0.5 min. Solvent B was then increased to 95% at t=5.5 min and maintained at that level until t=6.5 min. At t=7 min, solvent B was reduced back to 0%, which was maintained until t=9.5 min. The column oven was set to a temperature of 50°C, with a flow rate of 500 μL/min and an injection volume of 10 μL. The mass spectrometer operated in positive electrospray ionization mode with a scheduled multiple reaction monitoring (MRM) scan. Instrument parameters included an IonSpray voltage of 5500 V, a temperature of 500 °C, a curtain gas (CUR) setting of 20, an ion source gas 1 (GAS1) setting of 40, an ion source gas 2 (GAS2) setting of 50, and a collision gas (CAD) set to medium. The entrance potential (EP) was maintained at 10 V, while the declustering potential (DP), collision energy (CE), collision cell exit potential (CXP),

and the precursor ion (Q1) and fragment ion (Q3) settings were individually optimized for each analyte and its isotope-labelled internal standard (ISTD).

For the UHPLC separation of the 3-NPH panel targeting organic acids, ketones, and keto-acids, a different gradient method was used with solvent A (0.01% (v/v) formic acid in water) and solvent B (0.01% (v/v) formic acid in acetonitrile). The gradient started with 25% solvent B at minute 0, which increased to 65% at minute 6, then to 90% at minute 6.3, and finally to 100% at minute 6.5. Solvent B was held at 100% until the 7^{th} minute, then reduced to 25% at the 7.5^{th} minute, and maintained at that level until the 12.0^{th} minute. The column oven temperature was set to 40 °C, with a flow rate of 400 µL/min and a sample injection volume of 10 µL. The mass spectrometer operated in negative electrospray ionization mode with a scheduled MRM scan. The IonSpray voltage was set at −4500 V, with a temperature of 400 °C. The curtain gas (CUR), ion source gas 1 (GAS1), and ion source gas 2 (GAS2) were set at 20, 30, and 30, respectively, while the collision gas (CAD) was set to medium. The entrance potential (EP) was maintained at −10 V, and the DP, CE, CXP, Q1, and Q3 parameters were individually optimized for all analytes and isotope-labelled ISTDs.

6.3.3.4 Quantification

Individual seven-point calibration curves were generated for each analyte to quantify organic acids, amino acids, and biogenic amines and derivatives. The ratios of each analyte's signal intensity to its corresponding isotope-labelled internal standard were plotted against specific known concentrations using quadratic regression with a $1/x^2$ weighting. Lipids, acylcarnitines, and glucose were analysed semi-quantitatively using

a single-point calibration of a representative analyte, assuming linear regression through zero for compounds sharing the same core structure. All metabolite analyses were conducted using Analyst 1.7.2 (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA) and MultiQuant 3.0.3 (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA).

6.3.4 Statistical analysis

Statistical analysis was performed with MetaboAnalyst 6.0 (Pang et al., 2024). Metabolites with more than 20% missing values were excluded from further analysis. For the remaining metabolites, values below the limit of detection (LOD) were imputed using 1/5 of the minimum positive value of each variable. The data were log-transformed and auto-scaled. After filtration and normalization steps, available compounds were analysed for week 6 and week 12 faecal samples from both trials and week 2, week 6 and week 12 blood samples from Trial 2. Anova or t-test, and volcano plots using adjusted p-values were generated to address significant metabolites for control and MetS groups. Principal component analysis (PCA) and two-dimensional partial least squares discriminant analysis (2-D PLS-DA) score plots were used to compare plasma metabolite data across study groups. Differentiated metabolites were identified by a variable importance in projection (VIP) using a score cut-off of > 1.5. Heat maps of the top 50 significant metabolites (via t-test or ANOVA) were created via MetaboAnalyst 6.0 (Pang et al., 2024).

6.4 Results

A number of changes were detected in faecal samples for both trials as well as blood samples for trial 2, including organic acids, amino acids and derivatives, acylcarnitines, biogenic amines, phosphatidylcholines, nucleotides, indoles and derivatives, sphingolipids, sugars, lipids, ceramides, cholesterol esters, diacylglycerols, and triacylglycerols.

6.4.1 Comparison of faecal metabolome of control and MetS pigs at week 6 in Trial 1

A number of significant differences were observed between control and MetS pig faecal samples in Trial 1. PCA (Figure 6.1A) revealed significant separation of the faecal metabolomes from control pigs and MetS pigs at week 6 (PERMANOVA, p=0.001). The heatmap (Figure 6.1B) shows the top 50 metabolites with significant differences. The VIP plot (Figure 6.1C) shows that C18:1 (elaidic carnitine), kynurenic acid, C18 (stearoylcarnitine), histamine, C0 (l-carnitine), hex2cer(d18:1/22:0) and hex3cer(d18:1/18:0) had higher concentrations in HFD-fed pig (MetS group) faecal samples while 3-methyladipic acid, 2-hydroxyisobutyric acid, quinoline-4-carboxylic acid, 2-hydroxy-2-methylbutyric acid, 2-hydroxyisovaleric acid, N-acetyl-glutamic acid, pyruvic acid, N1-acetyl-lysine had higher concentrations in control group samples.

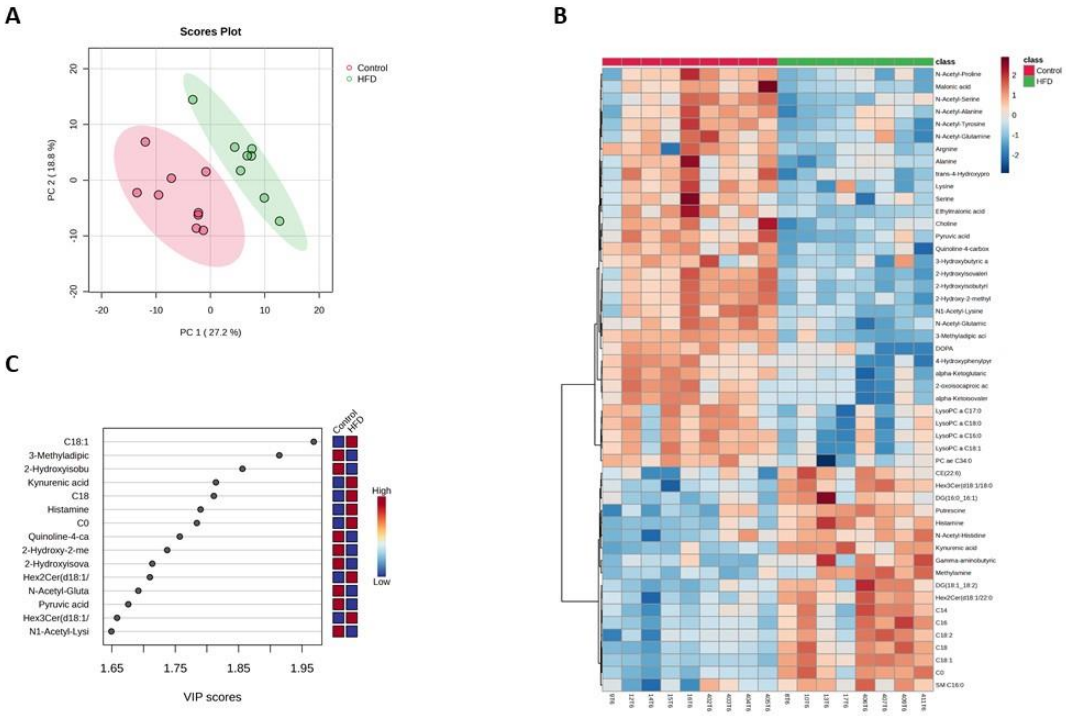

Figure 6.1 Multivariate analysis at week 6 (Trial 1). **A:** Principal Component Analysis (PCA) scatter plot for control and MetS pig samples at week 6. (Green dots = HFD-MetS, pink dots = control). PERMANOVA revealed significant separation (p=0.002). **B:** Heatmap representing the top 50 significant metabolites (t-test, FDR < 0.05) in the comparison of control and HFD-MetS groups. (Red: control; green: HFD-MetS). **C:** Rank of the top 15 metabolites identified by Partial Least Squares Discriminant Analysis (PLS-DA) according to the Variable Importance in Projection (VIP) score on the x-axis. The most discriminating metabolites are shown in descending order of their coefficient scores. The colour boxes indicate whether metabolite concentration is increased (red) or decreased (blue).

A volcano plot (Figure 6.2) was generated to illustrate the downregulated and upregulated metabolites in Trial 1. At week 6, the following metabolites were significantly higher in MetS pig faecal samples compared to control pigs: C18:1 (FDR=5.50E-06), C18 (FDR=0.00040726), kynurenic acid (FDR=0.000407), histamine (FDR=0.00055328), C0 (FDR=0.00055328), Hex2Cer(d18:1/22:0)

(FDR=0.001583), Hex3Cer(d18:1/18:0) (FDR=0.002985), methylamine (FDR=0.0053826), DG(18:1_18:2) (FDR=0.0064546), C16 (FDR=0.006737), putrescine (FDR=0.0073484), C18:2 (FDR=0.010756), DG(16:0_16:1) (FDR=0.0192), N-acetyl-histidine (FDR=0.0192), C14 (FDR=0.022317), gamma-aminobutyric acid (FDR=0.026906), CE(22:6) (FDR=0.026906), and DG(16:0_18:2) (FDR=0.045748). On the other hand, faecal samples from control pigs had higher levels of 3-methyladipic acid (FDR=3.87E-05), 2-hydroxyisobutyric acid (FDR=0.00019594), 2-hydroxyisovaleric acid (FDR=0.001583), N-acetyl-glutamic acid (FDR=0.0020051), pyruvic acid (FDR=0.0024183), N1-acetyl-lysine (FDR=0.003206), alpha-ketoglutaric acid (FDR=0.0035186), alpha-ketoisovaleric acid (FDR=0.0076129), choline (FDR=0.01035), DOPA (FDR=0.010756), malonic acid (FDR=0.010756), 2-oxoisocaproic acid (FDR=0.011253), 4-hydroxyphenylpyruvic acid (FDR=0.010756), trans-4-hydroxyproline (FDR=0.012996), N-acetyl-serine (FDR=0.019061), LysoPC a C18:1 (FDR=0.014061), PC ae C34:0 (FDR= 0.021808), alanine (FDR=0.021872), N-acetyl-proline (FDR=0.026014), N-Acetyl-Tyrosine (FDR=0.026906), 3-Hydroxybutyric acid (FDR=0.027211), dopamine (FDR=0.028549), N-acetyl-alanine (FDR=0.033278), LysoPC a C18:0 (FDR=0.036343), arginine (FDR=0.042466), benzoic acid (FDR=0.045556), LysoPC a C18:2 (FDR=0.04658), N-acetyl-aspartic acid (FDR=0.0473) compared to faecal samples from MetS pigs at week 6 of Trial 1.

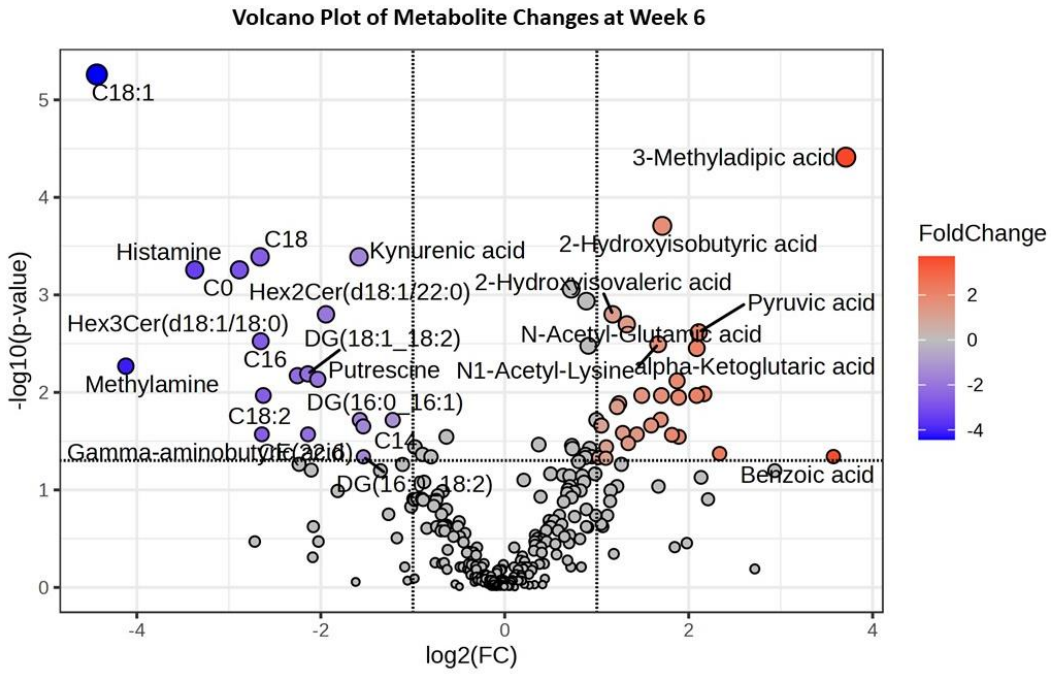

Figure 6.2 The volcano plot illustrates the significant fold changes in metabolite levels between control and MetS pig faecal samples at week 6 in Trial 1. Each point represents an individual metabolite. The x-axis shows the log2 fold change of metabolites, with positive values indicating higher levels in the control group and negative values indicating higher levels in the MetS group. The y-axis shows the -log10 of the false discovery rate (FDR), with higher values indicating greater statistical significance. Blue points represent metabolites that are significantly upregulated in the MetS group (FDR < 0.05); while red points represent metabolites with significantly, higher levels in the control group (FDR < 0.05). Points that do not meet the significance threshold are shown in grey. The dashed horizontal line represents the FDR threshold of 0.05.

6.4.2 Comparison of faecal metabolome of control and MetS pigs at week 12 in Trial 1

Faecal samples were compared from control and MetS pigs at week 12 of Trial 1. PCA (Figure 6.3A) shows significant separation between the groups (PERMANOVA,

p=0.016). The heatmap (Figure 6.3B) shows the top 50 metabolites with significant differences across the control and MetS pig faecal samples (t test, FDR<0.05).

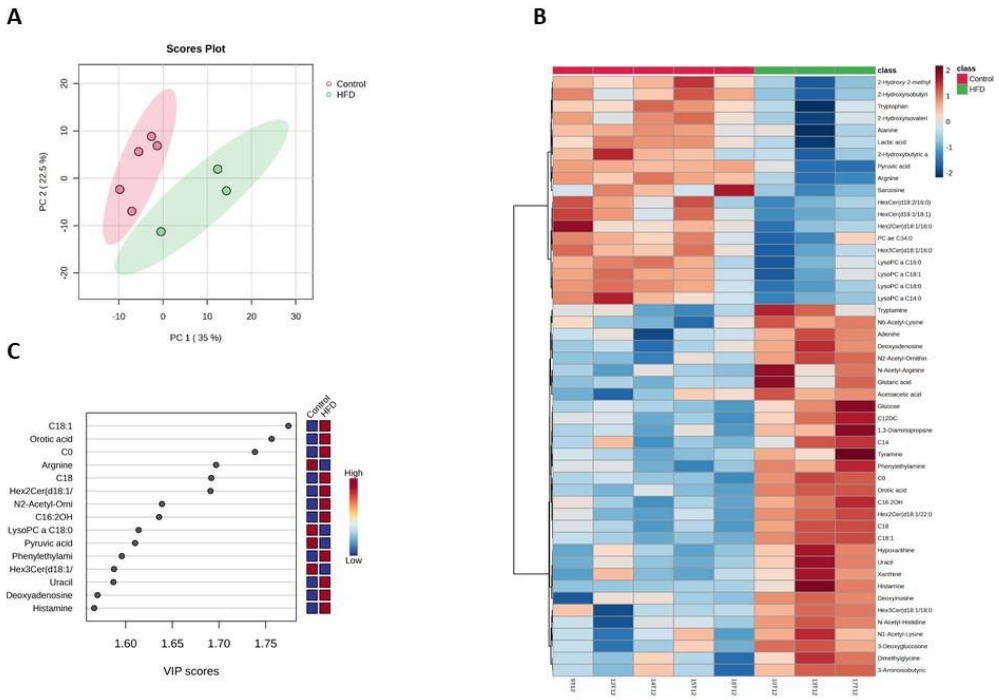

Figure 6.3 Multivariate analysis at week 12 of Trial 1. (**A)** Principal Component Analysis (PCA) scatter plot for control and MetS pig samples at Week 12. Green dots represent HFD-MetS and pink dots represent control samples. PERMANOVA analysis revealed significant separation between faecal samples from control and HFD-MetS pigs (p=0.012) (**B**) Representative Heatmap of top 50 significant metabolites (t-test, FDR<0.05) in the comparison of control and HFD-MetS (red: Control, green: MetS). (**C)** Rank of the top 15 metabolites identified by the PLS-DA according to the VIP score on the x-axis (VIP score>1.5). The most discriminating metabolites are shown in descending order of their coefficient scores. The color boxes indicate whether metabolite concentration is increased (red) or decreased (blue).

The VIP plot (Figure 6.3C) shows that C18:1, orotic acid, C0, C18, hex2cer (d18:1/22:0), N2-acetyl-ornithine, C16:2OH, phenylethylamine, uracil, deoxyadenosine and histamine had higher concentrations in HFD-fed pig (MetS

group) faecal samples while arginine, lysoPC a C18:0, pyruvic acid, and hex3Cer (d18:1/16:0) had higher concentrations in control group samples.

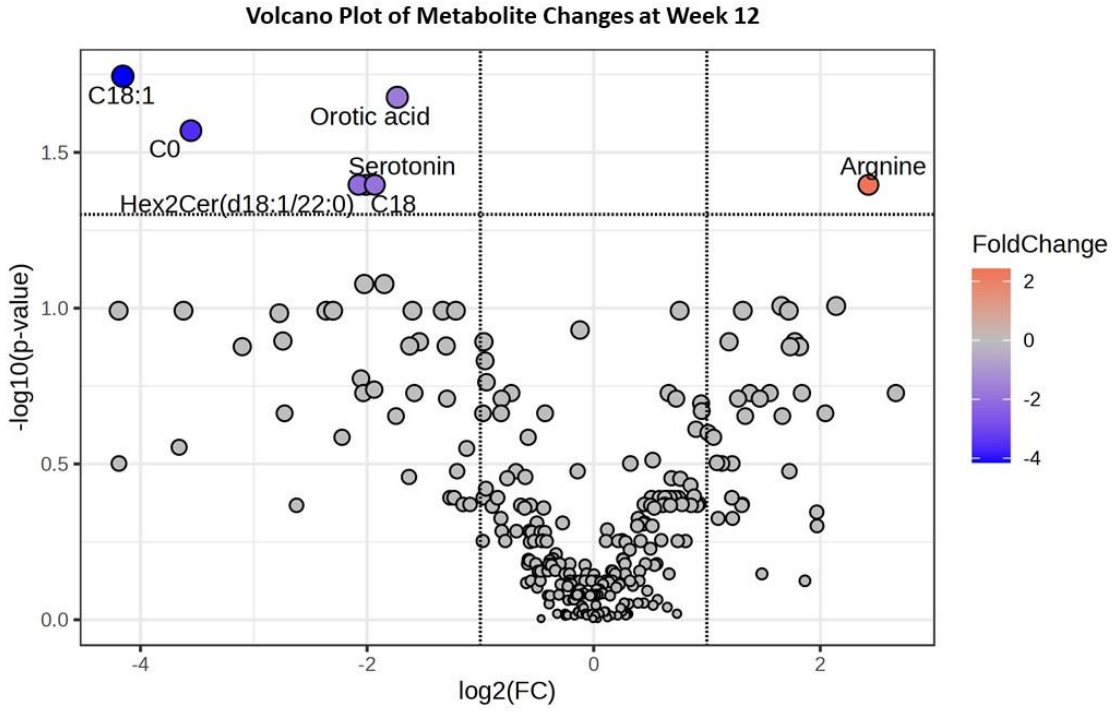

Figure 6.4 The volcano plot illustrates the significant fold changes in metabolite levels between control and MetS pig faecal samples at week 12. Each point represents an individual metabolite. The x-axis shows the log2 fold change of metabolites, with positive values indicating higher levels in the control group and negative values indicating higher levels in the MetS group. The y-axis shows the -log10 of the false discovery rate (FDR), with higher values indicating greater statistical significance. Blue points represent metabolites that are significantly upregulated in the MetS group (FDR < 0.05); while red points represent metabolites with significantly higher levels in the control group (FDR < 0.05). Points that do not meet the significance threshold are shown in grey. The dashed horizontal line represents the FDR threshold of 0.05.

A volcano plot (Figure 6.4) was generated to show upregulated and downregulated metabolites at the end of the intervention (week 12) in Trial 1. Faecal samples from MetS pigs had higher levels of C18:1 (FDR=0.018015), orotic acid (FDR=0.021044), C0 (FDR=0.026928), Hex2Cer (d18:1/22:0) (FDR=0.04019), C18 (FDR=0.04019), and serotonin (FDR=0.04019) compared to samples from control group. Arginine level was found to be significantly lower in faecal samples from MetS pigs at week 12 compared to samples from control pigs (FDR=0.04019).

Compared to week 6, several shifts in metabolite significance were observed at week 12. A subset of metabolites that discriminated between control and MetS pigs at week 6 were no longer significantly different by week 12, suggesting a shift in metabolic profiles over time. These included kynurenic acid, methylamine, putrescine, several diglycerides (DG(18:1_18:2), DG(16:0_16:1), DG(16:0_18:2)), N-acetyl-histidine, gamma-aminobutyric acid, CE(22:6), and multiple control-associated metabolites such as 3-methyladipic acid, 2-hydroxyisobutyric acid, pyruvic acid, N-acetyl-glutamic acid, and dopamine. Conversely, several metabolites became significantly altered only at week 12. In MetS pigs, levels of orotic acid, N2-acetyl-ornithine, C16:2OH, phenylethylamine, uracil, deoxyadenosine, and serotonin were elevated, while hex3Cer(d18:1/16:0) was higher in control pigs.

6.4.3 The effect of oral administration of *Lb mucosae* DPC6426 and fibre singly and in combination (synbiotic) on pig faecal metabolomics in Trial 2

At week 6 and week 12 of Trial 2, faecal samples were collected to perform targeted metabolomics analysis to reveal the effect of administration of *Lb. mucosae* DPC6426 and fibre singly and in combination.

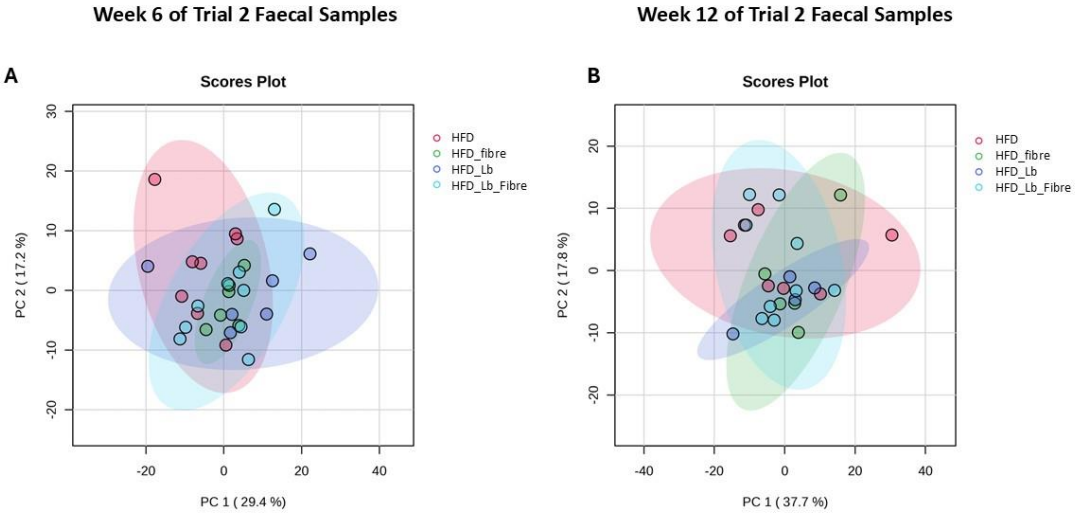

Figure 6.5 Principal Component Analysis (PCA) scatter plot for Trial 2 faecal samples at week 6 (A) and week 12 (B). The groups are high fat, sugar, and salt diet (HFD), HFD with fibre (HFD_fibre), HFD supplemented with *Lb mucosae* DPC6426 (HFD_Lb), and HFD supplemented with combination of fibre and *Lb mucosae* DPC6426 (HFD_Lb_fibre). PERMANOVA analysis did not reveal significant separation between faecal samples from groups ($p>0.05$).

PCA analysis did not reveal a significant separation between the treatment groups at week 6 (Figure 6.5A) and week 12 (Figure 6.5B) (PERMANOVA, $p>0.05$) of Trial 2. The VIP plot (Figure 6.6A) shows C12:1 (trans-2-dodecenoylcarnitine), C12

(dodecanoylcarnitine) and 2-Hydroxybutyric acid levels were higher in faecal samples from HFD_Lb_fibre pigs while Hex2Cer (d18:1/18:0), C5DC (glutarylcarnitine), C8 (octanoylcarnitine), PC aa C40:4, C4:1 (butenylcarnitine), LysoPC a C20:3, DG (18:1_20:4), C6 (hexanoylcarnitine) and CE(22:6) levels were higher in faecal samples from HFD_Lb pigs. Pigs that were fed with fibre (HFD_fibre) showed higher levels of C16:1OH, C16OH, and C4 at week 6 of Trial 2.

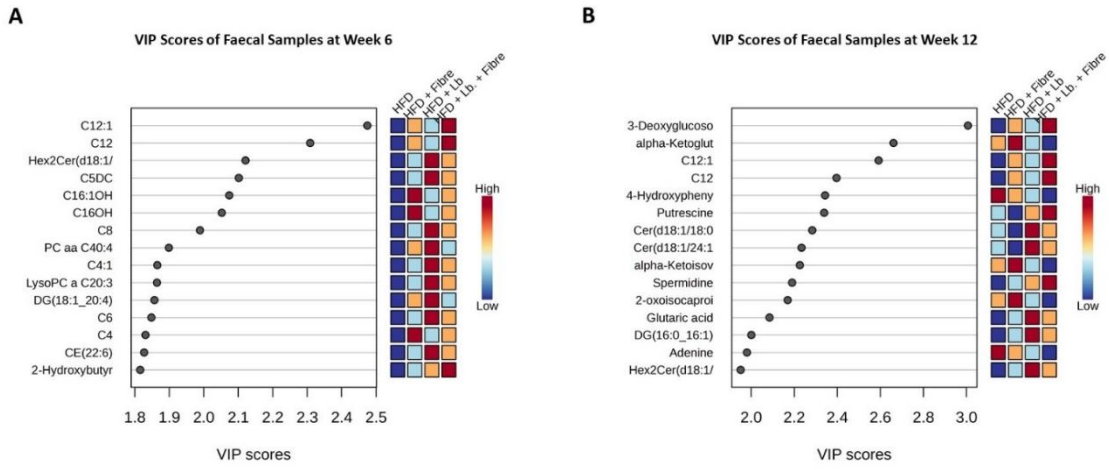

Figure 6.6 The plots represent rank of the top 15 metabolites identified by the PLS-DA according to the VIP score on the x-axis (VIP score>1.5) in Trial 2. **(A)** Top 15 VIP scores of faecal samples at week 6 and **(B)** Top 15 VIP scores of faecal samples at week 12. The most discriminating metabolites are shown in descending order of their coefficient scores. The color boxes indicate whether metabolite concentration is increased (red) or decreased (blue). The groups are high fat, sugar, and salt diet (HFD), HFD with fibre (HFD_fibre), HFD supplemented with *Lb mucosae* DPC6426 (HFD_Lb), and HFD supplemented with combination of fibre and *Lb mucosae* DPC6426 (HFD_Lb_fibre).

At week 12 (Figure 6.6B), 3-deoxyglucosone, C12:1, C12, putrescine and spermidine were higher in HFD_Lb_fibre group while Cer(d18:1/18:0), Cer(d18:1/24:1), glutaric acid, DG(16:0_16:1) and hex2cer(d18:1/20:0) levels were higher in HFD_Lb group.

HFD_fibre had higher levels of alpha-ketoglutaric acid, alpha-ketoisovaleric acid and 2-oxoisocaproic acid. HFD had higher levels of 4-hydroxyphenylpyruvic acid and adenine.

6.4.4 The synbiotic effect of *Lb mucosae* DPC6426 and fibre on serum metabolites in Trial 2

The serum metabolites were compared at week 2, week 6, and week 12 (Trial 2) for the four intervention groups including serum samples from HFD, HFD_fibre, HFD_Lb and HFD_Lb_fibre. PCA analysis (Figure 6.7) showed no significant separation between the groups (PERMANOVA, $p>0.05$). At week 2, the VIP plot (Figure 6.8A) shows alanine, 5-oxoproline, indole-3-propionic acid, N-acetyl-proline, lactic acid, and pipecolic acid were higher in HFD serum samples. Serum samples from HFD_fibre pigs showed higher levels of N-acetyl-alanine, PC ae C40:3, Cer(d18:1/22:0) and PC ae C40:2 compared to the other three groups. HFD_Lb_fibre group had higher levels of LysoPC a C17:0, PC ae C44:3, DG(18:1_20:4), C18 and LysoPC a C16:1.

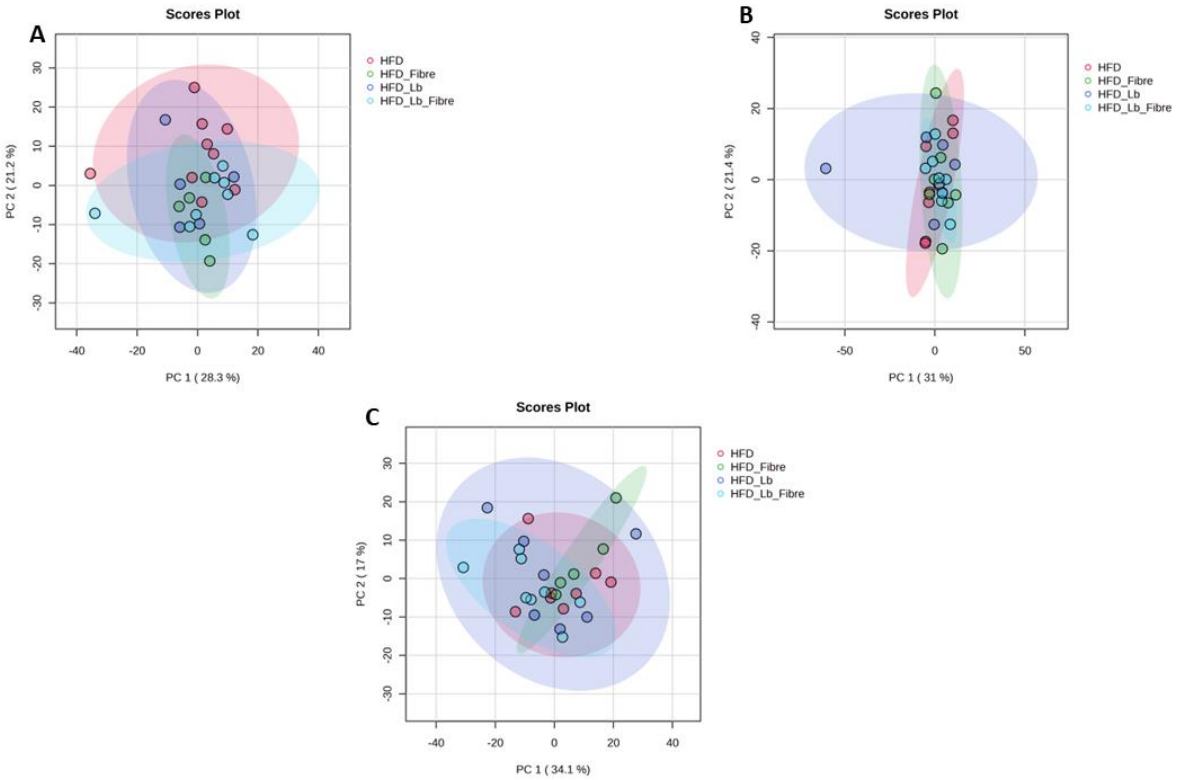

Figure 6.7 Principal Component Analysis (PCA) scatter plot for Trial 2 serum samples at week 2 (A), week 6 (B) and week 12 (C). The groups are high fat, sugar, and salt diet (HFD), HFD with fibre (HFD_fibre), HFD supplemented with *Lb mucosae* DPC6426 (HFD_Lb), and HFD supplemented with combination of fibre and *Lb mucosae* DPC6426 (HFD_Lb_fibre). PERMANOVA analysis did not reveal significant separation between faecal samples from groups ($p>0.05$).

At week 6, indole-3-propionic acid, DG(16:0_18:1), DG(16:0_18:2), TG(18:0_30:1), ethanolamine, and DG(16:0_20:0) levels were higher in serum samples from the HFD group (Figure 6.8B). N-acetyl-glycine, alpha-aminobutyric acid, alpha-ketoisovaleric acid, serine, methionine, 3-hydroxybutyric acid and phenylalanine levels were higher in HFD_Lb_fibre serum samples compared to others. Moreover, HFD_fibre samples had higher levels of Hex2Cer(d18:1/14:0) and p-hydroxyhippuric acid. At the end of the intervention (week 12), the VIP plot (Figure 6.8C) shows N2-acetyl-ornithine,

putrescine and urea levels were higher in HFD serum samples while LysoPC a C17:0, LysoPC a C16:0, LysoPC a C18:1, LysoPC a C20:4, PC ae C36:1, LysoPC a C16:1, lysoPC a C28:1, PC aa C26:0 were higher in HFD_Lb_fibre group than others. Besides, PC aa C24:0, LysoPC a C26:0, HexCer(d18:2/23:0) and TG(17:0_24:1) were higher in serum samples from HFD_Lb.

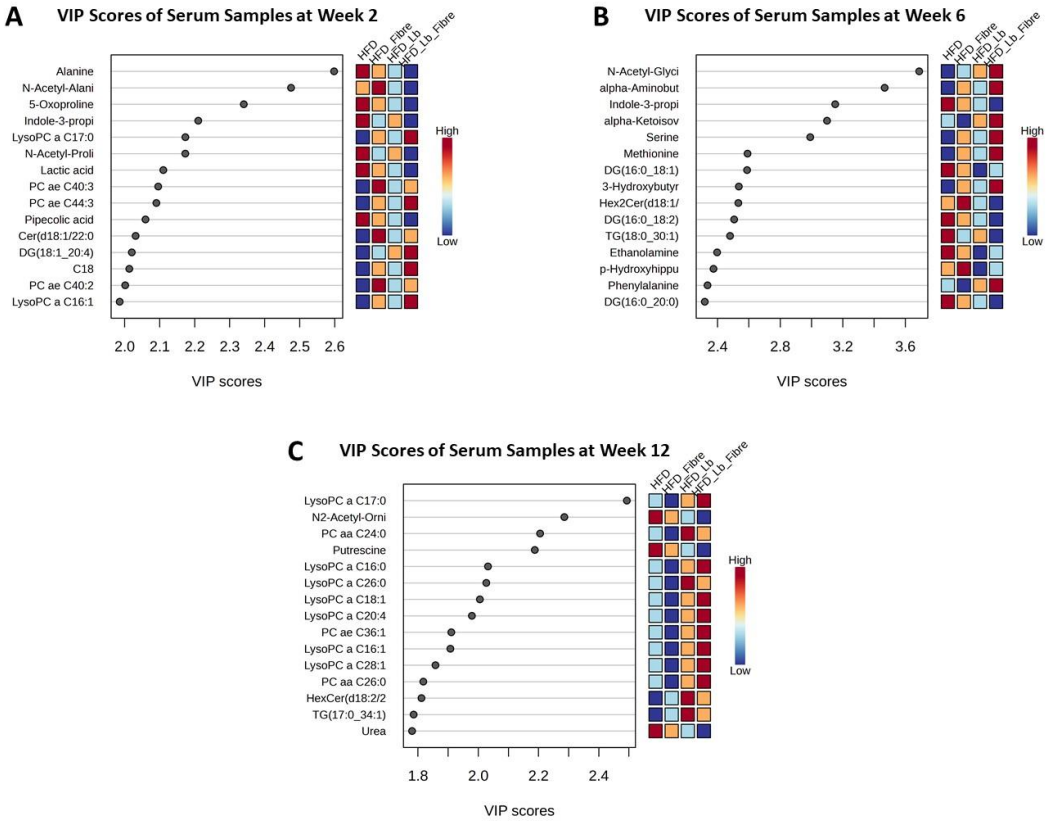

Figure 6.8 The plots represent rank of the top 15 metabolites identified by the PLS-DA according to the VIP score on the x-axis (VIP score>1.5) in Trial 2. **(A)** Top 15 VIP scores of serum samples at week 2, **(B)** Top 15 VIP scores of serum samples at week 6, **(C)** Top 15 VIP scores of serum samples at week 12. The most discriminating metabolites are shown in descending order of their coefficient scores. The color boxes indicate whether metabolite concentration is increased (red) or decreased (blue). The

groups are high fat, sugar, and salt diet (HFD), HFD with fibre (HFD_fibre), HFD supplemented with *Lb mucosae* DPC6426 (HFD_Lb), and HFD supplemented with combination of fibre and *Lb mucosae* DPC6426 (HFD_Lb_fibre).

6.5 Discussion

The present study investigated the impact of oral supplementation of *Lb. mucosae* DPC6426 and dietary fibre on metabolic profiles in a porcine model of MetS. We first compared faecal samples from Trial 1, the first porcine trial where we developed the MetS model using a high fat, sugar, and salt diet (O'Donovan et al., 2020). In Trial 1, faecal metabolomic analysis revealed significant differences between control and MetS pigs at weeks 6 and 12. At week 6, PCA demonstrated clear separation between the groups (PERMANOVA, p=0.001), with distinct metabolite profiles. MetS pigs had elevated levels of metabolites such as elaidic carnitine (C18:1), kynurenic acid, and histamine, while control pigs exhibited higher concentrations of metabolites including 3-methyladipic acid and pyruvic acid. Similarly, at week 12, significant separation between control and MetS pigs was observed (PERMANOVA, p=0.016). MetS pigs displayed increased concentrations of metabolites like orotic acid and serotonin, whereas control pigs had higher levels of arginine and pyruvic acid. Next, we examined faecal and blood samples from Trial 2 which included four different treatment groups: HFD, HFD_Lb (HFD supplemented with *Lb mucosae* DPC6426), HFD_fibre (HFD supplemented with fibre), and HFD_Lb_fibre (HFD supplemented with a combination of *Lb mucosae* DPC6426 and fibre). In Trial 2, the effects of the interventions on the faecal metabolome were investigated at both week 6 and week 12. PCA analysis revealed no significant separation between treatment groups at either time point (PERMANOVA, p>0.05). However, certain metabolites showed treatment-

related trends. Faecal samples from pigs receiving the synbiotic treatment exhibited higher levels of trans-2-dodecenoylcarnitine (C12:1) and 2-hydroxybutyric acid, whereas other metabolites, such as glutarylcarnitine (C5DC) and octanoylcarnitine (C8), were more elevated in other treatment groups. These results indicate that the synbiotic intervention may influence the faecal metabolome, though differences were not statistically significant.

6.5.1 Metabolite changes in HFD vs. control pigs (Trial 1)

6.5.1.1 Carnitine and acylcarnitines

Faecal metabolites such as C18:1 (elaidic carnitine), C18 (stearoylcarnitine), and C0 (L-carnitine) were significantly higher in the MetS group compared to control pigs in Trial 1. Carnitine and acylcarnitines play crucial roles in fatty acid metabolism, particularly in the transport of long-chain fatty acids into the mitochondria for β-oxidation (Longo et al., 2016). The elevated levels of these metabolites in the HFD group suggest an increased reliance on fatty acid oxidation due to excess dietary fat. Studies have reported that dysbiosis in the gut microbiota, often observed in metabolic disorders, can lead to increased faecal levels of carnitine and acylcarnitines, contributing to systemic inflammation and metabolic disturbances (Lloyd-Price et al., 2019, Smith et al., 2021b). A study reported high levels of elaidic carnitine in hepatic cirrhosis patients' faecal samples compared to healthy controls (Huang et al., 2013). Carnitines were found to promote the growth of *Escherichia coli* through anaerobic respiration (Lemons et al., 2024). The observed increase in C0 and other acylcarnitines in the HFD group aligns with these mechanisms, underscoring the link between altered lipid metabolism and MetS.

6.5.1.2 Kynurenic acid

Kynurenic acid, another metabolite found in higher concentrations in the faecal samples from MetS pigs compared to controls at week 6 in Trial 1, is a tryptophan metabolite involved in the kynurenine pathway. This pathway is crucial in modulating immune responses and maintaining the balance between neuroprotective and neurotoxic compounds (Alexander et al., 2012). Elevated levels of kynurenic acid have been associated with obesity, insulin resistance, and cardiovascular disease (Ala and Eftekhar, 2022, Tsuji et al., 2023). Kynurenine pathway metabolites are linked to neuroinflammatory processes and energy metabolism, which could play a role in the development of insulin resistance (Tsuji et al., 2023). The increased kynurenic acid in the MetS pigs could indicate enhanced tryptophan catabolism, which is often triggered by chronic low-grade inflammation, a hallmark of MetS and obesity (Lischka et al., 2022). Kynurenic acid has also been implicated in the regulation of energy homeostasis and appetite, with studies suggesting that it may contribute to the development of obesity by modulating central nervous system pathways (Favennec et al., 2015).

In addition, the kynurenine pathway is closely linked to the immune system, particularly in the production of NAD+ (nicotinamide adenine dinucleotide), which is essential for cellular energy metabolism and repair processes (Cervenka et al., 2017). Dysregulation of this pathway, as indicated by increased kynurenic acid levels, can lead to an imbalance in NAD+ production, contributing to the metabolic dysfunction observed in MetS. Moreover, kynurenic acid acts as an antagonist at the NMDA (N-methyl-D-aspartate) receptor, which plays a role in neuroinflammation and

neurodegeneration (Pathak et al., 2024). Therefore, the elevated kynurenic acid in HFD-fed pigs not only reflects metabolic disturbances but also suggests potential implications for brain health in the context of MetS.

6.5.1.3 Histamine

Histamine levels were also elevated in the HFD group in Trial 1, which is significant given histamine's role as a mediator of inflammation and immune responses. Histamine is produced by the decarboxylation of histidine, a reaction catalysed by histidine decarboxylase. Histamine production often rises in a dysbiotic gut, leading to increased intestinal permeability, which may exacerbate the low-grade inflammation typically observed in MetS (Sanchez-Perez et al., 2022). Increased intestinal permeability represents a significant factor in the pathophysiology of MetS, as it can lead to the translocation of lipopolysaccharides (LPS) and other inflammatory mediators into the systemic circulation (Cani et al., 2008). Notably, increased histamine levels in the MetS group may reflect alterations in gut microbiota composition and associated inflammation (Branco et al., 2018). Histamine has been shown to influence energy homeostasis by modulating appetite and glucose metabolism, with elevated levels potentially leading to dysregulation of these processes (Maslowski and Mackay, 2011).

Moreover, histamine plays a role in the regulation of adipose tissue function. Adipocytes express histamine receptors, and the activation of these receptors by histamine can influence lipolysis and adipogenesis (Provensi et al., 2016). In obesity, altered histamine signalling may contribute to adipose tissue dysfunction, promoting insulin resistance and systemic inflammation. The observed increase in histamine in

the HFD group thus highlights its potential role in exacerbating metabolic disturbances and inflammation in MetS.

6.5.1.4 Ceramides

Hexosylceramides, specifically Hex2Cer(d18:1/22:0) and Hex3Cer(d18:1/18:0), were found in higher concentrations in the HFD group in Trial 1. Ceramides are sphingolipids that play a critical role in cellular signalling, particularly in apoptosis, insulin resistance, and inflammation (Hannun and Obeid, 2008). Elevated ceramide levels have been linked to the development of insulin resistance, a key feature of MetS (Bikman and Summers, 2011). Ceramides interfere with insulin signalling by inhibiting the activation of Akt, a kinase essential for glucose uptake and metabolism (Holland and Summers, 2008). The increased levels of hexosylceramides in the HFD group suggest that a high-fat diet may enhance sphingolipid synthesis, contributing to insulin resistance and the progression of MetS.

Ceramides are also involved in the regulation of adipose tissue inflammation. They can activate inflammatory pathways such as NF-κB, leading to the production of pro-inflammatory cytokines and further promoting insulin resistance (Shoelson et al., 2006, Summers, 2006). Additionally, ceramides have been implicated in the development of hepatic steatosis and cardiovascular disease, both of which are common complications of MetS (Bikman and Summers, 2011). The observed increase in ceramides in HFD-fed pigs is consistent with these findings and underscores the role of sphingolipid metabolism in the pathophysiology of MetS.

6.5.1.5 Pyruvic acid and other energy metabolism-related metabolites

In contrast to the HFD group, the control group in Trial 1 had higher levels of faecal metabolites involved in energy metabolism, such as pyruvic acid, a key intermediate in glycolysis and essential for the production of ATP through oxidative phosphorylation (Mayes and Bender, 2003). The reduced levels of pyruvic acid in the HFD group suggest impaired glycolytic flux, likely due to the shift towards fatty acid oxidation as the primary energy source in response to excess dietary fat. This metabolic shift is characteristic of MetS, where glucose utilization is often compromised, leading to hyperglycaemia and insulin resistance (Shulman, 2000).

The control group in Trial 1 also had higher levels of 3-methyladipic acid, 2-hydroxyisobutyric acid, and N1-acetyl-lysine, which are indicative of normal mitochondrial function and energy production. These metabolites are involved in the catabolism of branched-chain amino acids (BCAAs), which are crucial for maintaining muscle mass and metabolic health (Nie et al., 2018). The observed differences in these metabolites between the control and HFD groups might highlight the metabolic dysregulation caused by a high-fat diet and the potential protective effects of maintaining a balanced diet.

6.5.1.6 Amino acid metabolism

Amino acid metabolism, particularly involving arginine, was significantly altered in the MetS group in Trial 1. Arginine is a precursor for nitric oxide (NO), a molecule that plays a critical role in vascular function and insulin signalling (Wu and Morris, 1998). The depletion of arginine observed in the HFD group at week 12 is concerning, as reduced NO production can lead to endothelial dysfunction, a common complication

of MetS and cardiovascular diseases (Forstermann and Sessa, 2012). Furthermore, arginine is involved in the urea cycle, which detoxifies ammonia produced during protein metabolism. Disruption of this cycle, as indicated by altered arginine levels, can contribute to metabolic acidosis and other metabolic disturbances in MetS (Morris, 2002). Moreover, arginine may play an important role in metabolic pathways and reduce pro-inflammatory cytokines and kidney function which will affect the development of MetS (Lent-Schochet et al., 2019).

6.5.1.7 Orotic acid

Orotic acid, another metabolite that was elevated in the HFD group at week 12 in Trail 1, is an intermediate in the *de novo* synthesis of pyrimidines, which are essential for DNA and RNA synthesis (Rosenfeldt et al., 1998). Elevated orotic acid levels have been associated with hepatic steatosis and liver dysfunction, both of which are common in MetS (Matilainen et al., 2020). The increase in orotic acid in the HFD group suggests that a high-fat diet may disrupt nucleotide metabolism, contributing to liver pathology and the overall metabolic disturbances observed in MetS.

6.5.2 Effects of *Lb mucosae* DPC6426 supplementation on faecal and blood metabolites in Trial 2

6.5.2.1 Faecal metabolite profiles

The HFD_Lb group in Trial 2 exhibited elevated levels of hexanoylcarnitine (C6) and octanoylcarnitine (C8) in faecal samples at week 6, indicating that supplementation with *Lb mucosae* DPC6426 may be influencing lipid metabolism and fatty acid oxidation processes by increasing the capacity for lipid catabolism, likely due to the

modulation of gut microbiota composition and function. Essentially, the presence of these acylcarnitines in the faeces may imply that the body is actively processing and breaking down fatty acids, leading to measurable changes in metabolic byproducts. A low-calorie diet intake for 12 weeks by overweight individuals significantly reduced visceral fat and increased plasma levels of medium- and long-chain acylcarnitines including hexanoylcarnitine and octanoylcarnitine during weight loss (Kang et al., 2018). Probiotic supplementation has been shown to have beneficial effects on lipid metabolism, including reducing serum cholesterol levels and modulating bile acid metabolism, which can contribute to improved metabolic health (Ooi and Liong, 2010).

The combination of fibre and *Lb mucosae* DPC6426 appeared to produce a synergistic effect, with higher levels of + and 3-deoxyglucosone observed in the HFD_Lb_fibre group by week 12 in the faecal metabolome in Trial 2. Spermidine is a polyamine that has been shown to extend lifespan and promote autophagy, a cellular process that removes damaged organelles and proteins (Eisenberg et al., 2009). The increased levels of spermidine in the combined intervention group suggest enhanced autophagic activity, which could help mitigate the cellular stress and inflammation associated with MetS (Madeo et al., 2018). Spermidine supplementation has been shown to protect against diet-induced obesity in animal models (Choksomngam et al., 2021). Another recent study showed that spermidine can improve gut barrier integrity in an obese mouse model (Ma et al., 2020).

3-Deoxyglucosone was also higher in the faecal metabolome of HFD_Lb_fibre groups at week 12 in Trial 2. A recent European cohort study investigated the dietary intake

of 3-deoxyglucosone and changes in body weight over time in adults. They showed an inverse association between 3-deoxyglucosone and body weight gain among younger and normal-weight participants (Debras et al., 2024).

6.5.2.2 Serum metabolite profiles

The analysis of serum metabolites revealed significant differences between the intervention groups in Trial 2 at week 2, week 6, and week 12. Specific metabolites, such as N-acetyl-glycine, serine, methionine, and phenylalanine were elevated in the HFD_Lb_fibre group at week 6 compared to the HFD group. These amino acids are involved in protein metabolism and may indicate improved nitrogen balance and reduced muscle breakdown, which are important for maintaining metabolic health in the context of a high-fat diet (Wang et al., 2013, Locasale, 2013). The higher levels of these amino acids suggest that the combination of fibre and *Lb. mucosae* DPC6426 may support better amino acid metabolism and redox balance, potentially mitigating some of the oxidative stress and inflammation associated with HFD-induced MetS.

Our study also revealed that the combination of fibre and *Lb mucosae* DPC6426 supplementation can reduce the amount of N2-acetylornithine significantly at week 12 (HFD_Lb_fibre - HFD, p=0.027). Increased N-acetylornithine was associated with the development of diabetes (Lillo et al., 2022). The metabolomics profile of plasma samples from South Asian Indian men showed an increase of the N-acetylornithine level in patients with type 2 diabetes-derived nephropathy that is further increased in diabetes-derived nephropathy (Devi et al., 2019). The increased level of N-acetylornithine may be because of an increased level of acetate in diabetes (Lillo et al., 2022).

Our study has also shown some serum LPC levels including LysoPC a C17:0 (p=0.003243), LysoPC a C16:0 (p=0.032668), LysoPC a C16:1 (p=0.047017) were increased significantly at week 12 with supplementation of fibre and *Lb mucosae* DPC6426. Until recent years, studies reported a positive correlation between serum LPC levels and the risk of cardiovascular diseases (Law et al., 2019). In contrast, recent studies have reported decreased LysoPC levels in disease states (Knuplez and Marsche, 2020). Importantly, a reduction in LPCs was associated with a risk of adverse outcomes in chronic kidney disease patients (Duranton et al., 2019). Decreased levels of LPC were observed in rheumatoid arthritis (Fuchs et al., 2005), diabetes (Barber et al., 2012), polycystic ovary syndrome (Sun et al., 2019), Alzheimer's disease (Grimm et al., 2011, Mulder et al., 2003), pulmonary arterial hypertension (Chen et al., 2020), aging (Semba et al., 2019), asthma (Ried et al., 2013) and liver cirrhosis, where they were associated with increased mortality risk (Krautbauer et al., 2016). Plasma LPC levels were found to be reduced in sepsis (Park et al., 2014) and correlated inversely with sepsis mortality (Drobnik et al., 2003) and in-hospital mortality in pneumonia (Cho et al., 2015). A recent study showed LPC (C17:0) improves HFD-induced hyperglycaemia and insulin resistance in a mouse study (Bao et al., 2022). Additionally, higher blood concentrations of LPC are positively correlated with the muscle insulin sensitivity index in diabetic patients (van der Kolk et al., 2019) and inversely correlate with impaired fasting glucose and diabetes incidence (Wang et al., 2018, Razquin et al., 2018, Zeng et al., 2019, Diamanti et al., 2019). Reduced plasma LPC levels were found in obese subjects and LPC was negatively correlated with body mass index and C-reactive protein level (Heimerl et al., 2014).

At week 12, PC aa C24:0 (p=0.0017813) and LysoPC 26:0 (p=0.011595) levels were higher in the HFD_Lb group, while they were lower in the HFD_Lb_fibre group. Additionally, both PC ae C36:1 (p=0.010409) and PC aa C26:0 levels were elevated in the HFD_Lb_fibre group. Phosphatidylcholine (PC) is one of the most abundant phospholipids which regulates lipid, lipoprotein and whole-body energy metabolism (van der Veen et al., 2017). A clinical study involving elderly participants found a link between a lower likelihood of having abnormal insulin resistance (as measured by HOMA-IR) and certain species of phosphatidylcholine (Semba et al., 2018). They found an inverse correlation between high plasma glucose levels and insulin resistance with phosphatidylcholine levels. These findings indicate that phosphatidylcholine species with longer chain lengths and unsaturated fatty acids may help protect against the risk of diabetes and insulin resistance. LPC and PC were also shown to have a anti-inflammatory effect on *in vitro* cell culture models (Treede et al., 2007).

Serum putrescine level was found to be significantly lower in the HFD_Lb_fibre group (ANOVA, p=0.041015) at week 12 but not at week 6. Elevated levels of putrescine are associated with metabolic dysregulations, often observed in individuals with obesity and metabolic syndrome (Fernandez-Garcia et al., 2019). Moreover, putrescine has been associated with promoting the proliferation of colon cancer cells (Farriol et al., 2001). The reduction in the samples following *Lb mucosae* DPC6426 and fibre supplementation suggests potential benefits in mitigating metabolic dysregulation associated with obesity. Given the link between elevated putrescine and metabolic syndrome as well as colon cancer proliferation, these findings highlight the importance of combined prebiotic and probiotic interventions in promoting metabolic health.

Similarly, serum urea levels were found to be lower in the HFD_Lb_fibre group than the HFD at week 12. Uric acid has been associated with increasing risk of cardiovascular disease and metabolic syndrome (Nejatinamini et al., 2015). Increased levels of urea in serum have been associated with obesity and may be correlated with insulin resistance (Li et al., 2021).

6.6 Conclusion

This study elucidates the significant impact of dietary interventions, specifically *Lb. mucosae* DPC6426 and dietary fibre, on metabolic profiles in a porcine model of metabolic syndrome. These data suggest that the synbiotic combination of *Lb. mucosae* DPC6426 and dietary fibre may exert its beneficial effects through multiple mechanisms. First, the synbiotic enhanced fatty acid oxidation and lipid metabolism, as indicated by elevated acylcarnitines such as hexanoylcarnitine and octanoylcarnitine, suggesting improved mitochondrial function. Additionally, the increased production of spermidine in the synbiotic group points to enhanced autophagy, which could mitigate cellular stress and inflammation commonly associated with metabolic syndrome. The reduction of N-acetyl-ornithine, a metabolite linked to diabetes, further supports the role of the synbiotic in improving insulin sensitivity. Together, these metabolic shifts highlight the potential for a multifaceted mechanism involving enhanced lipid catabolism, and modulation of inflammatory pathways. Future studies should explore the long-term impacts of these interventions and further elucidate the gut-cardiac axis to develop novel therapeutic strategies for metabolic syndrome management.

6.7 Acknowledgements

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6.8 References

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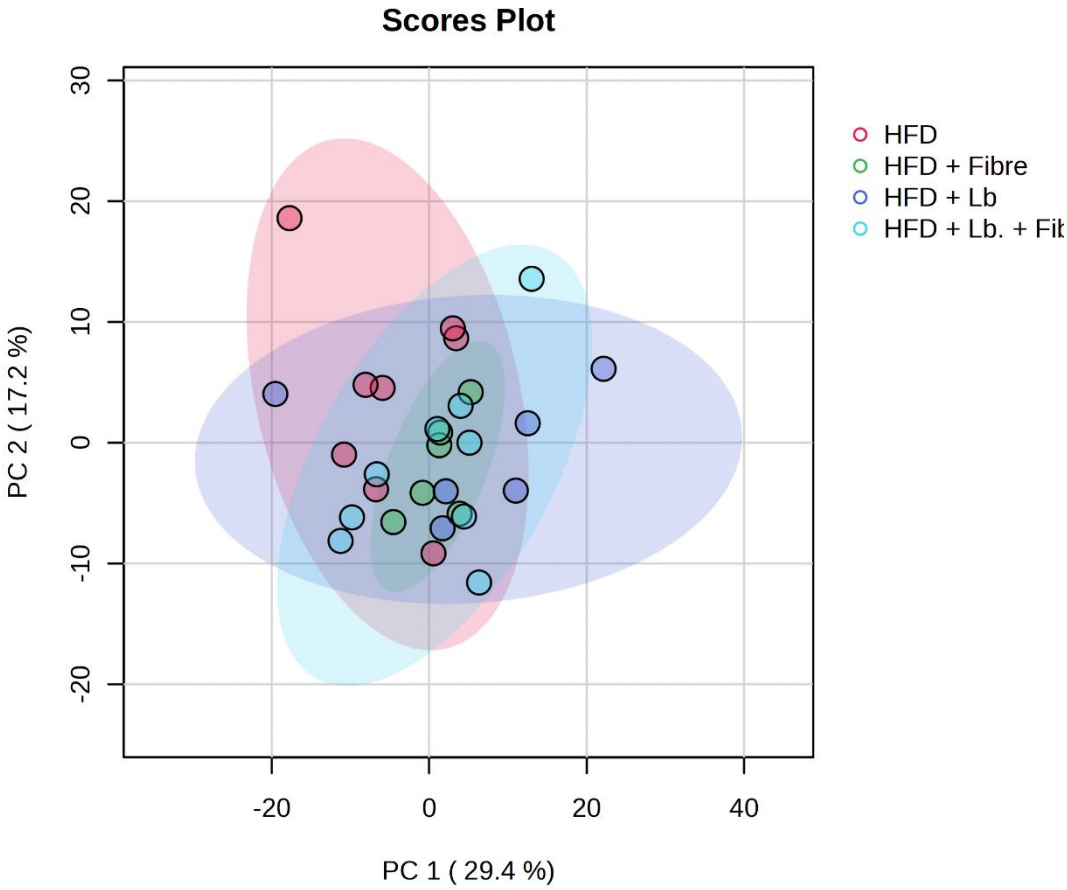

Chapter 7.1

Protocol for a Prospective Cohort Study Exploring the Gut Microbiota of Infants with Congenital Heart Disease Undergoing Cardiopulmonary Bypass (the GuMiBear study)

Fatma Koc is third author of this protocol which is published in:

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7.1 Abstract

The gut microbiota develops from birth and matures significantly during the first 24 months of life, playing a major role in infant health and development. The composition of the gut microbiota is influenced by several factors including mode of delivery, gestational age, feed type and treatment with antibiotics. Alterations in the pattern of gut microbiota development and composition can be associated with illness and compromised health outcomes. Infants diagnosed with 'congenital heart disease' (CHD) often require surgery involving cardiopulmonary bypass (CPB) early in life. The impact of this type of surgery on the integrity of the gut microbiome is poorly understood. In addition, these infants are at significant risk of developing the potentially devastating intestinal condition necrotising enterocolitis. This study will employ a prospective cohort study methodology to investigate the gut microbiota and urine metabolome of infants with CHD undergoing surgery involving CPB. Stool and urine samples, demographic and clinical data will be collected from eligible infants based at the National Centre for Paediatric Cardiac Surgery in Ireland. Shotgun metagenome sequencing will be performed on stool samples and urine metabolomic analysis will identify metabolic biomarkers. The impact of the underlying diagnosis, surgery involving CPB, and the influence of environmental factors will be explored. Data from healthy age-matched infants from the INFANTMET study will serve as a control for this study.

7.2 Introduction

7.2.1 What is currently known

The establishment of gut microbiota begins at birth and continues over the first years of life. Continued evolution of the gut microbiome after birth is governed by host factors such as both the adaptive and innate immune system, as well as external factors such as diet, medication and toxin exposure, and illness (Skillington et al., 2021). Understanding the role of the gut microbiome in metabolism, immune function and nutrition is gaining increasing recognition, as it is accepted that an altered colonisation has been associated with a higher risk of disease later in life (Cilieborg et al., 2012). In the critical first weeks and months of life, perturbations to the infant gut microbiome have implications for growth development and health (Healy et al., 2022).

7.2.2 The microbiome and systemic inflammation

It is evident that under certain conditions, disruption of the normal microbiota that colonise the intestinal tract can occur (Ohland and Jobin, 2015). These conditions include systemic inflammatory processes, which can result in intestinal inflammation, where proinflammatory bacteria can flourish, interacting with the intestinal epithelium to cause cytokine release, activating key inflammatory pathways increasing morbidity and prolonging critical illness (Cabrera-Perez et al., 2017). The pattern of cytokine release in patients undergoing cardiopulmonary bypass (CPB) is described as comparable to those released in systemic inflammation such as trauma and sepsis (Halter et al., 2005). However, the nature of gut microbiota compositional changes in infants undergoing surgery with CPB remains understudied. This research aims to address this knowledge gap to enhance our understanding and inform care practices.

7.2.3 Congenital heart disease and necrotising enterocolitis

Congenital heart disease (CHD) affects approximately 1 in every 100 babies born throughout the USA every year ((CDC), 2022). It is the most common congenital defect worldwide (van der Linde et al., 2011). A diagnosis of 'complex CHD' can include conditions such as hypoplastic left heart syndrome (HLHS); hypoplastic right heart syndrome, transposition of the great arteries requiring intervention in the first week of life, while CHD such as atrioventricular septal defect, tetralogy of Fallot may require corrective surgery in the first few months of life. CHD requiring surgery involving CPB presents a greater risk to patients. This increased risk is not limited directly to the surgery, compromised ventricular function or low cardiac output state, but includes the risk of developing necrotising enterocolitis (NEC).

There is a well-established connection between CHD and NEC, a potentially devastating intestinal condition of infancy (Martin et al., 2016). NEC carries a reported incidence of between 3% and 9% in infants with CHD with all-cause mortality rates as high as 38% in children with 'cardiogenic' NEC (Siano et al., 2019). While CHD remains one of the most common risk factors for NEC, the underlying pathophysiology of this association is complex (Kelleher et al., 2021). Growing evidence suggests that perturbations in the early-life gut microbiota composition increase the risk for NEC (Healy et al., 2022, Murphy et al., 2021). A significant association between episodes of low cardiac output and shock in the development of NEC is recognised (McElhinney et al., 2000, Carlo et al., 2007). It is reported that infants with certain types of CHD, mainly HLHS, may possess abnormal systemic vasculature contributing to the increased risk for NEC (Miller et al., 2014). Whether it is those, or

other causes of impaired perfusion to the gut, the resulting damage to the mucosal barrier can provide an entry point to bacteria provoking an inflammatory cascade and the devastating consequences that can ensue (Nino et al., 2016). The vulnerability of infants with CHD is enhanced during the course of surgical intervention involving CPB (Kelleher et al., 2021), and the role of the gut microbiome has received little research focus in this context.

7.2.4 Surgery involving CPB

Infants diagnosed with CHD are at risk of alterations to their intestinal homoeostasis, a further threat is presented in the context of surgery involving CPB (Kelleher et al., 2021). There is evidence to suggest intestinal ischaemia reperfusion injury occurs after CPB and contributes to epithelial barrier dysfunction (EBD) potentially exposing the bloodstream to bacteria or bacterial products (Typpo et al., 2015). Although alterations in gut barrier integrity and resident microbiota have been demonstrated (Healy et al., 2022). It is not fully understood what changes to the microbiome occur following CPB, and the nature and severity of EBD. While the gut microbiota in infants with CHD following CPB remains understudied, a small single centre case–control study recently identified significant gut microbiota perturbations in patients with CHD (Salomon et al., 2021). This case–control study highlighted that children with CHD had a disrupted gut microbiome at baseline with an over-representation of proinflammatory bacteria, this was further exacerbated by CPB. Samples were collected preoperatively and in a limited 24- and 48- hour time frame postoperatively. The significance of intraoperative variables including aortic cross clamp time and duration of CPB was not determined.

Our study proposes to address the knowledge gap and advance existing research by examining the gut microbiome of infants with CHD preoperatively, and at defined time points up to 2 years of life/postoperatively. This timeline will account for the recovery phase post cardiac surgery, including time to re-establish full feeds, wean from mechanical ventilation and circulatory support, and allow for surveillance of outcome measures including NEC, repeat surgery and mortality postoperatively. Comparisons will be made with healthy age-matched infants recruited as part of the INFANTMET study (Hill et al., 2017). As well as collecting intraoperative variables such as duration of CPB and aortic cross clamp time, a novel aspect of this research will be to profile the metabolites in urine to assess potential metabolic biomarkers and pathway changes. Our research will recruit patients in a National Centre for Paediatric Cardiac Surgery, where 40 open cardiac surgeries are performed on infants annually. We; therefore, anticipate active recruitment will ensure the proposed target sample of 50 participants is achievable. No additional invasive procedures will be required for sample collection, enhancing the acceptability of the research for consenting parents or carers. This project will investigate the subdivisions of the gut microbiota of infants with CHD, and environmental factors such as the influence of mode of delivery, preoperative fasting states and mode of feeding, and use of preoperative antibiotics. Understanding the status of the intestinal microbiome of infants with CHD and the effects of undergoing surgery with CPB is vital in informing best care practices to enhance patient outcomes.

7.2.5 Objectives and outcomes

The primary study objectives and outcomes are:

To characterise the gut microbiota composition of infants with specified CHD undergoing surgery with CPB at specific time points perioperatively.

To compare any differences in gut microbiota composition of infants who take part in this study at defined time points preoperatively and postoperatively and compare with the microbiota of healthy babies from the INFANTMET study at matching time points (Hill et al., 2017).

To characterise the urine metabolite profile of infants with specified CHD undergoing surgery with CPB and compare with healthy infants from the INFANTMET study (Hill et al., 2017).

Secondary objectives and outcomes:

To explore the influence of maternal and environmental factors on gut microbiome composition.

7.3 Methods

7.3.1 Study design

This study is a prospective cohort study of infants with CHD undergoing CPB at the National Centre for Paediatric Cardiac Surgery at Children's Health Ireland (CHI) at Crumlin, Dublin, Ireland. This single-site study will investigate the differences in the gut microbiome, metabolomics readouts and stress levels between infants with CHD undergoing CPB and healthy age-matched controls.

7.3.2 Participant selection

This study will involve the collection of demographic and clinical maternal and infant data from infants diagnosed with CHD scheduled for surgery involving CPB. CHD is typically diagnosed antenatally with fetal echocardiography performed routinely at 20–24 weeks gestation. Any child presenting with a murmur or features of cardiac conditions, or symptoms of CHD will be diagnosed using clinical examination, including palpation, auscultation, ECG and echocardiography. Cardiac diagnoses are classified according to cardiovascular physiology that is, left to right shunt, cyanosis with biventricular circulation, univentricular circulation and outflow tract obstruction, as presented in table 7.1. To be eligible for the study, the participants must meet the terms of the inclusion and exclusion criteria as presented in table 7.2.

Table 7.1 Classification of CHD subtypes

Group	**CHD group**	**CHD subtypes**
1	Left to right shunt	AVSD, VSD, large ASD
2	Cyanotic CHD with biventricular circulation	D-TGA/IVS, D-TGA/VSD
3	Cyanotic CHD with univentricular circulation	HLHS, HRHS (Tricuspid Atresia) Pulmonary Atresia
4	RVOT obstruction	Tetralogy of Fallot, dysplastic PS
5	LVOT obstruction	Shones syndrome, coarctation of aorta, interrupted aortic arch
6	Others	Ebstein anomaly, truncus arteriosus

ASD, atrial septal defect; AVSD, atrioventricular septal defect; CHD, congenital heart disease; CPB, cardiopulmonary bypass; DTGA, D-transposition of the great arteries,; DTGA-IVS, D-transposition of the great arteries with intact ventricular septum; HLHS, hypoplastic left heart syndrome; HRHS, hypoplastic right heart syndrome; LVOT, left ventricular outflow tract; PS, pulmonary stenosis; RVOT, right ventricular outflow tract; VSD, ventricular septal defect.

Table 7.2 Inclusion and exclusion criteria

Inclusion criteria	**Exclusion criteria**
Infants born full term (≥34 weeks +6 days) weeks gestation)	Stillbirth or live birth where the baby is born alive but dies shortly after
Infants diagnosed with *CHD and scheduled for surgery involving CPB	Infants who are born healthy with no underlying illness, syndrome, or chronic disease
Infants born in Ireland to allow sample follow up	Participation in another study
Ability of the participant's parent/carers (in the investigator's opinion) to comprehend the full nature and purpose of the study	Infants not undergoing surgery involving cardiopulmonary bypass
Consent to participate in the study and willingness to comply with the protocol and study restrictions by the participant's parent/carers	Infants where parents/carers do not give consent to participate in the study
Upper age limit of less than 6 months	Gastrointestinal pathology or intestinal surgery, excluding gastrostomy tube

*CHD includes Hypoplastic left heart syndrome (HLHS); Hypoplastic Right Heart Syndrome (HRHS), Transposition of the Great Arteries (TGA), Atrioventricular Septal Defect (AVSD), Tetralogy of Fallot.

7.3.3 Public and patient involvement statement

The mother of a child who had cardiac surgery as a baby was involved in reviewing the research questions, outcome measures and study literature at the study design phase. The public and patient involvement (PPI) representative did not participate in the recruitment or the conduct of the study due to competing demands and availability. The PPI representative has offered to support dissemination of the study results through their involvement in charitable foundations and child and parent support fora.

7.3.4 Recruitment

Participants meeting all inclusion criteria will be selected after admission to the hospital, outpatient clinic or cardiac day unit. Study-related information will be given in written form as well as explained by a member of the project team. No study-related activities will begin before the potentially eligible participants' parents/carers have signed the informed consent form (ICF). Participants parents/carers will be asked to refer to the privacy notice on the hospital website or they can receive a hardcopy if they wish. Signed ICFs will be stored safely in a locked cabinet in the research office.

7.3.5 Compensation

No compensation will be provided to the participants. There are no cost implications for the health service executive or to the participants. The management of patients and investigative tests will comply with current standards of care.

7.3.6 Study timeline

After completing recruitment procedures, that is, determining whether the patient meets the study inclusion criteria, discussing the study with the parents/caregivers and obtaining informed consent, clinical and demographic data will start to be collected. The study will be undertaken for a period of 2 years after the infant is initially recruited.

7.3.7 Demographic data

The infants' diagnosis including whether antenatal or postnatal diagnosis, comorbidities, date of birth, gestational age, sex, mode of delivery, Appearance, Pulse, Activity, Grimace, Respiration scores, birth weight, head circumference, mode and type of feeding pre and post operatively, antibiotics administered postdelivery, complications and antenatal events will be recorded (case record form located in online supplemental appendix 1).

7.3.8 Maternal data

Maternal history including age, antibiotics received, smoking status use of probiotics and significant antenatal events will be recorded.

7.3.9 Surgical course

The type of surgery performed, duration of CPB and cross-clamp time will be recorded. Antibiotic use and any intraoperative events will be recorded as well as clinical information including arterial blood gas data.

7.3.10 Postoperative data

Paediatric Index of Mortality score, duration of mechanical ventilation, haematology variables including haemoglobin and haematocrit, renal data, for example, blood urea nitrogen and creatinine, fluid balance and cardiovascular support including vasoactive inotrope score, as well as duration of stay in paediatric intensive care unit will be recorded.

7.3.11 Feeding information data

Feeding information including the type of feed and duration of the feed prior to and after surgery will be recorded. The date the patient is established on full feeds will be recorded. Full feeding is defined as when the patient no longer requires parenteral nutrition or intravenous fluids.

7.3.12 Discharge information data

Discharge information data: This will include the patient's status on discharge from paediatric intensive care unit (PICU), as well as length of PICU and hospital stay.

7.3.13 Complications

The occurrence of complications will be recorded, for example, the development of NEC. The timeline for recording NEC onset will be based on the initiation of triple antibiotic therapy, based on a full surgical review including clinical presentation, radiological and laboratory data. In addition, the occurrence of death, rehospitalisation for heart failure or cardiac problems will be included.

7.3.14 Subject withdrawal/exclusion

Under the Declaration of Helsinki, the research nurse will explain to the consenting adult that they have the right to withdraw from the study at any time and that this will in no way prejudice their future treatment. The reason for withdrawal will be recorded in the source documents and on the appropriate CRF. Consenting adults will be made aware that stored samples from individuals withdrawing from the study may have undergone processing and may be analysed in the study.

7.3.15 Regulatory procedures

The study is conducted following the version Fortaleza, Brazil, October 2013 of the Declaration of Helsinki 1964. The Protocol and the ICF have been approved by the Clinical Research Ethics Committee of Children's Health Ireland GEN/826/20. As biological samples will be procured in one institution and sent to another, a data-sharing agreement is in place between The Cardiology Department, CHI at Crumlin Hospital, and APC Microbiome Ireland, in Cork. This research is fully compliant with the guidelines as set out in The General Data Protection Regulation, the Irish Data Protection Acts 1988 to 2018 including Protection Act 2018 (Section 36(2)) (Health Research) Regulations 2018.

7.3.16 Data statement

Once collected, the anonymised demographic, clinical and laboratory analysis data as well as statistical codes will be uploaded to the open access Research Repository University College Dublin.

7.3.17 Sample collection and analysis

7.3.17.1 Faecal samples

Stool samples will be collected at the following time points: within 24 hours after birth (timepoint (TP) 1), within 24 hours presurgery (TP 2), 1-week postsurgery (TP 3), 4 weeks postsurgery (TP 4), 24 weeks postsurgery (TP 5), at 52 weeks (TP 6) and 2 years of age (TP 7). Information about antibiotic therapy administered before or during the stool collection will be recorded.

As the study site is the National Centre for Paediatric Cardiology, all infants diagnosed with CHD antenatally are transferred to the study site from the Maternity Unit for management of CHD. The first study stool specimen is typically obtained after the infant is transferred to the study site and informed consent obtained, which is typically within 24 hours of delivery.

The sample will be collected by the bedside nurse or the parent/carers and transferred to the laboratory on receipt of the sample during the weekdays or weekends. At night, the sample will be kept in the dedicated fridge and transferred to the laboratory within 4–5 hours for appropriate storage at −80°C until further analysis. A Standard Operating Procedure for sample collection when participant is no longer an in-patient is provided in online supplemental appendix 2.

7.3.17.2 Urine samples

Testing the urinary metabolomics of study participants will allow the potential identification of altered metabolomic profiles, and explore the role of microbiota in such alterations. Mirroring the INFANTMET study, urine samples will be collected at

4–8 weeks postsurgery for metabolomic analysis using Sterisets Uricol Urine Collection Pack (MedGuard, Ireland). The urine sample will be collected from the urinary catheter if the participant is catheterised. Alternatively, a pad will be placed in the diaper and used to collect an unsoiled urine sample from the infant. The pad will then be placed in a biohazard bag and frozen immediately at −80°C prior to processing. After all the sample collections are complete, they will be shipped to Teagasc Food Research, Moorepark, Ireland, using DHL overnight service for microbiome and metabolomics analyses. Styrofoam Saf-T-Pak STP-309 shipper box or equivalent will be used. DNA extraction will be performed on stool samples using the modification of the Repeated Bead Beating plus Column method (Yu and Morison, 2004). LC-MS (liquid chromatography-mass spectrometry) will be used for metabolomics analysis of urine (Hill et al., 2017).

7.3.17.3 Sample collection for discharged participant

Parents/carers will receive a sample collection discharge pack and a parent diary/instruction served as a reminder to collect due samples at different time points prior to discharge home. They will receive a follow-up text message or phone call to remind them on the due sample. The sample collection discharge pack consists of urine/stool collection containers with study code, sterile pad, syringe, zip-lock bag, gloves, biohazard bag and cooler bag. Parents/carers are asked to keep the collected sample at the dedicated section of their home freezer. They will transport the collected sample in the cooler bag provided when attending outpatient department for appointments. They will ask a member of the project team to transfer the collected

sample to the laboratory on arrival at the hospital. The study researcher is available at the dedicated contact number for any queries.

7.3.17.4 Adverse events and participant well-being

There are no expected safety concerns related to the study. All study participants will be under the care of the cardiology team at the hospital with access to psychological support, as well as nursing and medical professionals, social workers and chaplaincy.

7.3.17.5 Data collection and management

The study diaries, study dataset and paper/digital CRF systems will be used for recording data from each study participant. All the data collected in this study are pseudonymised, as each of the participants will be assigned a specific study code and on receipt, data will be referred to the study code. All study staff responsible for entering data into the CRF system received training in advance of the study commencement. This training included familiarity with the study diaries, study dataset and paper/digital CRF system and have completed good clinical practice in research training. They have individual access to the password protected study shared drive within the hospital. The study team will monitor the data/sample collection process. Any inconsistencies identified during the study will be presented as queries at the regular project team meeting.

7.3.18 Comparison data

Data collected as part of the INFANTMET Study will serve as a healthy control comparison for this study. INFANTMET compared the gut microbiota development of breastfed infants born via C-section or vaginally at full term or preterm at Cork

University Maternity Hospital. Ethical approval for sample collection by Cork University Hospital Research Ethics Committee (reference number ECM (w) 07/02/2012). One hundred and ninety-two infants were recruited to the INFANTMET study and stratified according to delivery mode and gestational age at birth. Faecal samples were collected from the infants at 1, 4, 8 and 24 weeks of age and years 1, 2 and 4 of life and stored under controlled conditions. Urine samples were collected at 4 weeks of age for metabolomic analysis and stored in a freezer at −80°C prior to processing. Samples were analysed in accordance with the analysis proposed for the GuMiBear study. Although INFANTMET study participants did not have CHD, they nonetheless serve as a valuable comparison group. The stool and urine samples collected as part of the GuMiBear study will be as closely time matched as possible to the INFANTMET study samples to capture the major developmental period of the early life gut microbiota.

7.3.19 Bioinformatics and statistical analysis

7.3.19.1 Sample size justification

Published research in this area is lacking. However, one case–control study by Salomon et al (Salomon et al., 2021) included 17 cases and 12 control participants and was sufficiently powered to determine a statistically significant difference in beta diversity in cases versus controls ($F=5.6$, $p<0.001$). Our study proposes to include 50 patients with CHD undergoing surgery with CPB and age matched controls, almost three times the Solomon et al study. We; therefore, anticipate that the proposed sample size is justified.

7.3.19.2 Demographic and clinical data

Demographic and clinical data and laboratory information will be tested for normality using the Shapiro-Wilk test. Descriptive statistics will be used to describe normally distributed data, and expressed as mean±SD. Continuous data not normally distributed will be reported as median and IQRs. Categorical variables will be expressed as counts and percentages. Groups will be compared using $\chi 2$ tests for categorical variables and independent-samples Student's t-tests for normally distributed continuous variables. For variables not normally distributed, the Mann-Whitney U test will be used. Comparison will include subgroup analysis of participants who experienced postoperative complications including NEC with those that did not. Comparisons will also include cyanotic vs acyanotic heart disease subgroup analysis, as well as mode and type of feeding preoperatively and postoperatively.

7.3.19.3 Microbiome analysis

Metagenomic shotgun sequencing data will be analysed using bioBakery suite of tools (https://huttenhower.sph.harvard.edu/biobakery_workflows/). Trimmed and human reads filtered using KneadData (V.0.7.2) with the default parameters. Quality controlled data will be taxonomically profiled at the species level with relative abundance by MetaPhlAn2. Functional profiling will be performed using HUMAnN3 and strain profiling using StrainPhlAn.

For alpha diversity analysis, samples will be rarefied to even depth and phyloseq::estimate richness will be used to calculate Chao1, Shannon and Simpson indices. Alpha diversity indices between groups will be univariately compared using the Wilcoxon rank sum test. A beta-diversity ordination will be generated using the

Aitchison distance and visualised using principal component analysis plot. The Adonis function in the vegan package will be used to implement a permutational multivariate analysis of variance to test whether samples cluster beyond that expected by sampling variability. MaAsLin2 (multivariate associations with linear models) will be used to investigate multivariable associations between sequencing data and clinical metadata. MaAsLin2 performs boosted, additive, linear models to detect associations while adjusting for confounding factors. Sparse canonical correlation analysis will be used to calculate the overall correlation between metabolites and microbes, and to identify strongly associated biomarkers. Pairwise Spearman's rank correlation analysis will also be performed.

7.4 Discussion

Despite an increasing awareness of the early-life critical window of microbiome development on the health and wellness of infants, there remains much to learn about the interactions of the microbiome with the infant host with CHD undergoing surgery involving CPB. This study is designed to address this knowledge gap, and incorporates a sound methodology with particular strengths enhancing the value of its findings. The specimen collection strategy occurring at multiple time points over a 2-year period in the proposed study will deepen our understanding of the temporal dynamics of the colonising microbiota, and their interactions with host physiology. The study design will account for maternal and perioperative variables to determine changes to the microbiome. Access to existing microbiome data from healthy age-matched infants provides a valuable opportunity to present high-quality comparative information. A limitation of this study may include the failure to recruit infants with CHD not identified antenatally, despite active fetal screening services. While multicentre trials

capturing sufficient case numbers of NEC cases will offer robust conclusions, this study will offer valuable evidence to support the influence of CHD and CPB on the microbiome and intestinal EBD. Future research can build on existing studies, and explore treatment strategies including recommendations for efficacious probiotic strain administration, including the supplements to promote a diverse gut microbiota to improve outcomes for this vulnerable population.

7.5 Ethics and Dissemination

This research study is ethically approved by the Clinical Research Ethics Committee of Children's Health Ireland (REC REF No: GEN/826/20). Study results will be available to patients with CHD and their families, carers, support networks, paediatric cardiology and microbiome societies and other researchers. Study findings will provide a deeper understanding of the gut microbiota of infants with CHD and inform perioperative management options including strategies to prioritise the integrity of the gut microbiota.

7.6 Status of Study

The trial is ongoing and as of 5 February 2023, 84% of the participants have been recruited. Laboratory analysis has been carried out on 25% of study samples.

7.7 Ethics Statements

Patient consent for publication

Not applicable.

7.8 Acknowledgments

All study participants, Paediatric Intensive Care Unit/Children's Heart Centre nurses for assisting in sample collection. All laboratory staff including Ms Irene Regan who have facilitated tracking and secure storage of study samples. Dr Adam James, Dr Ross Foley and the Consultant Paediatric Consultants, CHI at Crumlin for their support with this study.

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Chapter 7.2

Gut Microbiome in Children with Congenital Heart Disease after Cardiopulmonary Bypass Surgery (GuMiBear Study)

Fatma Koc is first author of this research paper which is published in:

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7.1 Abstract

The gut microbiome of infants with congenital heart disease (CHD) undergoing cardiopulmonary bypass surgery (CPB) is at risk of profound alteration. The aim of this study was to examine the gut microbiome pre- and post-bypass surgery to explore potential implications of altered gut biodiversity. A prospective cohort study involving infants with CHD who underwent CPB was performed. Faecal samples were collected from infants alongside the collection of demographic and clinical data in order to examine gut microbiome changes before and after surgery. 16S rRNA gene sequencing analysis was performed on DNA isolated from stool samples to determine changes in gut microbiome composition. Thirty-three patients were recruited, with samples from thirteen of these available for final analysis. Compared with healthy, matched controls, at a genus level, pre-operative samples for infants with CHD demonstrated a higher relative abundance of *Escherichia-Shigella* (31% vs 2–6%) and a lower relative abundance of *Bifidobacterium* (13% vs 40–60%). In post-operative samples, the relative abundance of *Escherichia-Shigella* (35%), *Enterococcus* (11%), *Akkermansia* (6%), and *Staphylococcus* (5%) were higher than pre-op samples. One infant developed post-operative necrotising-enterocolitis (NEC). They displayed a marked abundance of the *Enterococcus* (93%) genus pre-operatively. This study demonstrates that infants with CHD have an altered gut microbiome when compared with healthy controls and there might be a possible link between an abundance of virulent species and NEC.

7.2 Introduction

Congenital heart disease (CHD) refers to a group of structural or functional abnormalities in the heart that are present at birth (Hoffman, 1990). CHD is the most common group of birth defects (Magner et al., 2023), having an incidence of approximately 9 per 1000 live births (Mandalenakis et al., 2020, Rosamond et al., 2007). Lesions that are dependent on blood supply through the ductus-arteriosus are a group of critical congenital heart defects that typically require intervention in the neonatal period and are fatal if untreated. They carry an incidence of 0.6/1000 live births (Plana et al., 2018). Meanwhile, common conditions such as ventricular septal defects and tetralogy of Fallot require corrective surgery in infancy (Goldberg, 2015).

The gut microbiota, a dynamic and complex community of microorganisms residing in the gastrointestinal tract, plays a pivotal role in various aspects of human health and physiology including immune regulation and metabolism (Gerritsen et al., 2011). It undergoes its initial development at birth and dynamic changes occur during the first two years of life (Hill et al., 2017). This developmental period is characterized by a rapid evolution in the composition and diversity of the gut microbiota, influenced by factors such as mode of birth, diet, genetics, and environmental exposures (Borre et al., 2014). There are several factors that might alter the diversity of the gut microbiome in infants with CHD such as prolonged hospitalisation, mode of delivery, mode of feeding, antibiotic administration, and cardiopulmonary bypass (CPB) surgery (Kelleher et al., 2021, Huang et al., 2022b).

CPB is required in most surgeries for congenital heart defects performed in infancy (the notable exception being coarctation of the aorta) (Whiting et al., 2015). CPB is

recognized to trigger systemic inflammation (Halter et al., 2005). There is evidence indicating that the cytokines released after CPB resemble those released in the context of other systemic inflammatory conditions, such as sepsis and trauma (Halter et al., 2005, Gogos et al., 2000). The inflammatory response that follows CPB with cardiac surgery is postulated to contribute to post-operative morbidity. Alterations in the gut microbiome and gut barrier of infants with CHD who have undergone CPB have been demonstrated when compared with controls in a single small study (Salomon et al., 2021). Exploring the factors contributing to the inflammatory process through the regulation of the gut microbiome, intestinal epithelial barrier dysfunction (EBD), and the resulting metabolites could enhance understanding of systemic inflammation (Salomon et al., 2021, Typpo et al., 2015) which has numerous potential benefits. The inflammatory process following CPB may contribute to the development of low cardiac output syndrome (LCOS) (Salomon et al., 2023) and longer aortic cross-clamp duration has been identified as a risk factor for its development (Drennan et al., 2021). Approximately 20–25% of paediatric patients, and as much as 50% of neonates, are known to experience some degree of LCOS (Sinclair et al., 1995, Chandler and Kirsch, 2016, Salomon et al., 2023, Wessel, 2001, Cremer et al., 1996). Among this group, those who develop LCOS tend to face higher mortality rates, prolonged stays in the intensive care unit (ICU), and extended periods of mechanical ventilation (Du et al., 2020). To date, treatment efforts include the widespread prophylactic use of Milrinone in the post-operative period, and in some centres the addition of post-operative corticosteroids (Robert et al., 2015, Burkhardt et al., 2015).

NEC is a devastating intestinal condition of infancy (Kelleher et al., 2021). This condition arises when an immature or compromised gastrointestinal tract fails to

maintain the mucosal barrier, facilitating the breach by bacteria, resulting in an inflammatory cascade which may lead to ischemia and perforation (Fisher et al., 2015). A robust relationship exists between gut microbiota and NEC development (Kim and Claud, 2019). Studies have shown that preterm infants who develop NEC often exhibit microbial dysbalance, characterized by an imbalance in the composition of the gut microbiota (Tarr and Warner, 2016, Warner et al., 2016). Studies exploring the gut microbiota of infants with CHD compared to control subjects have been limited to date (Salomon et al., 2021, Huang et al., 2022b), and to our knowledge only one study has demonstrated a link between alterations in the gut microbiome and adverse clinical outcomes (Huang et al., 2022b). The relationship between NEC and the gut microbiome of infants with CHD has to date been underexplored.

In the Gut Microbiome in Children with Congenital Heart Disease after Cardiopulmonary Bypass (GuMiBear) study, the primary objective was to explore perturbations within the gut microbiota composition pre and post cardiopulmonary bypass (CPB) surgery in neonates affected by CHD when compared with healthy aged matched controls. This study aims to add to the small but growing body of literature in this domain. Full details of study design has been previously published in Magner et al. (2023) (Magner et al., 2023).

7.3 Materials and Methods

7.3.1 Study design

This is a prospective cohort study involving infants diagnosed with CHD who underwent CPB at the National Centre for Paediatric Cardiac Surgery at Children's Health Ireland (CHI) at Crumlin, Dublin, Ireland. In order to examine gut microbiota

changes before and after surgery, faecal samples were collected from infants alongside the collection of demographic and surgical information, which includes surgical procedure performed, bypass and cross-clamp time. The collection of both were described in our previously published protocol (Magner et al., 2023). In order to compare healthy gut microbiota with the current cohort, we used age-matched faecal samples from a previously collected cohort INFANTMET (Hill et al., 2017). This research study is ethically approved (REC REF No: GEN/826/20). Study design and sample collection have been illustrated in Figure 7.1.

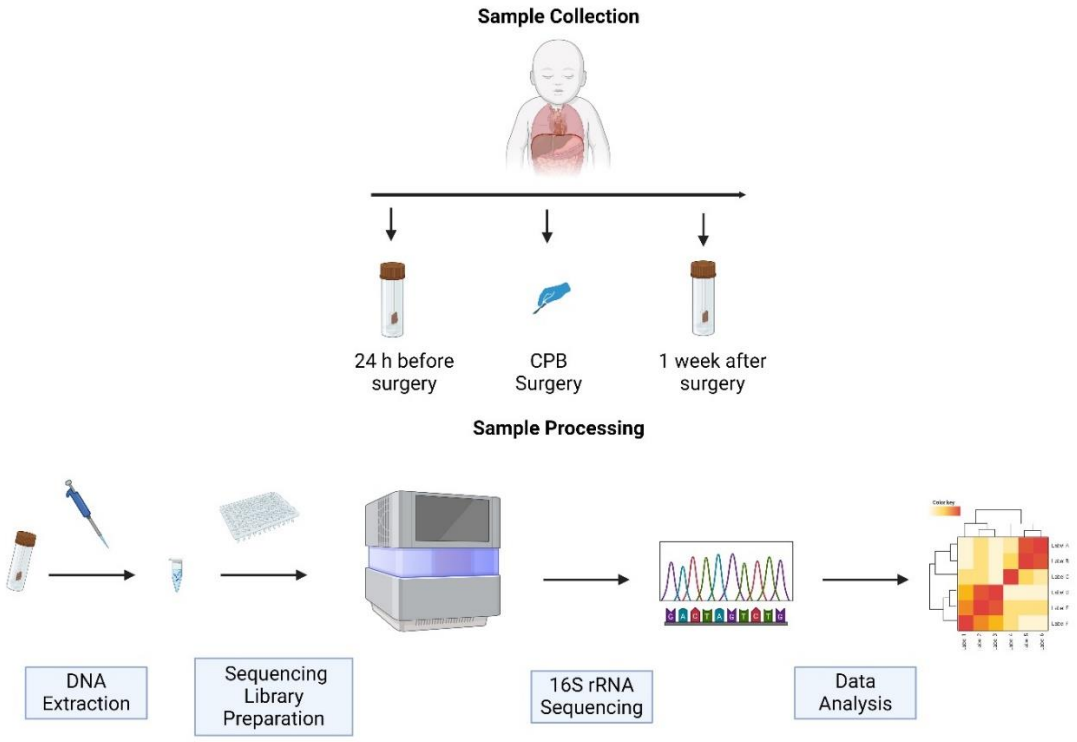

Figure 7.1 Figure illustrates the study design and the process of sample collection. Faecal samples were collected 24 h prior to the surgery and 1 week following the surgery. Faecal samples were subjected to DNA extraction and 16S rRNA gene sequencing.

7.3.2 Participant selection and recruitment

Participants for this study were selected according to the inclusion and exclusion criteria as shown in Table 7.1 (Magner et al., 2023).

Table 7.1 Inclusion and exclusion criteria. CHD, congenital heart disease; CPB: cardiopulmonary bypass.

Inclusion criteria	Exclusion criteria
	Stillbirth or live birth where the baby is born alive but dies shortly after
Infants diagnosed with CHD and scheduled for surgery involving CPB	Infants who are born healthy with no underlying illness, syndrome, or chronic disease
Infants born in Ireland to allow sample follow-up	Participation in another study
Ability of the participant's parent/carers (in the investigator's opinion) to comprehend the full nature and purpose of the study	Infants not undergoing surgery involving cardiopulmonary bypass
Consent to participate in the study and willingness to comply with the protocol and study restrictions by the participant's parent/carers	Infants where parents/ carers do not give consent to participate in the study
	Gastrointestinal pathology or intestinal surgery, excluding gastrostomy tube

7.3.3 Sample collection

Faecal samples were collected from participants pre-op (24 h before surgery) and post-op (1 week after the surgery) to examine gut microbiota composition. Faecal samples were collected in the Department of Cardiology, Children's Health Ireland (CHI) at Crumlin Hospital, Ireland by either the bedside nurse or the parent/caregivers and transferred to the laboratory upon receipt and preserved at -80 °C. The samples were then transferred to Teagasc Moorepark Research Centre, Ireland on dry ice in order to

process for 16S rRNA gene sequencing analysis. Faecal samples were stored at -80 °C until processing for microbiota analysis.

7.3.4 DNA extraction

DNA extraction from each faecal sample was performed using a repeated bead-beading method according to Yu and Morrison, 2004 (Yu and Morrison, 2004). Approximately 200 mg faecal sample was weighed in a sterile 2 mL screw cap tube containing different sizes of beads. The extraction was performed according to the previously published protocol (Koc et al., 2022).

7.3.5 16S rRNA gene sequencing library preparation

DNA extracts were prepared according to Illumina 16S Metagenomics Sequencing Library Preparation guidelines (Koc et al., 2022). All amplicons were sequenced at the Teagasc Next Generation DNA Sequencing Facility using the Illumina MiSeq platform with a MiSeq Reagent Kit v3 (Illumina, Inc. San Diego, USA) according to a previously described protocol (Koc et al., 2022).

7.3.6 Bioinformatics and statistical analysis

Sequenced reads were processed using the DADA2 pipeline, version 1.16 (Callahan et al., 2016a). Reads were trimmed and filtered using the following parameters; maxN = 0, maxEE = c (2, 2), rm.phix = TRUE, truncQ = 2, trimLeft = c (17, 21), truncLen = c (280,210) and. maxN = 0. The core sample inference algorithm was applied to the filtered and trimmed sequence data. An amplicon sequence variant (ASV) table was created and chimeric sequences were identified and removed. Taxonomy was assigned to the sequence variants using the IDTAXA taxonomic

classification method via the DECIPHER Bioconductor package and the SILVA SSU r138 2019 database (downloaded November 2022) (Wright, 2016, Quast et al., 2013). Data tables produced by the DADA2 pipeline were imported into the R package phyloseq and a phyloseq object was created for further analysis (Callahan et al., 2016b). The decontam R package was used to identify and remove putative contaminating ASVs using prevalence-based method. The prevalence of each sequence feature was compared to the prevalence in negative control (DNA extraction blank) to identify contaminants using the function "isContaminant (ps, method = "prevalence", neg = "is.neg", threshold = 0.35)" (Davis et al., 2018).

Alpha diversity refers to the diversity of species within a particular habitat or ecosystem. It provides insight into the richness and evenness of species within a single sample or location (Whittaker et al., 2001). Alpha diversity was examined using Shannon, Simpson, and Chao1 indices. The non-parametric Wilcoxon-Rank Sum statistical test (R function “pairwise.wilcox.test”) was used to assess the statistical significance of differences in alpha diversity indices. The Holm method was applied to adjust p values for multiple tests. Beta diversity quantifies the variation in species composition between different habitats or locations within a larger ecosystem, providing insight into the degree of similarity or dissimilarity among communities (Bevilacqua et al., 2023). Beta diversity was measured using the Aitchison distance by applying Principal Component Analysis (PCoA) to the centred log-ratio (CLR) transformed counts. Permutational multivariate analysis of variance (PERMANOVA) was used to examine if samples cluster beyond that expected by sampling variability using the adonis () function in vegan (Oksanen J, 2022). Differentially abundant features in microbiome data were tested using MaAsLin2 (Mallick et al., 2021). The

"fixedeffects" function used surgical information (pre and post-op), Benjamini Hochberg method was applied for p value correction. The p and q values were kept at default as $p < 0.05$ and $q < 0.25$ were considered statistically significant.

Demographic information, clinical and surgical data were analysed using IBM SPSS Statistics (version 29.0.1.0). The Shapiro Wilk test was to evaluate if data set was normally distributed and Student t test was used to compare groups which were normally distributed. Mann Whitney U test was used to evaluate any significant changes pre- and post op samples.

7.4 Results

7.4.1 Enrolment

A total of thirty three patients who were diagnosed with CHD were recruited to the study (Figure 7.2).

A total of thirteen eligible infants with pre-op and post op samples were finally included. Data from thirteen matched (gestational age, delivery mode, gender and sampling age) infants from the INFANTMET Study were used as healthy control comparison (Hill et al., 2017). INFANTMET compared the gut microbiota development of breastfed infants born via C-section or vaginally at full term or preterm at Cork University Maternity Hospital, Ireland. Informed written consent was obtained from the guardians of the participants.

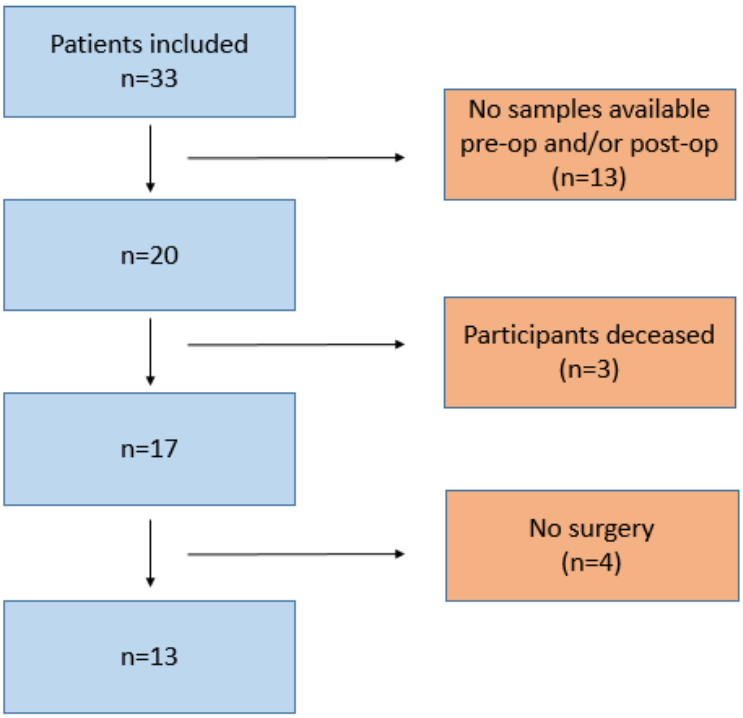

Figure 7.2 Illustrates the Study enrolment process. Patients were reviewed according to inclusion and exclusion criteria. There was no available faecal sample for thirteen infants. Three infants were deceased during the study and four infants had no surgery.

7.4.2 Demographic information

Birth weight ranged from 1.22 kg to 4.14 kg with a mean birth weight of 2.92 ± 0.24. Two infants were pre-term with birth weight less than 2.5 kg (1.68 and 1.22 kg, respectively). Participants' age ranged from 7 to 199 days, mean age of 86.23 ± 17.72 days. Gender comprised 8 female and 5 male infants. Gestational age ranged from 29 to 41 weeks and a median of 38 weeks. Bypass time differed for each patient ranging from 52 to 252 min with a mean of 126.58 ± 19.33 min. Cross-clamp time was between 20 and 174 min with mean of 80.76 ± 14.5 min.

The average Milrinone administration at day 1 was 0.52 ± 0.057 mcg/kg/min, day 2 was 0.45 ± 0.052 mcg/kg/min, at day 3 was 0.4 ± 0.059 mcg/kg/min. Morphine administration was 26.53 ± 2.22 mcg/kg/hour at day 1, 21.15 ± 2.66 mcg/kg/hour at day 2 and 14.3 ± 2.37 mcg/kg/hour at day 3. Median duration of stay in PICU was 4.5 ± 14.25 days. Two infants had a prolonged PICU admission (32 and 47 days), respectively resulting in a high standard deviation.

Nine infants were administered cefuroxime, one infant was given vancomycin + gentamycin, and one infant was given cefuroxime and piperacillin-tazobactam, one infant was given piperacillin-tazobactam, ceftazidime at post-op.

Table 7.2 Type of Feed after surgery.

Type of Feed (n=13)	
EBM (Expressed breast milk)	5
Formula	5
Both	3

Five infants were fed by expressed breast milk (EBM), five infants were fed by formula and 3 infants were mixed fed (Table 7.2).

7.4.3 Hospitalization and surgery

Arterial blood gas (ABG) pH and ABG pO2 levels were recorded before and after surgery. The average of ABG pH was 7.43 ± 0.18 and ABG pO2 was $93.6\text{mmHg} \pm 66\text{mmHg}$ before the surgery. The average of ABG pH was 7.31 ± 0.07 and ABG pO2 was $125.1\text{mmHg} \pm 64.4\text{mmHg}$ after the surgery.

7.4.4 Microbiota analysis

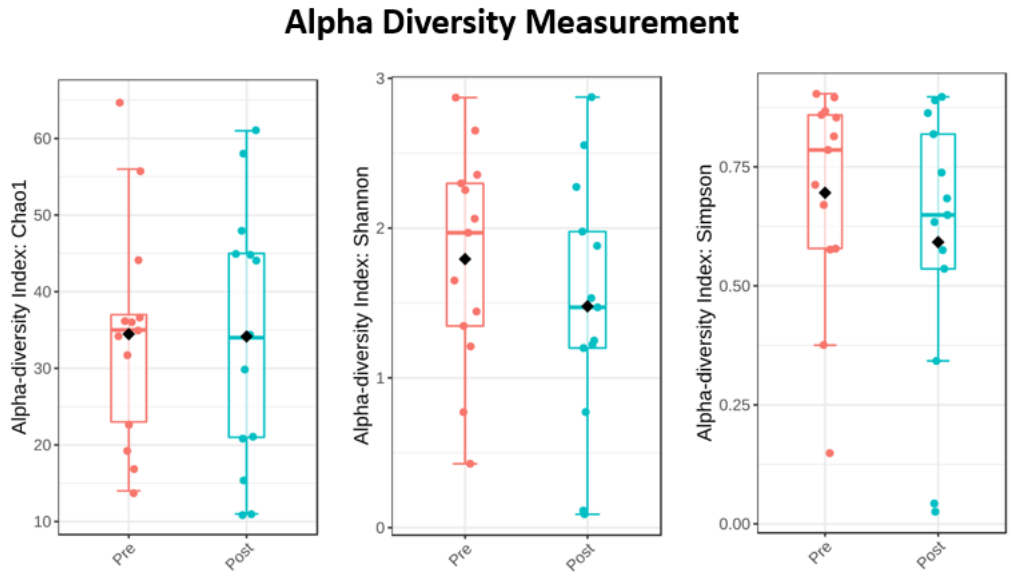

Figure 7.3 Box plots describing the Chao1, Shannon and Simpson between pre-op and post-op samples.

Faecal samples were analysed pre (n = 13) and post operatively (n = 13) to evaluate gut microbiota composition. Alpha diversity was evaluated to examine richness and evenness of bacterial species within each sample (Figure 7.3). No statistically significant differences in Chao1, Shannon and Simpson alpha diversity indices were found in the pre-op group compared with the post-op group.

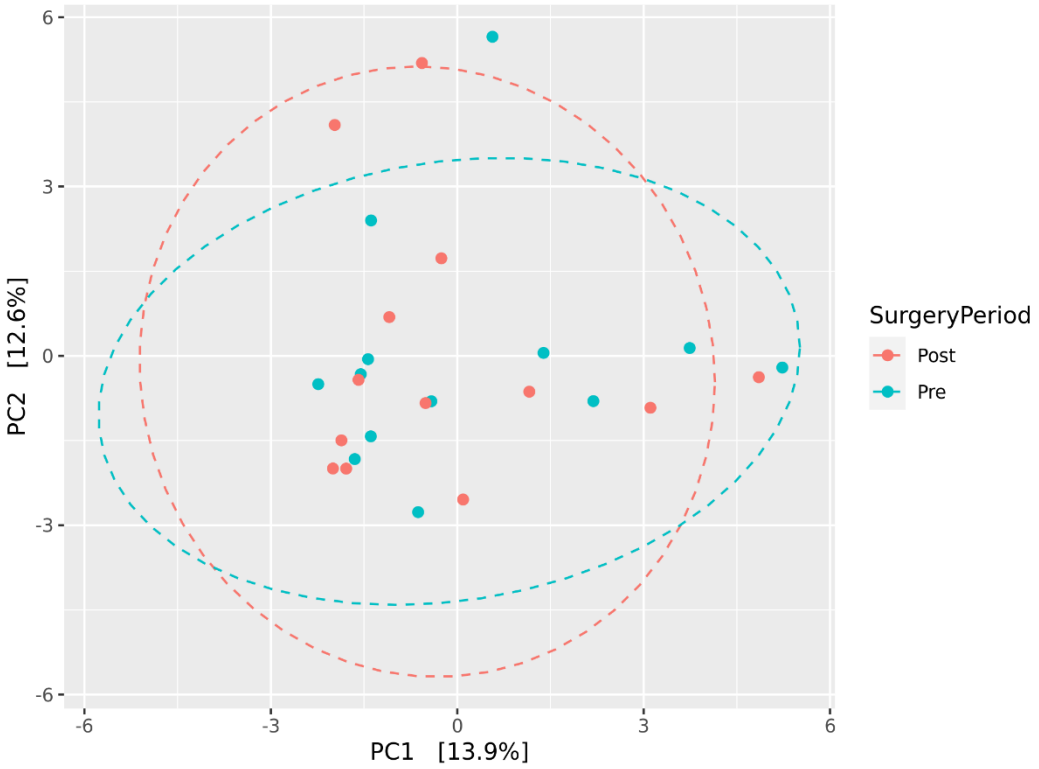

Figure 7.4 Principal component analysis (PCA) using Aitchison distance shows no significant separation between pre-op and post-op samples.

Principal component analysis (PCA) was used to evaluate beta diversity in pre-op and post-op samples (Figure 7.4). PERMANOVA was used to test if samples were clustering beyond the expecting variability. There was no significant separation between pre-op and post-op samples.

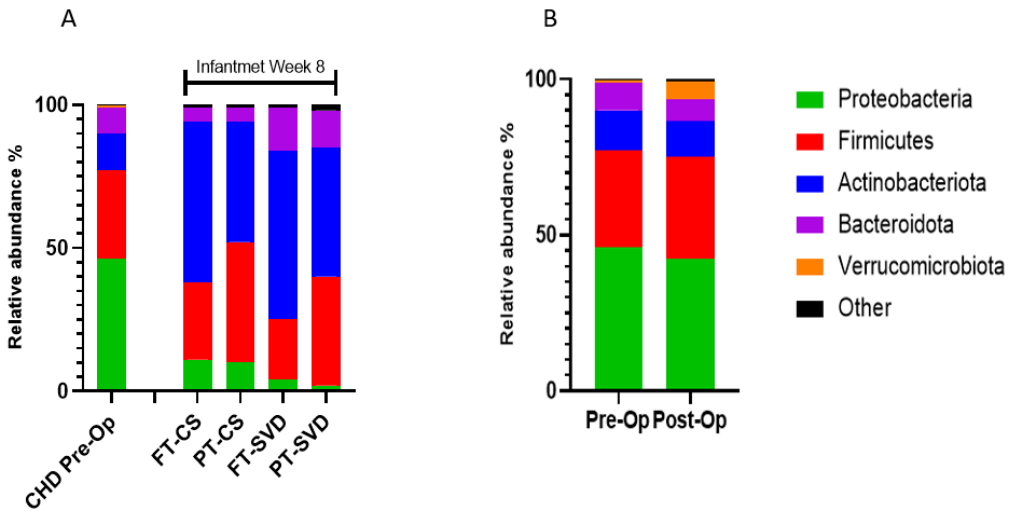

Figure 7.5 Graphic shows relative abundance at phylum level. Figure 7.5A, CHD Pre-Op samples from this study. FT-CS, PT-CS, FT-SVD, and PT-SVD displays samples from INFANTMET cohort. Figure 7.5B, the relative abundance of pre-op and post-op samples are shown.

Pre-op samples were compared with INFANTMET control samples at week 8 of life (Hill et al., 2017). Proteobacteria was found to be the most abundant phylum with 46% relative abundance followed by Firmicutes (31%), Actinobacteria (13%), and Bacteroidota (9%) in the CHD pre-op samples. By contrast, in the INFANTMET samples, the most abundant phylum was Actinobacteria (Figure 7.5A).

When pre-op samples were compared with post-op samples (Figure 7.5B), the relative abundance of Proteobacteria, Actinobacteria, and Bacteroidota were found to be slightly reduced in post-op samples (42%, 11%, and 7%, respectively). Firmicutes and Verrucomicrobiota were slightly elevated in post-op samples (33% and 6%, respectively).

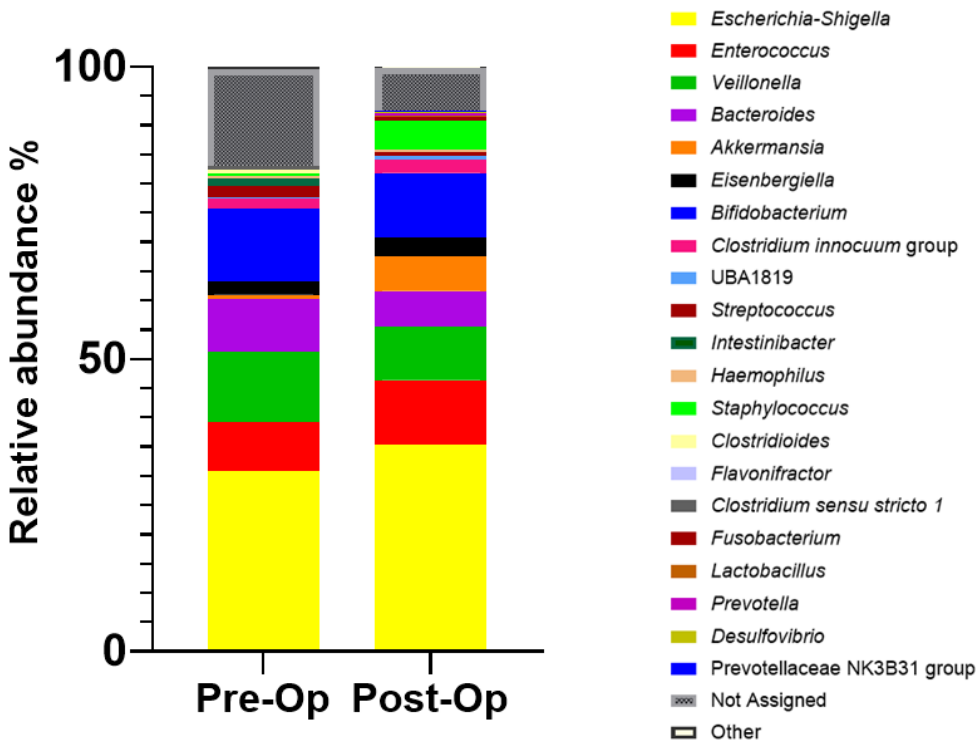

Figure 7.6 The graphic shows the relative abundance at genus level between pre-op and post-op samples.

At the genus level, notable alterations in microbial composition were observed between the preoperative (pre-op) and INFANTMET samples (Supplementary Figure 7.1). In the pre-op samples (Figure 7.6), *Escherichia-Shigella* (31%), *Bifidobacterium* (13%), *Veillonella* (12%), *Bacteroides* (9%), and *Enterococcus* (9%) emerged as the five most predominant genera. Furthermore, the relative abundance percentages for *Staphylococcus*, *Akkermansia*, *Lactobacillus*, *Prevotella*, *Fusobacterium*, and *Streptococcus* in the pre-op samples were 0.44%, 0.8%, 0.01%, 0.05%, 0.06%, and 2%, respectively. In contrast, the INFANTMET samples displayed a microbiota composition differing from that of CHD samples (Hill et al., 2017). In INFANTMET

samples, *Escherichia-Shigella* accounted for 2–6%, *Bifidobacterium* ranged from 40 to 60%, and *Veillonella* constituted 2% of the relative abundance.

Gut microbiota composition revealed distinct differences between pre-op and post-op samples at the genus level (Figure 7.6). Increased relative abundances of *Enterococcus* (11%), *Akkermansia* (6%), *Lactobacillus* (0.2%), and *Staphylococcus* (5%) were found in post-op samples. Conversely, higher relative abundances of *Escherichia-Shigella* (35%), *Veillonella* (9%), *Bacteroides* (6%), and *Streptococcus* (0.5%) were observed in the post-op samples.

Furthermore, MaAsLin2 was used to determine multivariable associations between clinical metadata and microbial features. No significant differences were identified following the Benjamin-Hochberg *p* value correction (q-value > 0.25).

7.4.5 NEC and gut microbiota

One infant (GMB12) developed NEC on post-op day 3. The age of the infant was 10 days, born at 37 weeks gestation. The patient underwent an arterial switch operation, aortic arch repair, VSD closure, ASD closure and left pulmonary artery patch plasty. Infant GMB12 was administered benzylpenicillin and gentamicin before the surgery, and post-surgery, a combination of cefuroxime, gentamicin, amoxicillin, and metronidazole was given as antibiotics. Bypass duration was 249 min and cross clamp duration was 129 min. He was on ventilation for 28 days. Mode of feeding was nasogastric tube and he was fed by EBM. He was in PICU for 32 days. The relative abundance of *Enterococcus* was the most dominant genus in the pre-op sample of the infant with the relative abundance of 93% (Figure 7.7). Following the surgery, *Staphylococcus* was the most abundant genus with relative abundance of 60%

followed by *Bacteroides* with 10%, *Fusobacterium* with 7.4%, and *Prevotella* with 7% and *Escherichia-Shigella* with 6%. Following surgery the relative abundance of *Enterococcus* level was less than 1% (Figure 7.7).

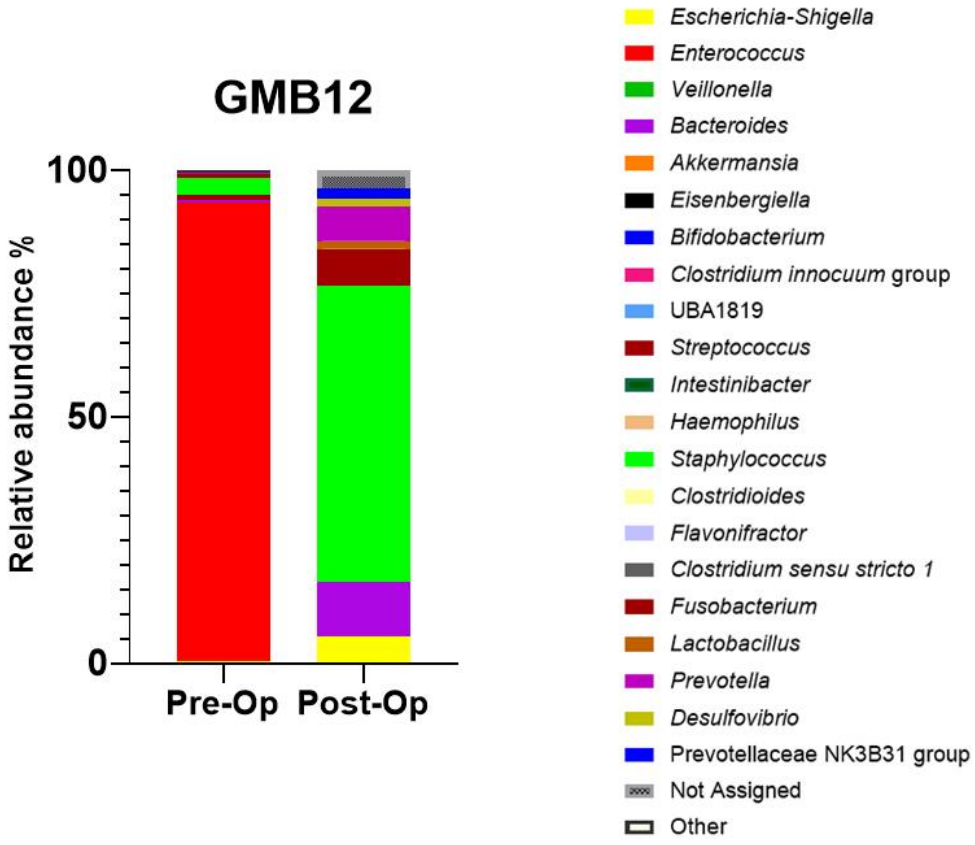

Figure 7.7 The bar chart represents relative abundance of genus levels in GMB12 pre and post-operatively.

7.5 Discussion

This prospective cohort study was conducted to explore gut microbiome alterations during CPB on infants who were diagnosed with CHD. 16S rRNA gene sequencing was performed on faecal samples from pre-surgery and post-surgery to explore alterations in gut microbiome. DNA samples from a previous longitudinal cohort study (INFANTMET) were used as age-matched healthy controls. One infant from the

current study developed NEC three days after the surgery. The microbiome data of the infant was explained exclusively.

Alpha diversity and beta diversity did not show any significant differences between pre-op and post-op samples. However, taxonomic analysis at phylum and genus levels displayed slightly changes between the pre-op and post-op samples based on percentages, however these differences were not statistically significant.

Before surgery, Proteobacteria was found to be the most dominant phylum in the gut microbiota of infants in this study. In INFANTMET cohort samples, the relative abundance of Proteobacteria was lower than pre-op samples in this study (Hill et al., 2017). The increase of Proteobacteria may be driven by the abundance of *Escherichia-Shigella* genera in samples. A 2021 study, where they compared the microbiota of CHD patients with control subjects, found higher relative abundance of Proteobacteria in pre-op samples than healthy infants (Salomon et al., 2021).

At the genus level, *Enterococcus*, *Escherichia-Shigella* and *Staphylococcus* were found to be higher in post-op samples than pre-op samples whereas *Bifidobacterium*, *Bacteroides* and *Veillonella* were found to be lower in post-op samples than pre-op samples. A similar trend was observed in a cohort study with lower relative abundance of *Bacteroides* in post-op than pre-op samples in infants (Salomon et al., 2021).

Escherichia and *Shigella* are known as opportunistic pathogenic bacteria and are associated with a range of infections mainly in gastro-intestinal diseases while *Escherichia coli* can be considered as a commensal microorganism (Koc et al., 2022). Increased levels of *Escherichia* were observed in gut dysbiosis such as small intestine bacterial overgrowth and inflammatory bowel disease (Petersen, 2022). *E. coli* that

produces lipopolysaccharide (LPS) has the potential to induce systemic inflammation, serving as a contributing factor to epithelial barrier dysfunction implicated in various diseases including NEC (Lei et al., 2014, Moore et al., 2016). LPS has been shown to have an important role in NEC development (Leaphart et al., 2007, Jilling et al., 2006).

The relative abundance of *Akkermansia* was found to be higher in post-op samples than pre-op samples. Recent studies showed that *Akkermansia* contains a strain named *A. municiphila* which is considered as a next generation probiotic (Jian et al., 2023, Lakshmanan et al., 2022). Studies showed that *Akkermansia* might be a potential beneficial bacteria to reduce the risk of developing cardiovascular diseases (Jian et al., 2023).

The infant who developed NEC following the surgery had a relative abundance of 93% *Enterococcus* in their gut microbiome. There are several bacterial strains found to be related to NEC yet there is no single strain found that is solely responsible (Coggins et al., 2015). The relationship between *Enterococcus faecalis* and NEC has been previously described in the preterm population (Delaplain et al., 2019). Earlier studies showed that in this population, patients who developed NEC tended to have lower percentages of *E. faecalis* than healthy controls but this did not reach statistical significance (Stewart et al., 2012, Normann et al., 2013). More recently, specific strains of *E. faecalis* have been shown to significantly increase NEC pathology (Delaplain et al., 2019). *Enterococcus* is the third most common nosocomial pathogen, and the rates of antibiotic resistance are increasing (Elizabeth Fiore, 2019). A recent study of neonates with CHD, demonstrated an abundance of enterococcal species, the presence of which was linked to adverse surgical outcomes and systemic inflammation

(Huang et al., 2022a). While this study is not powered to fully explore outcome data, it is notable that this patient had an abundance of enterococcal species and a challenging post-operative course in addition to the development of NEC.

After surgery, *Staphylococcus* was the most abundant genus in the gut microbiome of NEC patient, with a relative abundance of 60% followed by *Bacteroides* (11%), *Fusobacterium* (7.4%) and *Prevotella* (7%). Coagulase negative *Staphylococcus* was commonly found in NEC patients' gut microbiome and was shown to be a prominent pathogen in neonatal intensive care units and the majority cases of neonatal sepsis (Mollitt et al., 1988, Overturf et al., 1990, Heather J.L. Brooks, 2013). *Staphylococcus epidermidis* is likely to be a major contributor to NEC in infants (Dong et al., 2018). *S. epidermidis* was characterized by carriage of pathogenic factors such as *icaA*, IS256, SCC*mec,* and toxins which were found to induce mucosal necrosis and haemorrhage in the bowel (David W. Scheifele, 1987, P. Villari, 2000). *Staphylococcus aureus* is another strain of *Staphylococcus* genus which can damage the cell membrane via producing phenol-soluble modulins (Otto, 2014).

7.6 Study limitations

The initial composition of the gut microbiota among infants undergoing cardiac surgery in this cohort varied considerable possibly reflecting underlying differences in patient characteristics. Rapid changes in the gut microbiome occur over the first two years of life and the wide age range of our patients at time of initial sampling is one such explanation. This heterogeneity posed a challenge in examining changes in the gut microbiota pre and post operatively. Non-significant trends in the gut microbiota of infants undergoing CPB pre- and post-operatively were identified but these did not

reach statistical significance. With regards to post-operative samples, there is a wide variation in antibiotic administration which varies considerably between patients rendering analysis on the impact of antibiotic administration on the gut microbiome difficult. A larger cohort may uncover statistically significant changes and may also shed light on additional influences on the gut microbiota that may contribute to the development of NEC. Furthermore, healthy controls were matched based on gestational age, delivery mode, gender, and sampling age but not feeding type. Healthy controls were exclusively breastfed, while infants with CHD may have received either breast milk or formula. This is a potential confounding factor, and the possibility that it may have contributed to the observed gut microbiome alterations cannot be excluded. Longitudinal studies are imperative to monitor gut microbiota changes over time and identify when the gut microbiota aligns with that of a healthy cohort. In addition to the gut microbiota, investigating immunological parameters in patients may offer connections between immune-microbiota interactions and the onset of NEC.

7.7 Conclusion

This research uncovered notable differences in the gut microbiota of infants with CHD compared to healthy controls. However, in this small cohort no statistically significant differences were identified in pre- and post-operative samples. Administering probiotic strains after surgery could be a beneficial strategy to enhance infant gut health, potentially leading to improved overall well-being, reduced hospitalization duration, and a lower risk of developing NEC.

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7.10 References

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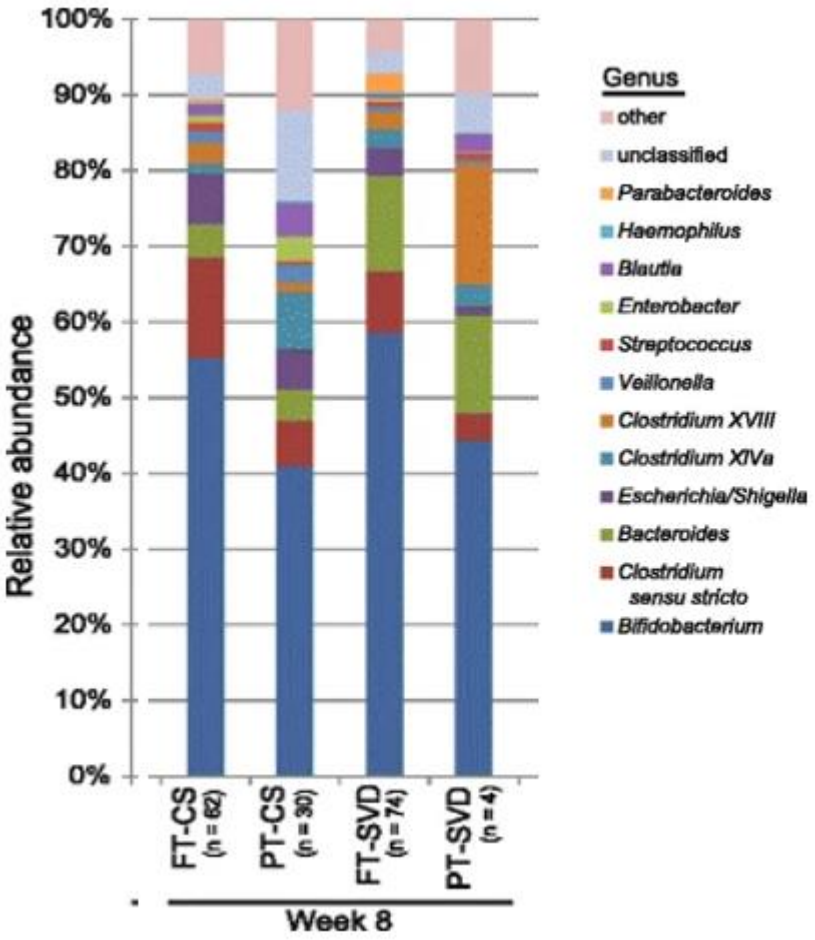
7.11 Supplementary Figures



Supplementary Figure 7.2 Bar chart shows relative abundance at genus level. FT-CS, PT-CS, FT-SVD, and PT-SVD displays samples from INFANTMET cohort.

Chapter 8

General Discussion

8.1 Discussion

The gut microbiota is increasingly recognized as a crucial determinant of health, influencing physiological processes such as metabolism, immune regulation, and gut barrier function (Li et al., 2024, Shen et al., 2024, Wang et al., 2024). Recent advancements in microbiome research have illuminated how dietary components, fermented foods, and microbial interventions can significantly alter gut microbiota composition and function (Zhu et al., 2024, Koc et al., 2024). This interplay is especially relevant in addressing disorders such as irritable bowel syndrome (IBS), metabolic syndrome (MetS), cardiovascular diseases and neonatal complications linked to congenital conditions (Debras et al., 2024, Salomon et al., 2021, Nordin et al., 2024). Through a focus on dietary interventions and microbiome-based therapeutics, this thesis seeks to investigate various strategies for utilizing gut microbiota modulation to enhance health outcomes. The studies encompass three primary themes: the development and assessment of functional foods (Chapters 2-5), the therapeutic efficacy of probiotics and synbiotics (Chapter 6), and the gut microbiome changes following neonatal surgical interventions (Chapter 7). This discussion combines the key findings from each chapter, delves into broader implications, and outlines directions for future research.

This body of work advances our understanding of the gut microbiome's role in health and disease, underscoring the necessity for personalized dietary and microbial interventions. As discussed in **Chapter 1.1**, various models have been reviewed which used study the gut microbiome, detailing their unique advantages, limitations, and specific applications. These models, ranging from *in vitro* systems to animal

experiments and human clinical trials, are crucial for unravelling the complex interactions that occur between microbes and their host. *In vitro* models provide controlled settings to examine microbial interactions and substrate effects, with systems such as batch and continuous cultures yielding foundational insights (Shin, 2013). Innovative platforms, such as SHIME and TIM-2, simulate different sections of the gastrointestinal tract, enhancing physiological relevance (Venema and van den Abbeele, 2013). Notably, the MicroMatrix™ represents a breakthrough in enabling high-throughput experimentation and precise variable control (Wiegmann et al., 2020). However, these models fall short of replicating the intricate host-microbiome interactions present *in vivo*, necessitating further validation through animal studies (Qi et al., 2023). Animal models fill this void, allowing researchers to investigate host-microbiome dynamics, disease mechanisms, and potential interventions. Rodents are commonly used due to their genetic similarities to humans and established methodologies; however, their physiological differences can limit direct applicability (Wos-Oxley et al., 2012). Pigs, with gastrointestinal and immune systems that closely resemble humans, provide invaluable insights for dietary intervention studies, probiotic efficacy, and chronic health outcomes (Hou et al., 2022). They also serve as better models for exploring human pathogens and vaccine effectiveness (Meurens et al., 2012). Non-human primates, while genetically and physiologically akin to humans, present significant ethical and logistical challenges (Phillips et al., 2014). Ultimately, human clinical trials remain the gold standard for elucidating the gut microbiome's influence on health (Swann et al., 2020). These trials provide direct evidence of how various interventions, including dietary modifications, prebiotics, probiotics, and synbiotics, can impact microbial composition and host health. Research has shown that

fibre-rich diets foster the growth of beneficial bacteria and elevate short-chain fatty acid (SCFA) production, leading to improved metabolic health and a reduction in inflammation (Gondalia et al., 2022, Li et al., 2024). Prebiotics, like inulin, selectively stimulate the growth of beneficial microbes, such as *Bifidobacterium*, enhancing gut barrier function and modulating immune responses (Rodriguez et al., 2022). The advent of multi-omics approaches has further refined our understanding of the systemic impacts of the gut microbiome, allowing for personalized nutrition strategies customized to individual microbiomes and metabolic phenotypes.

Dietary choices, particularly the intake of dietary fibre, significantly influence gut microbiota composition (Koç et al., 2020). As discussed in **Chapter 1.2**, dietary fibre plays an essential role as a key macronutrient for gut health, serving to nourish the gut microbiota (often described as a "virtual organ") (Mills et al., 2019). However, despite the recommended daily intake of 25-38 g, average consumption remains below 50% of this target (Koç et al., 2020). This shortfall contributes to imbalances in the gut microbiota, characterized by reduced microbial diversity and an increase in potentially pathogenic microorganisms. Such dysbiosis is closely associated with inflammation, a common factor underlying non-communicable diseases, including obesity, cardiovascular disease, type 2 diabetes, and colorectal cancer (Mills et al., 2019, Li et al., 2024). Increasing dietary fibre intake has been associated with reduced risk factors for these diseases and improved prognosis (Milajerdi et al., 2021, Waddell and Orfila, 2023, Yang et al., 2020). Research has highlighted the benefits of fibre-rich diets in promoting gut microbiota diversity and function, reinforcing its critical role in preventing inflammation-related conditions (Liu et al., 2022). However, existing

studies primarily demonstrate associative, rather than causal, links between fibre intake and health outcomes. A deeper understanding of the biological mechanisms underpinning these associations is needed to strengthen evidence-based recommendations.

The inter-individual variability in gut microbiota composition further complicates this relationship, emphasizing the need for personalized approaches to dietary fibre intake. These insights structured the experimental designs in Chapters 2, 3, 4, and 5, which investigated dietary interventions with enhanced prebiotic potential.

Chapter 2 assessed the effects of SHS treatment on wheat and oat bran, focusing on its impact on the human gut microbiota and SCFA production. SHS-treated and non-treated brans were subjected to *in vitro* digestion followed by *in vitro* colonic fermentation. Post-fermentation, 16S rRNA gene sequencing and SCFA analysis were performed. The SHS treatment notably increased the release of fermentable carbohydrates, including maltose and water-extractable arabinoxylans (WEAX), improving substrate accessibility for gut microbes (Hou et al., 2022). These changes were reflected in the altered microbial diversity and composition of faecal samples fermented with SHS-treated brans. Specifically, SHS treatment reduced the relative abundance of *Escherichia-Shigella*, genera associated with pathogenic effects, while increasing beneficial taxa such as *Bifidobacterium* and *Lactobacillus*, suggesting positive modulation of gut health (Sun et al., 2023). Notably, both *Bifidobacterium* and *Lactobacillus* were enriched in samples fermented with both treated and untreated brans, indicating that wheat and oat brans inherently support beneficial bacteria.

Moreover, the relative abundance of *Firmicutes*, a phylum associated with butyrate production and anti-inflammatory properties, was elevated in SHS-treated oat, SHS-treated wheat, and wheat bran fermentates compared to cellulose controls (Sun et al., 2023). Of particular interest, the *Lachnospiraceae* NK4A136 group, known for butyrate production, was enriched following SHS-treated bran fermentation. Additionally, *Pediococcus*, a lactic acid bacterium with known antimicrobial properties, was more prevalent in SHS-treated bran fermentates (Porto et al., 2017). This highlights the dual role of SHS treatment in enhancing both the functional and safety-promoting properties of dietary fibres.

Interestingly, oat bran, which contains higher beta-glucan levels (Renzetti et al., 2021), induced more pronounced microbiome shifts than wheat bran, indicating that the type of bran influences the outcome of SHS treatment. Overall, SHS treatment improved the fermentability and gut health potential of both bran types. These findings suggest that incorporating SHS-treated fibres into food products could support dietary strategies targeting gut health, including conditions like IBS and IBD.

Future studies should validate these effects through human clinical trials. Exploring additional fibre modification techniques such as high-pressure processing, microwave-assisted treatment, or enzymatic bioprocessing could offer complementary benefits (Hou et al., 2022). *In vivo* studies on diverse fibre types such as glucomannan, psyllium, or resistant starch could enhance our understanding of fibre bioavailability and functionality. Ultimately, integrating modified fibres into consumer products like breakfast cereals, snacks, or meal replacements could advance the development of personalised dietary strategies for managing gut-related disorders.

Chapters 3 and 4 explored dietary strategies targeting IBS through low-FODMAP formulations. In **Chapter 3,** we assessed the effects of various biscuit formulations on the gut microbiome, focusing on the potential of low-FODMAP, high-fibre biscuits to modulate gut health. Specifically, the LFP biscuits, low in FODMAPs and high in fibre, promoted greater gut microbiome diversity compared to BiFC biscuits, which were associated with higher levels of pathogenic *Escherichia-Shigella*. These bacteria are linked to IBS symptoms (Shukla et al., 2015). In contrast, LFP biscuits reduced *Escherichia-Shigella* relative abundance, suggesting their beneficial role in gut health. Additionally, WMC biscuits, associated with increased *Lactobacillus* relative abundance, demonstrated beneficial effects on gut health (Cooper et al., 2017). LFP biscuits reduced *Veillonella* levels, a genus implicated in IBS and oral diseases (Giacomini et al., 2023; Zhan et al., 2022), further highlighting their potential therapeutic effect. Despite the known reduction in *Bifidobacterium* levels in response to low-FODMAP diets (So et al., 2022), their role in IBS symptom management remains complex. Importantly, our results suggest that personalized dietary interventions, such as tailored low-FODMAP high-fibre formulations, could alleviate IBS symptoms by modulating the gut microbiome.

In **Chapter 4,** we expanded on these findings by examining the effects of sourdough breads fortified with specific lactic acid bacteria (LAB) strains (*L. plantarum* FST1.7, *L. paracasei* R3, and *P. pentosaceus* RYE106) on the gut microbiome. These breads, fermented *in vitro*, led to significant changes in microbial composition and SCFA production. Similar to our biscuit formulations, LAB fermentation resulted in elevated levels of *Agathobacter*, a key butyrate producer essential for gut health (McNabney and Henagan, 2017). Additionally, *Bacteroides* levels diminished in certain LAB-

fermented breads, suggesting protective effects against gut dysbiosis, which is often linked to IBS. The presence of *Limosilactobacillus* in SD-FST1.7 and the increase in *Bifidobacterium* in inulin-treated samples further support the therapeutic potential of these formulations for promoting gut health. SCFA analysis revealed that elevated levels of acetate, butyrate, and propionate in LAB-fermented breads could play a role in alleviating IBS symptoms by maintaining gut homeostasis and exerting anti-inflammatory effects.

Taken together, both chapters underscore the potential of dietary interventions, such as low-FODMAP, high-fibre biscuits and LAB-fermented low-FODMAP sourdough bread, in managing IBS symptoms through gut microbiome modulation. These findings suggest that personalized nutrition, targeting specific microbial populations and SCFA production, may be an effective strategy for IBS symptom relief. Future research should focus on human clinical trials to validate these results, exploring the long-term effects of these formulations on gut microbiota composition and functional health outcomes. Additionally, incorporating prebiotics like inulin or exploring alternative fermentation techniques could enhance the therapeutic potential of these foods, bridging the gap between research and real-world application.

Chapter 5 focused on the effects of traditional Turkish fermented food, tarhana, on the gut microbiota, using *in vitro* digestion, colonic fermentation, 16S rRNA gene sequencing, and SCFA analysis. Tarhana was made with four different flours (chickpea, purple potato, wheat, and einkorn) and fermented with either baker's yeast or sourdough. The study demonstrated that both flour type and fermentation method

significantly influenced microbial diversity, SCFA, and BCFA production, suggesting that tarhana can beneficially modulate gut microbiota.

Sourdough fermentation, in particular, enhanced beneficial bacteria like *Faecalibacterium* and *Roseburia*, linked to gut health and SCFA production, while reducing pathogenic genera such as *Escherichia-Shigella* and *Campylobacterota* (Lemons et al., 2024; Malinen et al., 2005). Both chickpea and purple potato flours increased SCFA levels, especially acetate, with sourdough fermentation further supporting gut health. Notably, *Akkermansia*, known for its anti-inflammatory effects, was enriched in sourdough-fermented samples, particularly those with wheat and einkorn flour, suggesting potential benefits for gut barrier integrity (Rodrigues et al., 2022). Additionally, *Bifidobacterium*, enriched in purple potato flour samples, further supports tarhana's role in promoting gut health (Sabate & Iglicki, 2022).

This study aligns with findings from earlier chapters, demonstrating that fermentation, especially sourdough, enhances the health-promoting properties of tarhana. Sourdough-fermented tarhana with chickpea or purple potato flour shows particular promise as a functional food for gastrointestinal health. Future research, including human clinical trials focused on populations with IBS or IBD, is needed to validate these effects. Exploring the use of alternative flours like buckwheat or millet, as well as tailoring tarhana to specific dietary needs (e.g., low-FODMAP or gluten-free versions), could expand its therapeutic potential for a broader population.

The models used thus far were all *in vitro* models and while they are critical for providing preliminary information on the impact of interventions on gut microbiota composition and functionality, *in vivo* models are vital when assessing the impact of

interventions on disease. Metabolic syndrome (MetS) which is a multi-faceted disorder potentially impacting >30% of the global population (Noubiap et al., 2022) and significantly increases the risk for cardiovascular diseases. Understanding the impact of diet and potential interventions on MetS is essential and pigs represent excellent models for this due to their physiological and anatomical similarities to humans. Furthermore, in addition to investigating the gut microbiome, metabolic profiling is crucial tool for understanding how diets and interventions impact health. Thus, in **Chapter 6**, we developed a porcine model of MetS to examine the faecal and serum metabolomic changes resulting from different treatments involving fibre and probiotic combinations. We conducted two trials, in Trial 1 we have developed MetS model using high fat, high sugar and high salt diet and compared their faecal metabolome with control pigs at week 6 and week 12. In Trial 1, faecal samples from MetS developed pigs indicated significant metabolic differences compared to control animals, both at 6 and 12 weeks of trial. Notably, the MetS pigs exhibited high levels of metabolites, such as elaidic carnitine, kynurenic acid, and histamine, suggesting disruptions in fatty acid metabolism and inflammatory pathways (Zheng et al., 2021). These shifts hint at alterations within the gut microbiome and an increased reliance on fatty acid oxidation, contributing to MetS-related dysfunctions. Disruptions in glucose uptake and insulin resistance were evident, with impaired insulin sensitivity and elevated glucose levels. These factors contribute to an imbalance between glucose and fatty acid oxidation, promoting ketogenesis and further compounding metabolic dysfunction. Altered glucose metabolism and the shift toward fatty acid oxidation are central features of MetS, contributing to insulin resistance, increased lipolysis, and ketosis, further aggravating the metabolic disturbances. In Trial 2, MetS developed

pigs have been administered dietary fibre, *Lb. mucosae* DPC6426 or synbiotics (*Lb. mucosae* DPC6426+dietary fibre). Their faecal metabolome were compared at week 6 and week 12 as well as serum metabolome at week 2, week 6 and week 12. In Trial 2, four treatment groups were evaluated, including *Lb. mucosae* DPC6426 and fibre supplementation. While no significant differences were noted across the groups, metabolites such as trans-2-dodecenoylcarnitine and 2-hydroxybutyric acid were more abundant in the synbiotic-treated pigs, indicating that *Lb. mucosae* DPC6426 and fibre supplementation could influence trends in the faecal metabolome, although without statistical significance. Key metabolites like carnitines and kynurenic acid were significantly elevated in the MetS group, highlighting a shift towards fatty acid oxidation and altered immune responses. Additionally, increased histamine levels may contribute to intestinal permeability and inflammation, potentially worsening MetS progression (Branco et al., 2018). Ceramides, another metabolite group, were also higher in the MetS pigs, linking them to insulin resistance and inflammation, thereby underscoring the metabolic disturbances observed in this model (Summers, 2006). Urea cycle disruptions, including altered arginine and ornithine concentrations, suggest defects in nitrogen metabolism that compound metabolic disturbances. While the synbiotic intervention did not significantly alter metabolite profiles in Trial 2, the trends suggest potential benefits in modifying gut microbiota and influencing metabolic pathways such as urea cycle. Synbiotic intervention resulted in lower urea levels in serum samples compared to MetS pigs. These findings offer insights into the biochemical changes associated with MetS and propose that interventions aimed at gut microbiota modulation could provide therapeutic potential for managing metabolic disorders. A key limitation of this study is the lack of comprehensive monitoring of

immune parameters and cardiac health. Since MetS is associated with chronic inflammation and cardiovascular risk, assessing immune cell profiles (e.g., cytokine levels, T-cell function) and cardiac parameters (e.g., blood pressure, lipid profiles) during disease progression could provide more comprehensive insights into the systemic effects of the interventions. Integrating these immune and cardiac parameters could help establish a clearer link between gut microbiota modulation, metabolic alterations, and disease progression. Future studies should aim to investigate longer-term effects of fibre and probiotic supplementation on the MetS metabolome, with a particular focus on refining the intervention strategies. For instance, exploring different probiotics strains or administering different dosage of dietary fibre may reveal more distinct effects on metabolic profiles. Additionally, integrating more advanced omics techniques, such as transcriptomics and proteomics, could provide a more comprehensive understanding of the molecular mechanisms driving the observed metabolic shifts and how they are modulated by interventions. A deeper exploration of the relationship between specific metabolites, such as carnitines, kynurenic acid, and histamine, and the progression of MetS could help identify biomarkers for early diagnosis and monitoring of the disease. Investigating the role of ceramides in insulin resistance and inflammation could also open new avenues for targeted therapeutic interventions. Finally, future research could also examine the interplay between gut microbiota, diet, and host immune responses in the context of MetS. Understanding how different dietary components influence the microbiome and, in turn, modulate metabolic pathways could lead to more personalized interventions that improve metabolic health and reduce the risk of chronic diseases associated with MetS.

Turning to our exploration of gut microbiome changes in infants with congenital heart disease (CHD) who have undergone cardiac bypass surgery, we detailed our sample collection protocol and study design in **Chapter 7.1**. This cohort was selected due to the high prevalence of gut dysbiosis and post-surgical complications like NEC among infants with CHD. The rationale for studying this cohort was based on the high prevalence of gut dysbiosis and post-surgical complications, such as necrotizing enterocolitis (NEC), in this group. Infants with CHD are at greater risk of microbial imbalances, which can exacerbate recovery and contribute to serious complications. By examining the gut microbiome changes in these infants, particularly in the context of surgical stress, we aimed to identify microbial patterns that could serve as biomarkers for early detection of complications such as NEC. This could inform the development of personalized probiotic or dietary interventions to improve postoperative recovery and reduce the risk of adverse outcomes. In **Chapter 7.2**, we analysed gut microbiome alterations in infants diagnosed with CHD who underwent cardiopulmonary bypass (CPB) surgery. The analysis compared pre- and post-surgery gut microbiota using 16S rRNA gene sequencing, alongside healthy controls identified in a previous study (INFANTMET) (Hill et al., 2017). Though no significant differences were identified in alpha or beta diversity, taxonomic analysis revealed trends in microbial shifts. Notably, Proteobacteria predominated pre-surgery in the study group, contrasting with lower levels in healthy controls. *Escherichia-Shigella* was identified as a likely driver of the increased Proteobacteria, aligning with findings from other CHD studies. At the genus level, post-surgery revealed elevated *Enterococcus*, *Escherichia-Shigella*, and *Staphylococcus*, while *Bifidobacterium*, *Bacteroides*, and *Veillonella* diminished. Increased *Escherichia* levels correlate with

gut dysbiosis, and previous studies suggest a potential role in systemic inflammation and necrotizing enterocolitis (NEC) development (J.L et al., 2013). One infant experienced NEC following surgery, with their gut microbiome pre-surgery showing a 93% abundance of *Enterococcus* a trend previously associated with negative surgical outcomes in other investigations (Wang et al., 2012). The infant who developed NEC following the surgery exhibited a gut microbiome dominated by *Staphylococcus*, a common pathogen linked to neonatal sepsis, particularly *Staphylococcus epidermidis* (Dong et al., 2018). Remarkably, *Akkermansia*, a potential next-generation probiotic, was found at higher levels post-surgery, possibly indicating beneficial effects, notably regarding cardiovascular health (Jian et al., 2023). The study encountered challenges due to patient heterogeneity, marked by variations in initial gut microbiota and antibiotic usage. The small cohort size limited the detection of statistically significant changes, and the exclusively breastfed healthy controls contrasted with the potentially formula-fed CHD infants, introducing a potential confounding factor. Although no significant differences were found, this study implies that microbiota variations in CHD infants could influence post-operative health outcomes, including the risk of NEC. The administration of probiotics following surgery presents a promising intervention to enhance gut health and mitigate complications. Emerging evidence also suggests that preoperative administration of probiotics may provide additional benefits. Probiotics administered prior to surgery could modulate the gut microbiota, potentially enhancing its resilience and functionality during the perioperative period. Studies have demonstrated that early probiotic supplementation can modulate immune responses, improve gut barrier function, and reduce systemic inflammation in infants, which are critical factors in preventing NEC (AlFaleh and Anabrees, 2014,

Underwood, 2019). Further studies, particularly those involving larger cohorts and longitudinal monitoring, will be crucial to corroborate these findings and investigate the immune-microbiota interactions linked to the development of NEC.

Across the studies presented, several crucial themes emerge that underscore the significance of gut microbiota modulation in disease management. First, dietary and technological interventions to regulate gut microbiota show great promise for addressing conditions such as IBS and metabolic syndrome. For instance, our findings on the effects of dietary fibres, probiotic strains, and functional food formulations illustrate how tailored dietary approaches can effectively influence microbiome composition and functionality. Second, the complexity of gut microbiome interactions with diet emphasizes the necessity for personalized strategies. Whether related to fibre type, food formulation, or selection of specific lactic acid bacteria (LAB) strains, the research highlights the importance of individualized approaches to nutrition. This is particularly relevant in our study of traditional fermented foods, like tarhana, which suggests that variations in fermentation methods can significantly affect gut health outcomes. Additionally, the integration of advanced fermentation models and innovative food processing techniques serves to bridge the gap between laboratory research and practical applications. These approaches not only facilitate the design of functional foods personalized to individual health needs but also enhance our understanding of metabolomic changes associated with dietary interventions. Furthermore, our exploration of gut microbiome alterations in infants undergoing cardiac surgery reveals how microbial diversity pre-surgery may impact post-operative health outcomes. This highlights the broader implications of microbiota modulation across different populations and health conditions, from infants with congenital heart

disease to adults with metabolic and gastrointestinal disorders. Future research should expand upon these findings by conducting rigorous clinical trials to validate the effectiveness of these interventions. Furthermore, investigating the molecular mechanisms that drive microbiota-diet interactions will be essential for expanding our understanding. Recognizing the role of inter-individual variability is also crucial, as it will aid in developing truly personalized nutrition strategies. By combining methodological rigor with practical applications, this collective body of work contributes to the overarching goal of leveraging the gut microbiome to improve health outcomes and manage a range of diseases more effectively.

8.2 Conclusion

The studies presented in this thesis provide compelling evidence that dietary and technological interventions can effectively modulate gut microbiota composition and functionality, offering promising strategies for managing conditions such as IBS, metabolic syndrome, and neonatal complications related to congenital heart disease. The findings underscore the necessity for personalized approaches in nutrition, revealing that factors such as fibre type, food formulation, and specific probiotic strains play essential roles in shaping individual gut health outcomes. Moreover, the research emphasizes the importance of integrating advanced fermentation techniques and innovative food processing methods to bridge the gap between laboratory findings and practical applications. This integration facilitates the development of functional foods tailored to meet individual health needs while also enhancing our understanding of the metabolic changes that occur in response to dietary interventions.

The implications of this work extend beyond dietary changes alone; they encompass a wide range of health conditions and populations. Our investigation into the gut microbiome alterations in infants undergoing cardiac surgery highlights the potential for microbial diversity to influence postoperative recovery, suggesting that microbiota modulation could play a vital role in improving health outcomes across diverse clinical scenarios.

As we look to the future, it is essential to conduct further clinical trials to validate the efficacy of these interventions and to deepen our understanding of the molecular mechanisms that underpin microbiota-diet interactions. Additionally, recognizing and addressing inter-individual variability will be pivotal in developing truly personalized nutrition strategies. This collective body of work not only enhances our understanding of the gut microbiome's influence on health but also contributes to the broader objective of leveraging microbiota modulation as a practical tool for improving health outcomes and managing a diverse range of diseases effectively. Through continued research and a focus on translation into clinical practice, we can harness the power of the gut microbiome to promote better health and well-being for individuals and communities alike.

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