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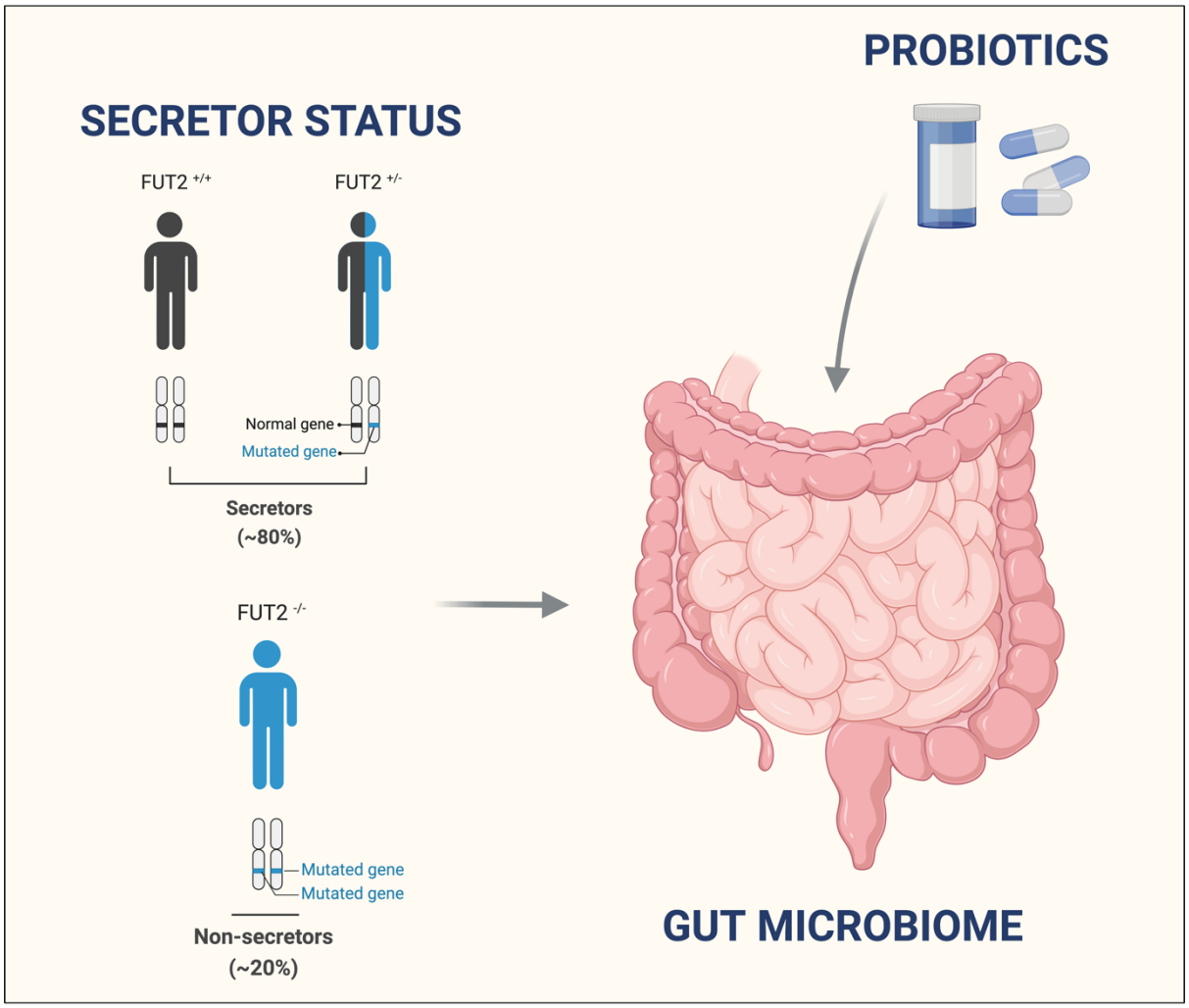
HOW DOES OUR MUCUS SHAPE BACTERIAL COLONIZATION?: EXPLORING MUCOSAL GLYCOSYLATION AND GUT MICROBIOME DYNAMICS

Yiming Wang BSc. MRes. (Biomedical Science)



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ABSTRACT

The profound influence that the gut microbiome exerts on diverse aspects of human physiology presents opportunities for therapeutic interventions. However, to be effective, the design of these interventions must account for variations in the nature of host-microbiome interactions between individuals. Amongst factors known to shape microbiome characteristics, human genetics is arguably the least well understood.

This thesis explores the influence of a common genetic variant that affects the production of mucosal α(1,2)-fucosylated glycans on mucosal secretions and occurs in around 20% of the human population. Specifically, it focuses on the relationship between expression of this trait and the retention and proliferation within the gut of common probiotic strains of *Bifidobacterium* species that differ in their ability to utilise α(1,2)-fucosylated glycans as an energy source. The investigation utilised a mouse model in which littermates varied in their ability to secrete α(1,2)-fucosylated glycans (*Fut2*$^{+/+}$ or *Fut2*$^{-/-}$) into which probiotic bacterial preparations were introduced by oral gavage. The consequences of gut microbiota disruption, a common trigger for probiotic use, was explored through antibiotic exposure prior to bacterial instillation. Probiotic dynamics and their relationships with host genotype and the characteristics and integrity of the gut microbiota were explored using a range of molecular and culture-based approaches. Amongst notable findings were significant differences in baseline gut microbiology, probiotic persistence, and impact of antibiotic disruption, according to *Fut2* genotype.

The findings described within this thesis establish an important association between a common human genetic polymorphism, the gut microbiome, and the potential to derive benefit from attempts to modulate host-microbiome interactions using probiotics. The insight gained into the influence of genetic polymorphisms and bacterial dynamics have profound and far-reaching consequences for the development and interpretation of probiotic interventions.

DECLARATION

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Yiming Wang

21 September 2023

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For those interested in this section, some patience is needed since summarising four—albeit extraordinary—years of life is no easy feat in such a small section. And readers beware: what follows is no ordinary list of acknowledgements. I intend to depart from conventional-style writing here to paint a candid, innovative assessment of a candidature well-lived.

Before jumping in, I first want to summarise my PhD life. Unlike others' PhD lives, which often border on hellish, mine was heaven-like compared to the two years prior (Masters by Research). I must admit I experienced some difficult moments embarking on this journey, but it was relatively smooth sailing afterwards. More importantly, I deeply believe the successes achieved during my PhD (here, I'm not referring to academic ones, which I will cover shortly) have been life-changing for the future. In these past four years, I met many people by destiny. I wish I could acknowledge each in just 1–3 sentences, as do other PhD students; however, their presence has significantly impacted me. To do them justice, I must introduce and thank each individually:

Prof. Geraint Rogers (Principal supervisor):

When meeting him in person, the first words that come to mind describing him before conversing might be 'handsome'. Thus was the first impression of three ladies in the lab who claimed they were attracted by his appearance (Geraint, if you are reading this paragraph, don't laugh, and I've no intention of telling you who they are for confidentiality reasons). His appealing appearance aside, his knowledge and expertise in clinical microbiology are markedly the more striking. A popular saying among youth in China translates as 'a man can

easily rely on his looks, yet will choose to succeed on merit', which perfectly sums up people like Geraint. Unlike the three ladies in the lab, his writing skills are what initially impressed me—I'd read his publications before we met him in person. I still recall how expert and professional his revisions were to my research proposal compared to those of two other potential supervisors. Thanks to that research proposal, I made the most resolute decision of my life to date: to be guided by him and renounce two other scholarship offers (Monash University and University of Queensland). Looking back after four years, it's a decision I will never regret.

To my eyes, Geraint is a supportive, empathetic, flexible and respectful supervisor. He supports each of his students differently, making them less stressed during their PhD journeys. As his student, I've learned much from him, far more than he could imagine. I will carry more than a few of these lessons, I sincerely believe, into my life post-PhD. First and foremost was establishing critical thinking. Like most people born and who grow up in China, we are taught by either teachers or parents what we should do and the type of people we should become from an early age, and we are not allowed to question the rationality of that. The presence of my supervisor, Geraint, opened other doors for me, helping me realise things in the world are neither purely black nor white, and I should think critically and have the courage to question authority. I honed this with each of our academic conversations and in chilled catch-ups about my personal life. The second thing I learned from him is to think of questions logically, moving from rationale to the answer. He may be unaware that how Asian people think and how Westerners think are different. In Asian populations, we normally find answers by starting from the end result to the rationale. It means when we read interesting results in a paper or accidentally unearth our own interesting results, we go searching for what happened to contribute to such results. However, most Westerners first prefer a sound rationale before spending time working on the project. Both ways now make sense to us. Using a Western thinking style, we can complete jobs more efficiently. I believe such a thinking style will make my future work more efficient, especially for work requiring little innovation. Last, he taught me

how to present my work to future managers/reporting people. A concise and target-driven summary is what readers look for instead of a bunch of work that confuses them.

I sincerely thank him for all his time and efforts in guiding me during my PhD journey and for his counsel on my future life, which he probably had no inkling of. I would say I feel so lucky to have him as my supervisor.

Dr. Steven Taylor (Associate supervisor):

After these PhD years, I am a little concerned my luck has already run out, given I met Geraint and Steven during this time. Steven was the first person I knew in this lab. I still remember the day he sat alongside me, working through different projects and how thoughtful he was to sketch each project's general background on a piece of paper. Thanks to this, the early stages of my PhD journey were nowhere near as difficult as they could have been. I am aware he is relatively new to supervising; perhaps I'm the first PhD student in his academic career. If that's the case, as his first student, I think he is a qualified supervisor. He supports all students differently when it comes to research work. Also, I consider him an excellent researcher. Many researchers easily get stuck on fascinating findings, focusing, thus, on the details. Steven represents another calibre of researchers who (like Geraint) never lose sight of the bigger picture on projects, avoiding becoming distracted by findings. This is a merit many researchers lack but is super important, as how each research study contributes to science or clinical settings matters more than what we find from each research study.

Steven is not only a supervisor to me but also a friend. In his personal life, he is a kind man who adopted a super cute dog, Sox, with his partner, Chloe. I've visited Sox many times and can tell this dog leads an extremely happy life in their home. I believe I am a witness to his and Chloe's love story. Chloe is the nicest, kindest, most friendly and thoughtful person one could ever meet. I was so happy for them when they announced they were getting married. I offer them my best wishes here in the acknowledgements section. Steven: I am still working

on gifts for your wedding; one will be hidden in this thesis. It's not yet ready, but it will be before I send off my final copy to the school. I hope you both like it.

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Dr Jocelyn Choo

Should anyone come to Flinders Medical Centre, where the lab is located, Jocelyn is easily recognisable among all staff working there, as she carries at least 4 different bags to the office most mornings.

While not my supervisor, she is like a big sister or guardian angel, supporting me whenever I need help. Moreover, she is a superb researcher who specialises in all lab techniques. As lab manager, she organised it brilliantly. She is one of the lab's greatest assets, and I've no doubt all her hard work will pay off.

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Last but not the least, I wish to thank Dr Abigail Taylor at Love Words Editing for expertly copyediting my thesis in line with Flinders University guidelines.

Thesis Tributes: Faces of Influence

This art, conceived by Yiming and skilfully brought to life by my friend Xin Qi

RESEARCH WORK

Research work prior to PhD but published during PhD

- Dong, H[#]., **Wang, Y[#].**, Zhang, X[#]., Zhang, X., Qian, Y., Ding, H., & Zhang, S*. (2019). Stabilization of brain mast cells alleviates LPS-induced neuroinflammation by inhibiting microglia activation. *Frontiers in cellular neuroscience*, *13*, 191. (IF=5.3)

- Lee, S. A., **Wang, Y.,** Liu, F., Riordan, S. M., Liu, L., & Zhang, L*. (2020). Escherichia coli K12 upregulates programmed cell death ligand 1 (PD-L1) expression in gamma interferon-sensitized intestinal epithelial cells via the NF-κB pathway. *Infection and immunity*, *89*(1), 10-1128. (IF=3.1)

Research work related to PhD and published during PhD:

- **Wang, Y.,** Taylor, S. L., Choo, J. M., Papanicolas, L. E., Keating, R., Hindmarsh, K., Thomoson, M. R., Morgan, L., Rogers, B. Geraint*., & Burr, L. D*. (2022). Carriage and Transmission of Macrolide Resistance Genes in Patients With Chronic Respiratory Conditions and Their Close Contacts. *Chest*, *162*(1), 56-65. (IF=11.4)

Manuscripts in progress:

- **Wang, Y.,** Choo, J. M., Richard, A., Papanicolas, L. E., Wesselingh, S., Taylor, S. L*., & Rogers, B. Geraint*. (2024) Intestinal persistence of *Bifidobacterium infantis* is determined by interaction of host genetics and antibiotic exposure. (Submitted to *ISME Journal*, Minor Revision, Under review)

[#] These authors shared the first author

* Corresponding or Senior author

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CHAPTER 1. INTRODUCTION

1.1 Preface

It is increasingly evident that the community of microbes colonising the human gastrointestinal (GI) tract (the gut microbiome) plays an integral and modifiable role in host health. The ability of therapeutic interventions to modulate host health relies on our understanding of the complex host–microbiome interactions.

To introduce these concepts and their interrelationship, I cover the following sections in Chapter 1 (see Figure 1.1):

- 1.3 Gut physiology
- 1.4 Gut microbiome
- 1.4 Interaction between host and gut microbiome
- 1.5 Manipulation of the host–microbiome relationship

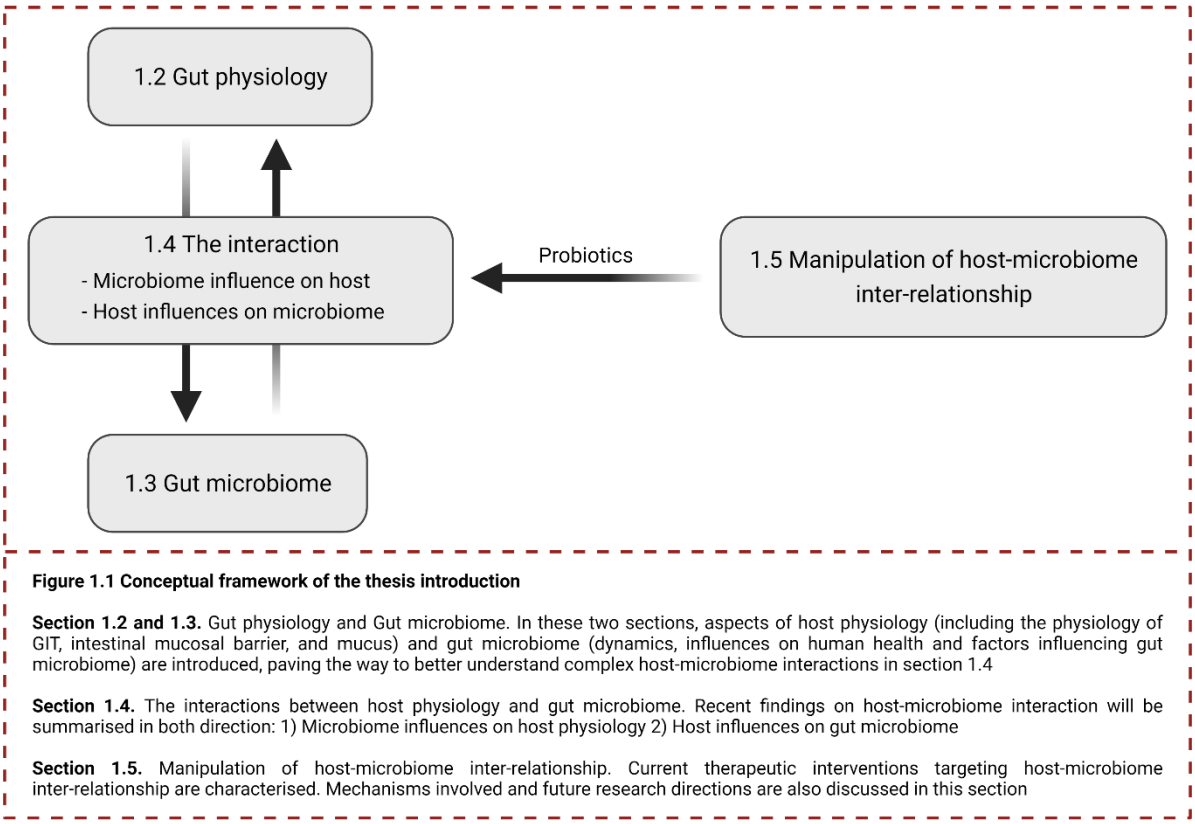


Figure 1.1 Conceptual framework of the thesis introduction

Section 1.2 and 1.3. Gut physiology and Gut microbiome. In these two sections, aspects of host physiology (including the physiology of GIT, intestinal mucosal barrier, and mucus) and gut microbiome (dynamics, influences on human health and factors influencing gut microbiome) are introduced, paving the way to better understand complex host-microbiome interactions in section 1.4

Section 1.4. The interactions between host physiology and gut microbiome. Recent findings on host-microbiome interaction will be summarised in both direction: 1) Microbiome influences on host physiology 2) Host influences on gut microbiome

Section 1.5. Manipulation of host-microbiome inter-relationship. Current therapeutic interventions targeting host-microbiome inter-relationship are characterised. Mechanisms involved and future research directions are also discussed in this section

Figure 1.1 Conceptual framework of the thesis Introduction

In this thesis, I explore the host–microbiome interaction. Several variables were utilised to represent different components of host–microbiome interactions to achieve this, specifically:

- genetic polymorphisms in the fucosyltransferase 2 (*Fut2*) gene to explore the impact of genetic mutations in mucin glycosylation on gut microbiome and *Fut2*-associated species
- probiotic interventions to indicate how bacterial supplementation modifies the host–microbiome interrelationship
- antibiotics to disrupt gut microbiome and minimise the impact of baseline gut microbiome when assessing bacterial supplementation effects on host–microbiome interrelationship.

1.2 Glossary & Abbreviations

1.2.1 Glossary

Given researchers used terms differently, the purpose of the glossary is to define their meaning as they are used in this thesis.

- **Antibiotics:** extrinsic drugs to disrupt the gut microbiome
- **Probiotic intervention:** a single bacterial intervention to colonise the gut
- **Host–microbiome interactions:** the interaction between the gut microbiome and host–gut physiology. The thesis specifically focuses on gut microbiome and mucin glycosylation
- **Secretor status:** an individual's genetic and physiological trait that determines whether they secrete certain blood group antigens into their bodily fluids, including saliva, mucus, tears, and other secretions
- ***Fut2*:** the secretor gene that encodes the enzyme α-1,2-fucosyltransferase, which facilitates attachment of the L-fucose monosaccharide to specific O-linked glycan chains, producing α(1,2)-fucosylated glycans

To enhance comprehension of the abbreviated terminologies utilized within this document, a comprehensive list of abbreviations is provided below (See next page).

1.2.2 Abbreviation list

Abbreviation	Full name
GI tract	gastrointestinal tract
GALT	gut-associated lymphoid tissue
sIgA	secretory immunoglobulin A
AMPs	antimicrobial proteins
IBD	inflammatory bowel disease
NAFLD	non-alcoholic fatty liver disease
SCFAs	short-chain fatty acids
CDI	*C. difficile* infection
MAVs	microbiome-associated genetic variants
GWAS	genome-wide association studies
NOD2	nucleotide-binding oligomerization domain-containing protein 2
IBS	irritable bowel syndrome
HMOs	human milk oligosaccharides
FMT	faecal microbiota transplantation
KO	knockout
WT	wildtype
HET	heterozygous
sgRNA	single-stranded guided RNA
UEA1	Ulex europaeus Agglutinin 1
MRS-CS	De Man, Rogosa and Sharpe broth/agar supplemented with cysteine-HCl and sodium ascorbate
CFU	colony forming unit
OD	optical density
SOP	standard operating procedure
PBS	phosphate buffered saline
ENA	European Nucleotide Archive
nMDS	non-metric multidimensional scaling
CT	cycle threshold
PERMANOVA	permutational multivariate ANOVA
SIMPER	similarity Percentage
LEfSe	linear discriminant analysis effect size
ANOISM	analysis of similarities
GHs	glycosyl hydrolases
AUC	area under the curve
2'-FL	2'-Fucosyllactose
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight

1.3 Gut and mucosal physiology

1.3.1 Human gastrointestinal (GI) tract

The human GI tract is a large communicable interface connecting the human body to the environment. Due to its complexity, it is usually divided into the upper and lower GI tracts. Each site comprises many organs. The upper GI tract includes the mouth, pharynx, oesophagus, stomach and duodenum (the first part of the small intestine). The remaining small intestine organs—the jejunum, ileum, colon, rectum and anus—are in the lower GI tract.

Due to the presence of these organs, our GI tract plays a critical role in nutrition absorption. The process of nutrition absorption occurs in the small intestine. To ensure efficiency, food digestion, a mechanical and biochemical process to break down foods into small particles, is an important step before nutrition absorption. Once food is digested, those nutrient molecules, including fats, proteins and carbohydrates, can travel through the intestinal epithelium via paracellular or transcellular pathways and enter the bloodstream for use. All other food materials that cannot be absorbed or utilised will be expelled through the rectum and anus.

Besides nutrition absorption, the GI tract is responsible for maintaining immune homeostasis.[1] However, maintenance of immune homeostasis is never an easy task. The immense GI tract surface area allows for nutrient absorption and is a repository for large microorganism communities, including innocuous and pathogenic microbes. Our GI tract, therefore, needs to protect the human body from pathogens while at the same time providing an environment tolerant to those harmless microbes. Once antigens are detected, innate and adaptive immune responses are quickly mounted with the help of antigen-presenting cells such as dendritic cells, macrophages and other immune cells that reside in gut-associated lymphoid tissue (GALT). Such an immune response could be either immunogenic against pathogens or tolerant to commensal microbes through multiple immune tolerance mechanisms, like producing secretory immunoglobulin A (sIgA) or inducing Th2 responses. Disrupting immune

homeostasis leads to chronic intestinal inflammation and other gut-related disorders like coeliac disease.[2,3]

1.3.2 Intestinal mucosal barrier

The human GI tract forms a physical and immunological barrier that separates the environment and internal host milieu while allowing luminal interaction. This barrier is referred to as the intestinal mucosal barrier. It comprises several elements that are important in allowing nutrient absorption and preventing the entry of harmful substances, such as luminal antigens and pathogens.

The mucus layer is the top part that separates luminal contents from the epithelial compartment of the intestine. The main components include glycosylated proteins secreted by goblet cells and water. An important part of the barrier, it not only prevents gut microbiota from contacting epithelial cells with the help of antimicrobial proteins (AMPs) and sIgA[4], but also facilitates the passage of luminal contents longitudinally. In the colon, two layers (inner and outer) are formed, and microbiota is mainly confined to the external layer. However, the mucus layer is diffused in the small intestine and does not form a double layer. Beneath the mucus layer is the intestinal epithelium, consisting of five distinct cell types originating from pluripotent stem cells residing in the crypts[4]. These include enterocytes, goblet cells, enteroendocrine cells, Paneth cells and microfold cells. These cells and the mucus layer contribute to absorbing nutrients, transporting substances and preventing the entry of pathogens.

Many external factors, like alcohol, medication, diet, smoking and stress, impact the gut homeostasis[4] and intestinal barrier integrity[5]. Defects of the intestinal mucosal barrier were associated not only with GI-related disorders such as coeliac disease[6], inflammatory bowel disease (IBD)[7] and colon carcinoma[8]. They were also found to be related to external intestinal disorders, such as type 1 diabetes[9], multiple sclerosis[10], non-alcoholic fatty liver disease (NAFLD)[11], obesity[12] and Parkinson's disease[13].

1.3.3 Mucus

Intestinal mucus is an organised and complicated agglomerate of structural glycoprotein networks with the decoration of host-specific glycans. In this structure, each glycoprotein part covering the epithelium is called mucin. The mucin comprises two types: secreted mucins (gel-forming mucin) and membrane-bound mucins. Membrane-bound mucins are essential components of cell-surface epithelial glycocalyx that build the connection between cells and the external environment. Conversely, the secreted mucins are important for the mucus barrier formation. These mucins include around 20 members and are coded by different mucin genes[14]. Of the secreted mucins, MUC2 is the most abundant in the small intestine and colon; MUC5AC and MUC6 are commonly found in the stomach and ileum. The membrane-bound mucins in the gut include MUC1, MUC3A/B, MUC4, MUC12, MUC13, MUC15, MUC17, MUC20 and MUC21[15]. A commonly shared feature of all mucins is a protein core that consists of amino acid residues proline (Pro), threonine (Thr) and serine (Ser), also referred as the proline-threonine-serine (PTS) rich domain[16].

As described in the previous section, human mucus is the first line of defence as a part of the intestinal mucosal barrier. Indeed, in our GI tract, mucus layers produced by goblet cells play important roles in maintaining intestinal homeostasis. As a bridge between the epithelium and luminal environment, the mucus layer serves as a repository for luminal microorganisms, providing them the niches and nutrients for passage and colonisation. Although the mucus itself is not impenetrable to microorganisms, the release of AMPs and peptides in the mucus, such as defensins, lysozyme and phospholipase A2-IIA, would prevent translocating these microorganisms across the mucosal barrier via a range of mechanisms[17]. Unlike the colon, our small intestine is covered by a single mucus layer. Given the discontinuous and penetrable nature of mucus, this antimicrobial defence mechanism is essential and critical in the small intestine. The protection role of mucus is confirmed by reports on many intestinal pathogens such as *Salmonella*[18], *Shigella flexneri*[19] and *Citrobacter rodentium*[20]. Other than its protective role, human mucus also facilitates the uptake of small molecules, such as ions and nutrients,

across the epithelial border. At the same time, it serves as a lubricator to help luminal material pass through the intestinal channel. The nutrient uptake mainly occurs in the small intestine, attributing to the discontinuity of the mucus layer and the release of digestive enzymes located at the membrane of epithelial cells. Mucus defects, such as the alteration of mucosal integrity, have been associated with many pathological conditions[21], such as IBD, genetic-based abnormalities and hyperglycemia.

1.3.4 Glycosylation

The gastrointestinal (GI) tract is rich in glycosylated proteins and lipids, wherein glycosylation, the process of adding sugar chains to proteins, represents a fundamental and highly conserved type of protein modification. This process is ubiquitous across the human body. Two primary forms of glycosylation observed in secreted or membrane-bound proteins are N-linked, which attaches sugars to asparagine residues, and mucin-type O-linked glycosylation, where sugars are attached to serine or threonine residues[22]. Particularly prominent in mucins, mucin-type O-glycosylation, commonly referred to as O-glycosylation, plays a pivotal role in their structure and function. Both murine and human mucins predominantly feature O-glycans consisting of N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) residues, forming the core structure[22]. These O-glycans can incorporate additional sugar residues such as fucose, galactose, and sialic acid, thereby contributing to the diverse repertoire of mucin glycoforms.

Fucosylation, a type of glycosylation, involves the attachment of fucose to glycans either terminally or internally and holds significant importance due to its diverse functional roles. Understanding the spatial distribution and prevalence of fucose within the GI tract is essential for comprehending its biological implications. Specifically, in humans, distinct fucosylation patterns are observed along the GI tract, with the ileum showcasing pronounced fucosylation, while other regions in GI tract exhibit varying levels alongside increased sialic acid and sulfate residues[23-25]. Comparatively, mouse mucins present different fucosylation patterns, notably with lower levels in the small intestine compared to humans. Despite this interspecies

difference, the prevalence of fucosylated glycans between human gut and murine gut remains relatively consistent, evidenced by comparable proportions in human fetal intestinal mucins and murine intestine studies. Specifically, of the 118 oligosaccharide structures identified in fetal intestinal mucins, 44% (52/118) are fucosylated[25]. This data aligns closely with findings from murine intestine studies, where 45% (21/47) of the primary O-linked oligosaccharides are fucosylated[26].

In humans, numerous genes orchestrate fucosylation and the metabolism of fucose-containing glycans, with many falling within the fucosyltransferases (FUTs) family[22]. Notably, the human genome harbors a total of 13 *Fut* genes, each encoding distinct fucosyltransferase enzymes responsible for adding fucose to the glycan chain via different linkages[22]. Among these, the *Fut2* gene stands out prominently, as it catalyzes the addition of an α(1,2)-linked fucose to disaccharide precursors, generating Lewis^b, Lewis^y, and H antigens (Table 1.1). These antigens constitute pivotal components of the histo-blood group system ABO, where the notable prevalence of ABH and Lewis antigens in humans holds significance. These antigens adorn not only various intestinal epithelial tissues but also bodily secretions, nasal epithelium, trachea, the lower genitourinary tract, and mucosal surfaces. Among all ABH and Lewis antigens, *Fut2*-dependent determinants (Lewis^b and H antigens) are exclusive to both the ileum and cecum, while other antigens (Lewis^x and Lewis^a determinants) manifest increasing expression along the intestinal tract. This juxtaposition is intriguing, considering that in mice, *Fut2* transferase is detected in the colon but not the small intestine. While the prevalence of *Fut2*-dependent fucosylated glycans in murine intestinal mucins remains insufficiently characterized, two *Fut2*-dependent fucosylated neutral oligosaccharide structures were identified, accounting for 22% (2/9)[26]. In humans, the prevalence of *Fut2*-dependent fucosylated glycans stands at 15% (18/118), with 18 *Fut2*-dependent fucosylated oligosaccharide structures detected in fetal intestinal mucins[25]. Despite relatively low prevalence rate, their presence bears substantial implications for human health, as elaborated

in detail in section 1.7. The identified oligosaccharides in mucin that were impacted by *Fut2* gene were listed in the Table 1.2.

Table 1.1 Structure of *Fut2*-dependent histo-blood antigens.

Histo-blood antigens	Structure
H-1 antigen	Fucα1-2Galβ1-3GlcNAcβ-
H-2 antigen	Fucα1-2Galβ1-4GlcNAcβ-
H-3 antigen	Fucα1-2Galβ1-3GalNAcβ-
Lewis^{b}	Fucα1-2Galβ1-3(-Fucα1-4)GlcNAcβ-
Lewis^{y}	Fucα1-2Galβ1-4(-Fucα1-3)GlcNAcβ-

The *Fut2* gene is responsible for transferring fucose residues (represented by red triangles) onto galactose molecules (depicted as yellow circles) through an α(1,2)-linkage. This process results in the formation of the terminal epitope Fucα2-Galβ-R. Following this, the FUT3 enzyme's activity leads to the creation of the Lewis b antigen. Additionally, various glycosyltransferases have the ability to further modify the H-antigen by adding GalNAc or Gal residues, thereby generating the A or B blood group antigens

Table 1.2 Comparison of identified oligosaccharides in mucin that were impacted by *Fut2* gene between human and mouse.

Oligosaccharide Type	Human	Mouse
Neutral oligosaccharide	Fuc→2Gal→3GalNAcol (Fuc→2)Gal→GlcNAc→3GalNAcol (Fuc→2)Gal→(GlcNAc→6)GalNAcol (Fuc→2)Gal→3(Fuc→4)GlcNAc→3GalNAcol (Fuc→2)Gal→3[(Fuc3/4)GlcNAc→6]GalNAcol (Fuc→2)Gal→4GlcNAc→3Gal→3GalNAcol (Fuc→2)Gal→3(Gal→4GlcNAc→6)GalNAcol (Fuc→2)Gal→GlcNAc→(Fuc2)Gal→3GalNAcol (Fuc→2)Gal→3[(Fuc→2)Gal→4GlcNAc→6]GalNAcol (Fuc→2)Gal→GlcNAc→3(Gal→GlcNAc→6)GalNAcol (Fuc→2)Gal→3[(Fuc2)Gal→(Fuc3/4)GlcNAc→6]GalNAcol (Fuc→2)Gal→(Fuc3/4)GlcNAc→(Fuc→2)Gal→3GalNAcol (Fuc→2)Gal→3[HexNAc→(Fuc→2)Gal→GlcNAc→6]GalNAcol (Fuc→2)Gal→3GlcNAc→3[(Fuc→2)Gal→4GlcNAc→6]GalNAcol	Fuc→2Gal→3GalNAcol (Fuc→2)Gal→(GlcNAc→6)GalNAcol
Acidic oligosaccharides	(Fuc→2)Gal→4GlcNAc→3(NeuAc→6)GalNAcol (Fuc→2)Gal→3(Fuc→4)GlcNAc→3(NeuAc→6)GalNAcol (Fuc→2)Gal→4($SO3^-$)GlcNAc→3GalNAcol (Fuc→2)Gal→3[($SO3^-$)GlcNAc→6]GalNAcol	N/A

1.4 Gut microbiome

Starting from the seventeenth century, when Antonie van Leeuwenhoek first observed and described single-celled organisms using a microscope, microorganisms, as human symbionts, successfully attracted researcher attention on health-related projects, gradually becoming an important research area. With sequencing technology development and the interpretation capability of gene sequences, our interests are no longer limited to the features of single microorganisms (e.g., morphology, physiology, genetics and behaviours). They have expanded to the interactions and relationships between multiple microorganisms in a particular environment.

The terminology 'microbiome' was created to refer to the community of microorganisms that live in or on a particular environment, such as soil, plants, or animals. It encompasses the microorganisms, their genetic material, and their host environment interactions. In this thesis, I focus on the gut microbiome. It is a dynamic and complex community of trillions of microorganisms that reside in our digestive system, particularly in the large intestine. These microorganisms coexist in a delicate balance and are pivotal to human health, playing roles in digestion and immunity and affecting our mood and behaviour. From the moment we are born, when microbes first colonise our bodies during birth, this microbial community starts to establish and continues to evolve throughout our lives.

Our gut microbiome is essential; it plays important roles in our lives regarding the immunological and microbiological functions it brings[3,27,28]. For example, the microorganisms in our gut could help breakdown the complex carbohydrates from our diets, synthesise essential vitamins, protect epithelial cells of the gut, train and refine the immune system and resist pathogen colonisation. More importantly, the gut microbiome impacts our host health, specifically initiating or developing many disorders like obesity[29], IBDs[30], NAFLD[31], rheumatoid arthritis[32] and neurodegenerative disorders[33] are associated with disrupting a healthy gut microbiome, widely summarised in different reviews[34-36].

1.4.1 Compositional and functional dynamics of microbiome

Our gut microbiome is diverse and complex. Its distribution varies spatially, cross-sectionally and temporally.

1.4.1.1 Spatial distribution

As mentioned in Section 1.3, the human GI tract is a lengthy tube that extends from the mouth to the anus, and the gut microbiome composition varies along this length. Different GI tract regions provide distinct niches for microbial communities. The oral cavity is the initial site of contact between food and microbes. It has a unique microbiome with bacteria[37,38]. Common bacterial genera found in the mouth include *Streptococcus*, *Neisseria*, *Veillonella*, *Actinomyce* and *Fusobacterium*. Given the individual heterogeneity, the type and number of bacteria can vary from person to person and depend on each individual's dental health, oral hygiene and diet. Unlike the oral cavity, the acidic environment in the stomach makes it less friendly to most microorganisms. However, certain bacteria like *Helicobacter pylori* can thrive in this environment and are associated with ulcers and other stomach diseases[39-42]. The small intestine is also an important part of the digestive tract. Various factors, including pH, oxygen levels, nutrient availability, and host secretions like bile, influence the spatial distribution of bacteria within the small intestine. As a result, bacterial density and composition vary longitudinally (from the duodenum to the ileum). The duodenum is the section close to the stomach. Due to the inflow of stomach acid and digestive enzymes, the duodenum is more hostile to bacterial growth. Common inhabitants[43,44] include *Lactobacillus*, *Streptococcus* and *Enterococcus*.

Further down the small intestine is the jejunum. Here, the pH becomes more neutral; thus, the environment is more friendly to bacteria. While *Lactobacillus* and *Enterococcus* are still dominant, other species like *Escherichia coli* (*E. coli*) might become more noticeable[45]. As the last part of the small intestine, the ileum has much higher bacterial density, reaching up to 10^7–10^8 bacteria per millilitre. Besides the bacterial load, the microbiome composition is richer than other sections of small intestines. More anaerobic genera[46], such as *Bacteroides* and

Clostridium, are able to colonise the ileum. Adjacent to the ileum is the cecum, located at the junction beween the ileum and the colon. The cecum hosts a diverse microbial community[47,48], with dominant bacteria including *Bacteroides*, *Firmicutes*, *Fusobacterium*, and *Prevotella*. Following the small intestine, the large intestine (colon) has the highest bacterial density of any GI tract area, reaching 10^{11}–10^{12} cells per gram of content. Predominant genera[49] include *Bacteroides*, *Clostridium*, *Eubacterium*, *Ruminococcus* and *Faecalibacterium*. *Bifidobacterium* is also present in the colon, especially in breastfed infants. Bacteria composition and concentration can vary between the ascending, transverse, descending and sigmoid regions of the colon, reflecting variations in transit time, pH and substrate availability. The last section of the GI tract is the rectum and anus. While the bacterial profile is similar to the colon, the rectum and anus might have higher relative concentrations of certain aerotolerant and facultative anaerobic species due to increased oxygen exposure. These bacteria[49] include *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Proteus* and *Klebsiella*.

1.4.1.2 Cross-sectional distribution

Another aspect of understanding the spatial organisation of the gut microbiome is to examine it cross-sectionally, distinguishing the gut lumen, mucus layer and epithelium. Most fermentative processes occur in the gut lumen, converting dietary fibres and other substrates into short-chain fatty acids (SCFAs) and other metabolites. Given this, the gut lumen is a good place for bacteria to grow. The predominant bacteria in the colon's lumen[49] include *Bacteroides*, *Clostridium* and some *Eubacterium* species. Compared with gut lumen, gut bacteria colonising the epithelium are less condensed. This is mainly due to the mucus layer protection and production of antimicrobial molecules. However, some tough bacteria can penetrate the mucus layers and colonise the epithelium. These bacteria[49] include both pathogenic ones (e.g., *E. coli*, *Salmonella* and *Shigella*, renowned for invading epithelial cells, leading to GI diseases) and commensal ones (e.g., segmented Filamentous Bacteria (SFB), *Akkermansia*, *Bacteroides* and *Lactobacillus*). Between the gut lumen and the epithelial cells is the mucus layer, which has been covered in section 1.3.2; it provides a protective barrier

and a habitat for bacteria. The mucus is organised into two layers in the colon: a denser and relatively sterile inner layer and a looser outer layer that houses various bacteria. Bacteria commonly found in the mucus layer include *Akkermansia*, *Bacteroides*, *Ruminococcus*, *Lactobacillus*, *Faecalibacterium*, *Roseburia* and *Helicobacter*. Some of these bacteria can benefit from the glycoproteins present in the mucus for better colonisation or adaptation, which will be further discussed in this chapter.

1.4.1.3 Temporal distribution

The structure and composition of the gut microbiome are dynamic and vary significantly over the individual's lifespan. In general, the gut of newborns is low in diversity but changes rapidly in the first few days of life. The common initial colonisers depend on the delivery modes, which have been well-summarised in an integrative review paper[50]. For infants born vaginally, their gastrointestinal tract is commonly colonized by the bacteria originating from their mother's vaginal and faecal flora. However, for Caesarean-delivered babies, skin-associated bacteria, such as *Staphylococcus*, *Corynebacterium* and *Propionibacterium*, can more often colonise their gut due to lack of exposure to typical vaginal bacteria[51,52]. As babies grow to the early infancy stage, their gut is predominantly colonised by *Bifidobacterium*. This phenomenon primarily stems from the advantageous utilization of human milk oligosaccharides (HMOs). The metabolites generated through the utilization of HMOs offer abundant carbon sources, facilitating the proliferation and establishment of *Bifidobacterium* within the gut ecosystem[53,54].

Compared with breastfed infants, formula-fed infants generally have a more diverse gut microbial community, including other bacteria like *Bacteroides*, *Clostridium* and Streptococcus[55]. Introducing solid foods significantly changes the gut microbiome[56], increasing diversity and introducing bacteria involved in the metabolism of complex carbohydrates. By the time children are about three years old, their gut microbiome starts resembling that of adults in composition but may still differ in relative abundance. *Bacteroides* and *Firmicutes*, primarily *Clostridia*, tend to dominate[57], helping digest complex carbohydrates. Diet, antibiotic use and environment continue to shape the gut microbiome during this stage.

The gut microbiome stabilises in healthy adults but is still subject to fluctuations due to factors like diet, disease, medication and lifestyle, which will be described in section 1.4.2. Bacteroidetes (e.g., *Bacteroides*) and Firmicutes (e.g., *Clostridium*, *Lactobacillus*) are the primary phyla present[58]. Other groups like *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* are present in smaller amounts. The ratio of Bacteroidetes to Firmicutes has been a point of interest in studies relating to obesity, though the exact implications are still debated[59,60]. Gut microbiome diversity tends to decrease in older age[61]. There is often a decline in beneficial microbes like *Bifidobacterium*[62] and an increase in potentially pathogenic bacteria like *Clostridium difficile* (*C. difficile*)[63]. Factors like diet changes, reduced mobility, chronic medications and increased health issues can influence the gut microbiome in the elderly[64]. Further, a shift towards a more pro-inflammatory state is often observed[61].

1.4.1.4 Perturbations, resilience and reconstitution

While our human gut works hard to keep the microbiome stable and healthy, various perturbations can destabilise the gut microbial community, leading to transient or long-term alterations in the composition and function of the gut bacteria. These alterations could potentially impact host health. Antibiotic exposure is the most significant disruptor of all perturbations, which can dramatically reduce microbial diversity[65]. Following antibiotic exposure, many beneficial commensal bacteria can be depleted completely, and those opportunistic pathogens could utilise the niche spaces and overgrow, leading to many health conditions like antibiotic-associated diarrhoea or *C. difficile* infections (CDIs)[66]. Besides, the long-term or repeated usage of antibiotics might also cause long-term shifts in the microbiome and antibiotic resistance development[67-70]. Besides antibiotics, the host diet is also a disruptor of the gut microbiome. Rapid changes in diet can shift the microbiome composition within days[71]. High-fat, high-sugar diets can reduce microbial diversity and favour bacteria linked to obesity and inflammation[72]. Not all diet changes bring negative consequences. For example, diets high in dietary fibre could promote the growth of beneficial bacteria that ferment fibres into SCFAs, which have anti-inflammatory properties[73,74]. Apart from these two major

perturbations (antibiotics exposure and diet change), others are similarly important, significantly impacting the gut microbial community, which have been well summarised[75]. These perturbations include infections, psychological and physical stress, non-antibiotic medications, surgery and lifestyle habits (smoking, alcohol consumption and drug use).

Despite the perturbations, our indigenous gut microbiome can recover and return to a state of relative stability and functionality after being perturbed or disrupted. This capacity has been referred to as gut microbiome resilience[75]. Mechanisms behind this resilience include functional redundancy (the presence of other species or strains in the microbial community that can perform the same function, ensuring continued ecosystem stability when one species is diminished due to perturbation), microbial interactions (e.g., the inhibition of pathogen growth by antimicrobial compounds produced by beneficial bacteria), colonisation resistances (prevention of pathogen overgrowth or colonisation through competition for resources or niche spaces) and microbial metabolic flexibility (the capability of gut microbes to switch their metabolic pathways on the available nutrients, allowing them to adapt to changing conditions after perturbations).

As previously mentioned, many perturbations are detrimental to the gut microbial community. Antibiotic exposure is one of them. Short courses of antibiotics can lead to significant changes in the microbiota composition[65,76]. While some species bounce back within days to weeks, others may take months. Some species might never fully recover to their pre-antibiotic levels. The process of restoring the composition and function of an individual's gut microbiome is called microbiome reconstitution. Compared with narrow-spectrum antibiotics, broad-spectrum antibiotics, which affect a wide range of bacteria, usually have a more profound and lasting impact on the gut microbiome[77].

Similarly, repeated or prolonged antibiotic use can lead to long-term alterations in the gut microbiome. Both make recovery to their original state more challenging. Currently, three approaches can facilitate effective microbiome reconstitution: dietary interventions, probiotic

or synbiotic supplementation and faecal microbiome transplantation (FMT). These will be further discussed in Section 1.5.4.

1.4.2 Factors influencing the gut microbiome

As mentioned in the previous section, our gut microbiome is influenced by wide-ranging factors (see Figure 1.2). These factors can be broadly categorised into two main groups: extrinsic and intrinsic.

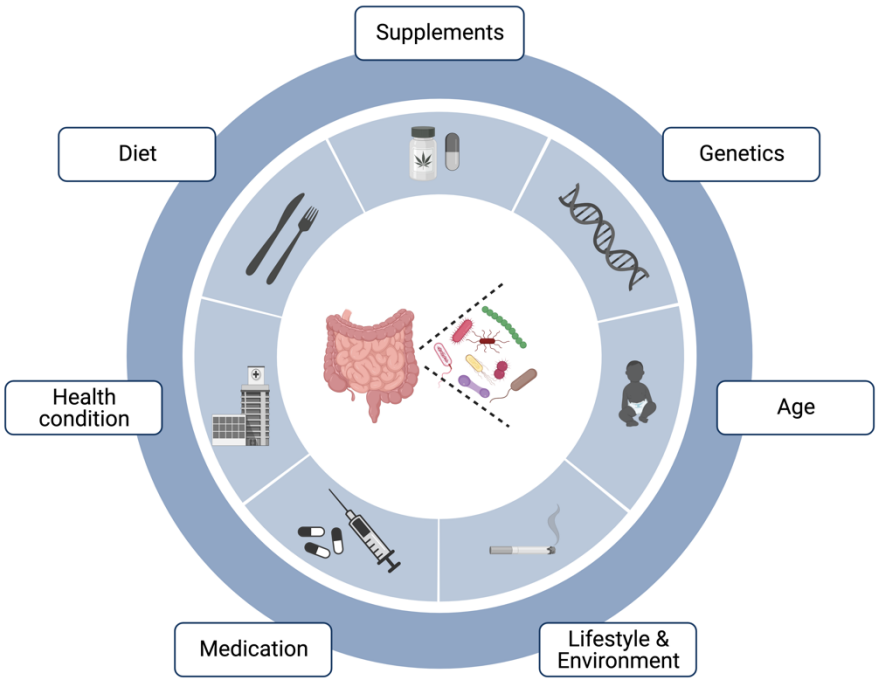


Figure 0.2 Factors that influence the gut microbiome

Of these seven factors, genetics and age are intrinsic factors that could influence gut microbiome while other five factors are extrinsic factors.

Extrinsic factors refer to those external or environmental factors that can influence the gut microbiome, including diets, lifestyle, medications, and environmental exposures[78-80]. Of these extrinsic factors, diet is one of the most significant that impacts the gut microbiome's composition and function. Different dietary components like fibre, complex carbohydrates and proteins serve as substrates, which specific gut microbes can utilise for colonisation. Fibre, for example, supports the growth of fibre-degrading bacteria (e.g., *Bifidobacterium*, *Faecalibacterium*, *Ruminococcus*, *Lactobacillus*, *Akkermansia* and *Roseburia*), promoting a more diverse gut microbial community[81]. Other than serving as energy sources, diets can alter the gut environment by adjusting metabolites[82]. An example is SCFAs[82]. It is a type of metabolite produced by gut microbes after fermentation. SCFAs usually introduce various health benefits, such as anti-inflammatory effects. These benefits more or less alter the gut environment where gut microbes reside, influencing the structure and composition of the gut microbiome.

Unlike extrinsic factors, intrinsic factors are inherent to the individual and are typically less modifiable. Primary intrinsic factors that play important roles in shaping gut microbial communities include host genetics, age, sex, host physiology and metabolism. Of these, host genetics is an underestimated factor. Recently, several host genes were found to be associated with gut microbiome[83-89]. Those well-characterised include the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) gene and the lactase (LCT) gene. Mutations in NOD2 are linked to Crohn's disease[90], a type of IBD. Changes in the NOD2 gene can influence the gut microbiota composition, with some studies suggesting decreased microbial diversity in mutation carriers[90,91]. Similarly, the LCT gene plays a significant role in human health. Its variants determine lactose tolerance or intolerance in humans. Lactose intolerance can lead to increased lactose fermentation in the colon, impacting the microbiota composition[84]. Other than these two genes, more gut microbiome-associated genetic variants (MAVs) have been found in recent microbiome genome-wide association studies (GWAS) [83-89]. The *Fut2* gene is the most famous among these loci. It is a gene that secretes the ABO

histo-blood group antigens in the mucosa. GWAS studies have found its strong association with the abundance of several taxa in the gut, such as the *Ruminococcus torques* genus group[92] and Bacteroides[93]. The abundance alteration of these taxa by the *Fut2* gene contributes to the entire gut microbial community changes, which many reports have confirmed[94-96].

1.4.3 Influences of the gut microbiome on human health

Our gut microbiome plays a crucial role in human health, impacting various physiology, metabolism and immune function aspects. The disruption in the composition and functions of the gut microbiome can have far-reaching consequences on host health, influencing various physiological processes and increasing the risk of several health conditions, referred to as gut dysbiosis. The health conditions triggered by gut dysbiosis include GI disorders (e.g., IBDs[97] and irritable bowel syndrome (IBS)[98,99]), immune system–related disorders (e.g., allergies[100,101] and autoimmune diseases[102,103]), metabolic disorders (e.g., obesity[104], type 2 diabetes[105]) and neurological disorders (e.g., Parkinson's diseases[106,107], Alzheimer's disease[108] and multiple sclerosis[109]). Further, gut dysbiosis may also disrupt the integrity of the gut epithelial barrier, leading to increased intestinal permeability[110,111]. This may allow harmful substances to enter the bloodstream, triggering immune responses and inflammation. On top of that, long-term dysbiosis has been associated with an increased risk of colorectal cancer[112,113].

1.5 The host–microbiome interaction

The interaction between host and gut microbiome is a dynamic and symbiotic relationship that plays a pivotal role in health and disease. A balance of mutual benefits shapes this intricate relationship: the host provides a habitat and nutrients for the microbes. In return, the microbes assist in digestion, produce essential vitamins, modulate the immune system and protect against pathogens. As previously stated in Section 1.4.4, this interaction's significance is clinically profound. The microbiome imbalance has been linked to a myriad of health issues, ranging from GI disorders like IBD and IBS to systemic conditions like obesity, diabetes, allergies and even neuropsychiatric disorders. Moreover, the microbiome influences drug metabolism and can impact the efficacy and toxicity of various medications, highlighting its role in pharmacology and personalised medicine.

Given its clinical significance, a better understanding of the host–microbiome is the first step before harnessing the power of the microbiome can revolutionise therapeutic strategies. Due to technological advances, multidisciplinary approaches have been developed in recent decades, allowing us to decipher interactions between the host and microbiome. Metagenomic sequencing (e.g., 16S sequencing and shotgun metagenomic sequencing) is the most common approach. Researchers can employ it to identify and quantify the microbial species present in a sample without the need to culture them. It provides a snapshot of microbial diversity and the potential functional capabilities of the community based on their genetic content. Other than metagenomic sequencing, transcriptomics (for the host) and metatranscriptomics (for the microbiome) are often employed to study transcribed genes, providing insights into active metabolic processes and interactions. Metabolomics is used to study the metabolic products in a sample, shedding light on the actual metabolic activities of the microbiome and its impact on the host's metabolic profile. Together with the traditional methods (in vivo and in vitro models), these study approaches will allow us to deepen our understanding of the host–microbiome relationship.

1.5.1 Microbiome influences on host physiology

It is widely acknowledged that our gut microbiome profoundly influences host physiology. This influence can be dissected into various aspects.

First, the gut microbiome is crucial in maintaining and regulating intestinal barriers. As covered in Section 1.3.2, the intestinal barrier is a protective line of defence that prevents the translocation of harmful entities from the intestine into the bloodstream while allowing nutrient absorption. This barrier consists of several components, including tight junctions, mucus layers and the underlying epithelial cells that undergo constant renewal due to stem cell proliferation. Our gut microbiome interacts with each of them. In detail, the beneficial microbes in the gut microbial community produce SCFAs like butyrate, which enhance tight junction assembly and function[114]. Conversely, when the balance of the gut microbiome is disrupted, as seen in dysbiosis, pathogenic microbes can produce toxins and metabolites that weaken tight junctions, leading to increased intestinal permeability, commonly termed 'leaky gut'. Further, certain gut microbes like *A. muciniphila* stimulate goblet cells to produce mucus[115], ensuring its adequacy. At the same time, some resident bacteria feed on mucus glycans as an energy source—the balance between mucus production and degradation by a healthy microbiome maintains the dynamic in equilibrium. However, in dysbiosis states, excessive mucus degradation can occur, thinning the protective mucus barrier and exposing the epithelium to potential harm. Besides, our gut microbiome promotes the regenerative process of the intestinal epithelium[116]. SCFAs, especially butyrate, provide energy for colonocytes and have been shown to promote the differentiation and proliferation of epithelial cells[117]. Additionally, specific microbial metabolites can activate signalling pathways that drive stem cell proliferation and differentiation[118], ensuring a continuous barrier renewal. In contrast, when the balance of healthy gut microbiome is disrupted, the regenerative potential of the epithelial layer may be compromised, leading to potential breaches in the barrier function.

Second, our gut microbiome can modulate the host immune system, playing a balancing act between inflammatory and tolerant immune responses. This was achieved primarily through

producing various metabolites and their intricate interactions with immune pathways. One of the most well-known example is SCFAs derived from the fermentation of dietary fibres by commensal bacteria. Butyrate, in particular, promotes the differentiation of regulatory T cells (Tregs) in the colon[119,120], vital for maintaining immune tolerance and preventing autoimmune reactions. Other than impacting the T cells, SCFAs are also reported to be able to influence the function of dendritic cells[121], skewing them towards a more tolerogenic profile, which can downregulate inflammatory responses. SCFAs can inhibit the activity of histone deacetylases (HDACs), leading to epigenetic changes that can reduce the expression of pro-inflammatory genes[122]. Apart from the metabolites produced by the gut microbiome, certain gut microbes (e.g., *E. coli*) themselves[123] or their components (e.g., lipopolysaccharides)[124] can be pro-inflammatory. Their overgrowth can stimulate the production of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α) and activate effector T cells, leading to inflammatory responses against pathogens. However, a healthy and balanced gut microbiome generally promotes immune tolerance via various mechanisms to prevent overreactions to harmless antigens, such as food particles and beneficial microbes.

Moreover, our gut microbiome plays a pivotal role in protecting the host from pathogen overgrowth and subsequent infections caused by pathogenic microorganisms. This protective role is achieved through many mechanisms. One is via direct competition for nutrients and space. The rapid consumption of nutrients from host diets makes the gut environment less hospitable for incoming pathogens. Similarly, the specific binding sites that the gut epithelium offers microbes to adhere to can be predominantly occupied by commensal microbes, making it difficult for exogenous pathogens to gain a foothold. Other than direct competition for nutrients and space, certain bacteria (e.g., probiotic lactic acid bacteria in the gut) can produce substances like bacteriocins and hydrogen peroxide that directly inhibit or kill potential invaders[125,126].

1.5.2 Host influences on the gut microbiome

The relationship between the gut microbiome and its human host is reciprocal. While it is well-established that our resident microbes impact various facets of our health, it is often overlooked that the host also shape gut microbial communities. One crucial aspect of this influence stems from our genetic makeup. As mentioned in Section 1.4.2, many intrinsic factors, including host genetics, age, sex, host physiology and metabolism, shape the gut microbiome's structure and composition. Of these host factors, the *Fut2* gene-related secretor status is a fascinating example of how genetic variations can influence the gut microbiome and host health, which is the main focus of this section.

The *Fut2* gene encodes the α(1,2)-fucosyltransferase enzyme and is responsible for adding fucose molecules to glycan structures on the surface of mucosal epithelial cells. These fucosylated structures are binding sites or energy sources for certain gut bacteria. When individuals have functional *Fut2* alleles (secretors), these fucosylated structures are expressed on the mucosal surfaces of the GI tract, respiratory tract and other mucous membranes, allowing interactions with specific bacteria. In contrast, non-secretors, which carry non-functional *Fut2* alleles, lack these fucosylated structures on their mucosal surfaces. As a result, their mucosal surfaces interact differently with bacteria, leading to distinct microbial communities in their gastrointestinal tracts. Recent studies have shown that secretors exhibit higher α-diversity in their gut microbiomes than non-secretors[94,95]. This increased diversity can be attributed to the presence of α(1,2)-fucosylated glycans on the intestinal epithelial cells in secretors. These glycans serve as attachment sites for a broader range of bacterial species, promoting the colonisation of various microbial taxa. Other than α-diversity, the gut microbiome composition also differed between secretors and non-secretors[94,96]. This result can be reflected in the taxa abundance difference between secretors and non-secretors. Secretors have been found to harbour greater amounts of *Bifidobacterium*, *Lactobacillus*, *Roseburia*, *Faecalibacterium*, *Ruminococcus* and *Bacteroides*. In contrast, non-secretors

were found to harbour greater amounts of *Prevotella*, *Alistipes*, *Parabacteroides* and *Escherichia/Shigella*.

Despite the microbiome differences between secretors and non-secretors, the mechanisms contributing to the microbiome differences are not fully understood. The glycan utilisation theory is the most popular mechanism discussed in recent decades (see Figure 1.3). As previously mentioned, α(1,2)-fucosyltransferase, encoded by *Fut2*, is expressed by multiple epithelial cell types and facilitates attachment of the L-fucose monosaccharide to specific O-linked glycan chains, producing α(1,2)-fucosylated glycans[127,128]. This type of glycan can be degraded and further utilised by gut microbes. The degradation process usually involves a special type of enzyme α(1,2)-L-fucosidase produced by certain gut microbes such as *Bifidobacterium* and *Bacteroides*. In other words, secretors, which express these antigens in their gut mucus, provide specific food sources for bacteria that can metabolise these structures. As a result, bacteria that can utilise these carbohydrates will naturally thrive better in secretors than non-secretors.

Besides serving as an energy source, the glycan present on secretors' mucosal surfaces can also serve as binding sites for bacterial adhesion, impacting colonisation patterns. This adhesion is particularly important in early life when the microbiome is just establishing. There has been increasing recognition[129] of the pivotal role played by specific gut microbes, particularly *Bifidobacterium infantis*, in early life and their utilization of Human Milk Oligosaccharides (HMOs)[130]. *B. infantis* is highly abundant in the gut during infancy and possesses specialized enzymes to digest HMOs, which are abundant in human breast milk. This symbiotic relationship between *B. infantis* and HMOs is crucial for the establishment of a healthy infant gut microbiome. This is because those early colonisers, such as *B. infantis*, adhering to the binding site can utilise the competitive colonisation advantage, thus shaping the subsequent microbial community structure. However, the significance of this interaction may vary depending on factors such as maternal secretor status and feeding practices. In

cases where the mother is a secretor, providing breast milk rich in fucosylated HMOs, the impact of *Fut2* deficiency in infants may be mitigated to some extent, as *B. infantis* can utilize these HMOs that containing α(1,2)-fucose such as 2′-fucosyllactose (2'-FL). Conversely, in *Fut2*-deficient, non-secretor infants, the absence of fucosylated HMOs in breast milk may underscore the importance of epithelial α(1,2)-fucose in facilitating the colonization of *B. infantis*. Furthermore, with the prevalent use of infant formula, which lacks HMOs, the role of *B. infantis* in early gut colonization and the importance of HMOs become even more pronounced.

While many microbiology studies support the glycan utilisation theory[131-135], these mainly focus on the capability of certain glycan degrading bacteria to produce α(1,2)-L-fucosidase and the utilisation of L-fucose (the metabolites of glycan after degradation) by these bacteria, serving as indirect evidence. More direct evidence on how glycan variation impacts the gut microbial community using an *in vivo* model is needed.

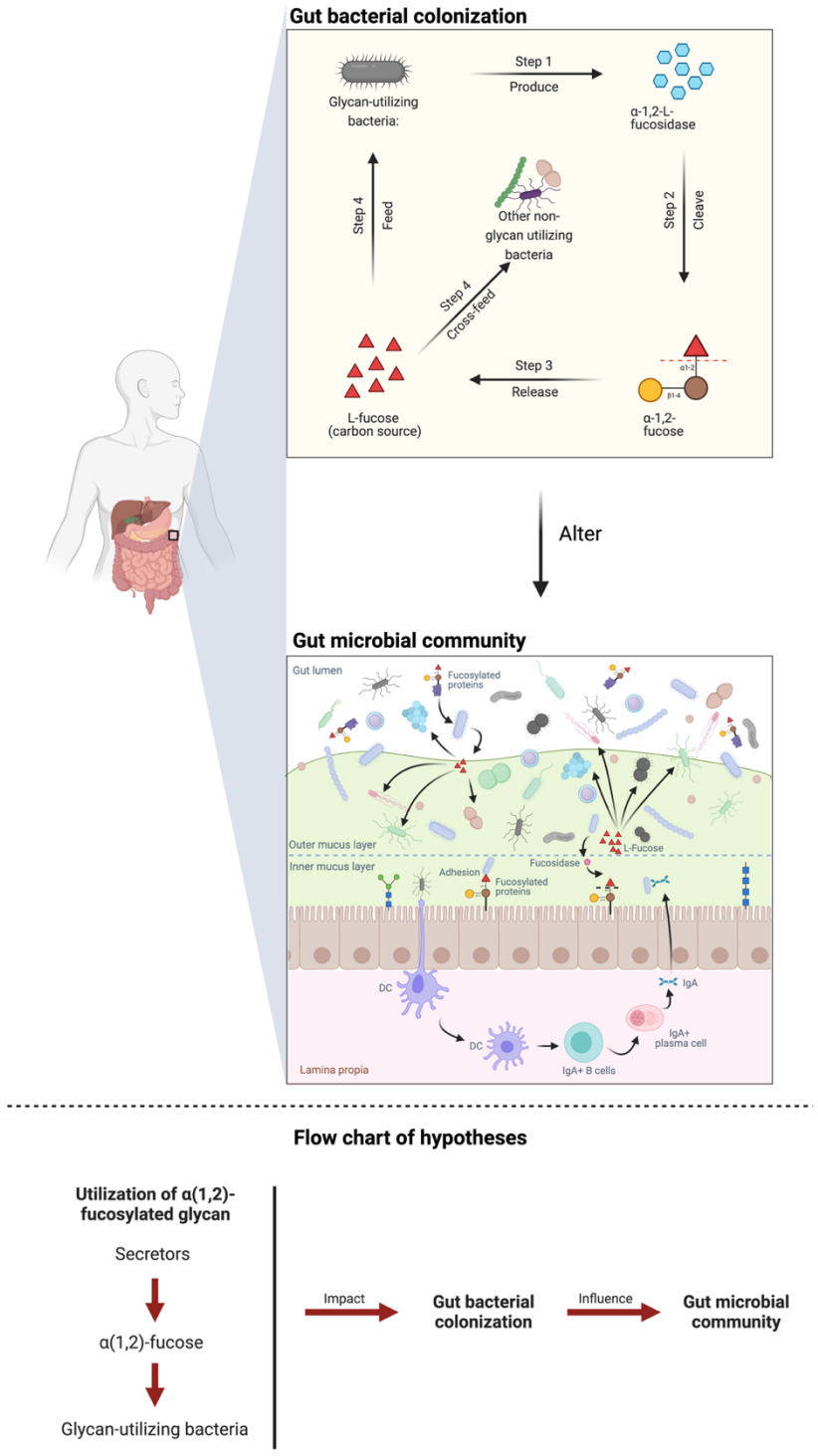


Figure 0.3 Potential mechanisms of how α(1,2)-fucosylated glycans influence gut microbial community

Glycan degrading bacteria in the human gut degrade the α(1,2)-fucosylated glycans, releasing the free L-Fucose, which can be utilized by these bacteria or crossed-fed by other glycan utilizing bacteria in the gut, thereby influencing gut microbial community

1.6 Manipulation of the host–microbiome interrelationship

As discussed, the gut microbiome is a dynamic interface between our environment and physiology. A healthy microbiome underpins various facets of our wellbeing, while dysbiosis can lead to various health challenges. Fortunately, the growing understanding of the host–microbiome interrelationship has birthed multiple interventions that have benefited thousands of patients suffering from gut dysbiosis–induced disorders. This section mainly focuses on currently available interventions to tune the host–microbiome interrelationship.

In recent decades, there has been a growing interest in interventions to modify the microbiome to improve health or treat various disorders. Available interventions currently include antibiotic therapy, dietary interventions, FMT and probiotic supplementation.

1.6.1 Antibiotics

Antibiotic exposure is a common intervention designed to treat bacterial infections by targeting, killing or inhibiting the growth of pathogenic bacteria that cause infection. While antibiotics can be lifesaving when used to treat bacterial infections, antibiotic exposure also negatively affects both gut microbiome and host health. First, it significantly reduces gut microbiome diversity[136,137] due to its non-selective feature of eliminating bacteria, including pathogenic, commensal and beneficial ones. This reduced microbial diversity can make the gut microbiome less resilient and less able to perform its functions effectively[138]. The loss of diversity can persist for an extended period, even after antibiotic therapy has ceased[136]. Second, it creates an ecological vacuum in the gut when antibiotics suppress dominant bacterial species. This vacuum is a representation of gut dysbiosis[66,139], leading to medical disorders such as GI symptoms, antibiotic-associated diarrhoea[140] and increased susceptibility to infections[141,142]. Third, it will alter gut microbiome composition—changes in gut microbiome composition can alter metabolic processes[143,144], nutrient absorption[145] and immune responses[146,147], contributing to conditions like obesity[148-150] and metabolic syndrome[151]. Last, long-term antibiotic exposure might also result in the development of antibiotic-resistant bacterial strains, including pathogenic ones[152]. An example is CDI,

considered as one of the most difficult infections to treat. Given the above, antibiotic therapy is undeniably valuable in treating bacterial infections. However, its impact on the host–microbiome interrelationship is profound and multifaceted, highlighting the necessity for prudent and cautious antibiotic use in clinical settings.

1.6.2 Dietary interventions

Of all interventions, dietary interventions are a gentle but powerful and direct way to modulate the host–microbiome interrelationship. This is because dietary components, such as carbohydrates, dietary fibre, proteins and fats, serve as substrates for microbial growth and metabolism in the gut. Considering different individuals have different diets, the composition of each individual's diet also differs, directly influencing the microbes that thrive in the gut. For example, a diet rich in dietary fibre provides substrates for fibre-fermenting bacteria, promoting their growth[81]. Conversely, diets high in sugar and processed foods can promote the growth of bacteria associated with inflammation[153]. Moreover, dietary interventions can also impact the diversity and composition of the gut microbiome, and the shift in the gut microbiome can be fast. Diets rich in a variety of fruit, vegetables and fibres are reported to be associated with increased microbial diversity[154], a marker of a healthy gut. Other than its impact on the growth of gut microbes and the entire microbial community, dietary interventions were found to impact host health through the gut microbiome. Certain dietary components, like omega-3 fatty acids from fish, can reduce gut inflammation[155]. This anti-inflammatory effect could be mediated by modulating the microbiome to favour anti-inflammatory species growth. Dietary intervention is an effective approach to modulating the host–microbiome interrelationship. However, the application of dietary interventions in modulating the host–microbiome interrelationship is still in its infancy. Many research gaps and questions remain to be further explored. For example, why do similar dietary interventions lead to differing microbial shifts among individuals? How do various dietary components interact synergistically or antagonistically to influence the microbiome? Which dietary-induced microbial changes are transient, and which lead to long-term shifts in microbiota composition? A better understanding of the complex interplay

between diet, the microbiome and host health offers a promising avenue for personalised nutrition and therapeutic strategies in various health conditions.

1.6.3 Faecal microbiota transplantation (FMT)

FMT is a straightforward, easily understood intervention compared to the previous two interventions. As its name suggests, it is a procedure that involves the transfer of faecal material from a healthy donor into the GI tract of a recipient. This intervention is developed to treat specific conditions associated with gut dysbiosis, such as recurrent CDI[156] and IBD[157]. Recipients can restore a balanced microbial community through this special intervention. Given that a healthy gut microbiome offers resistance against pathogenic microbial invasions, the restored gut microbial community can resist the colonisation of pathogens such as *C. difficile*. Besides, by altering the microbial community structure, FMT can also influence other host health aspects, such as the immune response and host metabolism. While FMT has shown efficacy in specific clinical scenarios, it has limitations and challenges. Due to the low efficacy of FMT on other conditions, the most established application of FMT is limited to CDI and IBD. Ensuring donor safety and determining the optimal delivery methods still require a great deal of work.

1.6.4 Probiotic therapy

Unlike other interventions, probiotic therapy is generally considered safe for most individuals. Public confidence in the safety derives from the nature of this therapy, as this intervention is based on the idea that introducing specific strains of beneficial microorganisms into the gut can have various health benefits. Probiotic bacteria currently used are mainly from two genera: *Bifidobacterium* and *Lactobacillus*. However, other beneficial species, including *Streptococcus thermophilus*, *Saccharomyces boulardii*, *Bacillus coagulans* and *E. coli* Nissle 1917, are also being used.

The principal concept underlying the use of probiotics is that introducing live commensal bacteria can re-establish physiological homeostasis by modifying the composition or

behaviour of the gut microbiota or by directly providing regulatory cues to the host. Once probiotics are supplemented, most probiotic bacteria transiently inhabit the gut, integrating the host microbial community. This colonisation can influence the overall structure and composition of the indigenous gut microbiome. Other than impacting the existing microbiome, the exogenous probiotic bacteria can produce and secrete a variety of metabolites, such as SCFAs. The produced metabolites play important roles in nourishing gut epithelial cells, modulating inflammation and influencing overall gut health. In situations of gut dysbiosis, exogenous probiotics can help restore balance by suppressing harmful pathogens or promoting the growth of beneficial microbes. The benefits are achieved from either outcompeting pathogens for nutrients or attachment sites on the gut epithelium or producing antimicrobial substances (e.g., bacteriocins) that directly inhibit the growth of harmful bacteria. Aside from the impact on gut microbes and the microbial community, probiotics were found to be able to interact with the host. For example, previous studies have found that probiotics can shape the host mucosal immune system[158], enhancing the production of protective immunoglobulins, modulating cytokine production and promoting immune cell activity (e.g., macrophages and T lymphocytes). They can also produce substances that enhance mucin production or promote the expression of tight junction proteins in the gut lining[159]. Given the above benefits, the clinical application[158] of probiotic therapy is wide, which covers GI disorders (e.g., antibiotic-associated diarrhoea, infectious diarrhoea, IBS and IBD), infections (e.g., urinary tract infections, vaginal infections and upper respiratory tract infections), allergic disorders (atopic dermatitis) and mental health (depression and anxiety)[158].

While probiotic therapy has garnered significant attention for its potential benefits in various health conditions, its limitations should be considered when evaluating usage. Of all the limitations, the largest is the inconsistent or variable clinical outcomes in different clinical trials, which has been raised[160]. The inconsistency of the clinical outcomes could be attributed to various reasons and possibilities. Strain specificity is one of them. The efficacy of probiotics is often strain-specific, meaning the beneficial effects seen with one strain may not apply to

others, even within the same species. Besides, the individual heterogeneity could also contribute to the variable clinical outcomes. Specifically, each individual has a special indigenous microbiome, and probiotic effectiveness depends on the individual's gut microbiome.

In some cases, the host's microbiome may not provide the necessary environment for probiotics to thrive and exert their beneficial effects. This has been well studied in a research paper where humans feature a person-specific gut mucosal colonisation resistance to probiotics[161]. Further, the genetic variation among the population might impact the variable clinical outcomes by conditionally limiting the growth of these probiotics. Take *Fut2* (previously discussed) as an example. The presence of the functional *Fut2* gene makes α(1,2)-fucosylated glycans available to glycan-utilising bacteria for better colonisation. *Bifidobacterium* is one such glycan-utilising bacteria that has been well-characterised. It can degrade the glycan by producing the α(1,2)-L-fucosidases and utilise the metabolite L-fucose as an energy source or niche space afterwards. Other than *Bifidobacterium*, *Lactobacillus* is more abundant in the secretor's gut, indicating its potential glycan utilisation capability. Because approximately one-fifth of the global population cannot express α(1,2)-fucosylated glycans in the gut, the genetic variation on glycan fucosylation could be one possibility that explains the variable clinical outcomes.

There are other obvious limitations and challenges besides inconsistent clinical outcomes. Not all probiotic strains can survive the harsh acidic environment of the stomach or the bile acids in the small intestine, and even when they reach the intestine, survival and colonisation are transient, which have been demonstrated in many studies. This indicates that the introduced strains may not persist in the gut, and their benefits can diminish once probiotic supplementation is discontinued. Other than survivability and persistence, the optimal dosage and timing of probiotic therapy can vary widely among individuals and conditions. Determining the right dose and regimen can be challenging, and too much or too little may not produce the

desired results. Moreover, there are also safety concerns. While probiotics are generally considered safe for healthy individuals, there is a risk of infection and sepsis caused by probiotic bacteria for immunocompromised patients and the elderly[162-166].

Probiotic therapy is a promising intervention to modulate the host–microbiome interrelationship, bringing clinical benefits. Its efficacy varies among individuals and depends on multiple factors, which should be further explored.

1.7 Dissertation aims

As described in the Introduction's previous sections, the complex host–gut microbiome interaction plays an important role in host health. Of all the variables that could potentially influence the interrelationship, host genetic variation in mucosal glycosylation is an important one that has not been fully investigated. Thus, the overarching aim of this dissertation is to characterise how host genetic variation that affects mucosal glycosylation impacts the microbial community of the gut and the ability of bacteria to colonise the gut.

Many genetic mutations can impact mucosal glycosylation. Mutations to the α(1,2)-fucosyltransferase gene, *Fut2,* are common in the human population, accounting for ~20% of the population worldwide, and impact the glycosylation of most secreted mucosal proteins and lipids. Individuals lacking functional *Fut2* alleles, termed 'non-secretors,' exhibit altered susceptibility to a spectrum of infectious and chronic inflammatory conditions, with notable variations observed across ethnicities. Extensive epidemiological studies have delineated contrasting susceptibilities between secretors and non-secretors to viral and bacterial pathogens[167-176]. Secretors display heightened susceptibility to viral infections, including HIV, influenza, and norovirus[167,170,171,173,174,176], while exhibiting reduced susceptibility to bacterial pathogens such as *Streptococcus pneumoniae* and *Salmonella enterica* serovar Typhimurium[168,172,175,177]. Furthermore, associations between *Fut2* deficiency and chronic inflammatory diseases, including chronic pancreatitis, asthma, type 1 diabetes, and psoriasis, have been elucidated[178-181], highlighting the multifaceted role of *Fut2* in modulating immune responses and inflammatory processes.

Mechanistically, *Fut2* deficiency influences disease susceptibility through diverse pathways, including modulation of pathogen adherence and alteration of the commensal gut microbiota. Murine studies have demonstrated the protective role of *Fut2* against bacterial infections such as *S. Typhimurium*, *Enterococcus faecalis*, and *Citrobacter rodentium*, mediated by α(1,2)-fucosylated glycans' effects on the gut microbiota composition and colonization resistance.

Additionally, *Fut2*-dependent fucosylated glycans serve as essential nutrients for commensal microbes, facilitating host recovery following intestinal stress. Despite significant progress in elucidating the associations between *Fut2* deficiency and disease susceptibility, our understanding of the underlying mechanisms remains incomplete. Factors such as environmental exposures and host-microbiota interactions contribute to the complex interplay between *Fut2*, mucosal glycans, and disease outcomes. Further research, including large-scale cohort studies with detailed metadata, is warranted to decipher the intricate relationships between *Fut2* deficiency and disease susceptibility across diverse populations.

The overarching aim of this dissertation is to better understand how host genetic variation that affects mucosal glycosylation impacts the microbial community of the gut and the ability of bacteria to colonise the gut.

Due to the importance of the *Fut2* gene, polymorphisms in *Fut2* were selected to represent host genetic variation. This thesis uses a mouse *Fut2* knockout (KO) model and in vitro bacterial growth studies to explore the impact of mucosal glycosylation on gut microbial community characteristics and bacterial colonisation in the gut.

Thus, in this doctoral thesis, I hypothesised that host genetic variation in mucosal glycosylation profoundly impacts the gut microbiome, contributing to an altered host–microbiome interrelationship. The hypothesis was subdivided into three smaller hypotheses:

- **Hypothesis 1 (Chapter 3):** the *Fut2* gene function impacts the gut microbiome.
- **Hypothesis 2 (Chapter 4):** the *Fut2* gene function influences gut bacterial colonisation.
- **Hypothesis 3 (Chapter 5):** supplementing α(1,2)-fucosylated glycans impacts the gut microbial community and growth of glycan-utilising bacteria.

Three experimental models were developed to test the above hypotheses:

Experimental model 1 (*In vivo*):

The first experimental model was designed to assess the impact of the *Fut2* gene on the gut microbial community. A transgenic *Fut2* KO mouse line was developed by knocking out one copy of the *Fut2* gene from mice using CRISPR/Cas9 technology (Location: NC_000073.7 on Chromosome 7). Baseline murine faeces were collected from $Fut2^{+/+}$ (WT) and $Fut2^{-/-}$ (KO) mice. The extracted DNA were subjected to 16S sequencing and sequencing analysis.

Experimental model 2 (*In vivo*):

In the second experimental model, both glycan-utilising *Bifidobacterium* species that is capable of utilising α(1,2)-fucosylated glycans and non-glycan-utilising *Bifidobacterium* species were given to mice as supplements. To assess the impact of α(1,2)-fucosylated glycans on probiotic colonisation, faeces collected at different timepoints after gavage were subjected to DNA extraction and qPCR. Survival rates and abundance levels across follow-up days were compared between WT and KO mice.

Experimental model 3 (*In vitro*):

In the third experimental model, baseline faeces collected from mice were incubated in minimal media (mBasal) with/without supplementation of α(1,2)-fucosylated glycans under anaerobic condition at 37˚ C. To evaluate the association of glycan utilisation by gut microbes with microbial community and bacterial colonisation, the cultured samples were subjected to 16S sequencing and growth rate analysis.

CHAPTER 2. METHODS AND MATERIALS

2.1 Summary

In this Chapter 2, the methods and materials used in this PhD project were provided. Details were presented based on the following structure:

- KEY RESOURCES TABLES
- DATA AVAILABILITY
- METHOD DETAILS
    - Mice
    - Bacterial strains
    - Oral gavage
    - Sample collection
    - DNA extraction
    - 16S rRNA gene amplicon sequencing
    - Bioinformatic processing on 16S sequencing results
    - Microbiome characterisation
    - Quantification of *Bifidobacterium* and total bacterial load
    - *In vitro* glycan utilisation assay
- QUANTIFICATION AND STATISTICAL ANALYSIS

2.2 Key resources table

Table 2.1 *Summary of material used in this project*

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibiotics		
Ampicillin	Sigma-Aldrich	N1876
Neomycin	Sigma-Aldrich	A1593
Bacterial and Virus strains		
Bifidobacterium bifidum	Japan Collection of Microorganisms	JCM 1255
Bifidobacterium breve	Japan Collection of Microorganisms	JCM 1192
Bifidobacterium longum subspecies infantis	Japan Collection of Microorganisms	JCM 1222
Chemicals, Peptides, and Recombinant Proteins		
2'-Fucosyllactose (98% purity)	Layer Origin, New York, USA	PureHMO
Lactobacilli MRS Agar 500G	BD, New Jersey, USA	Cat# 288210
Lactobacilli MRS Broth 500g	BD, New Jersey, USA	Cat# 288130
SYBR Green qPCR mix with Rox	Thermo Fisher, Massachusetts, USA	Cat# A25777
Critical Commercial Assays		
DNeasy PowerLyzer PowerSoil Kit	QIAGEN, Hilden, Germany	Cat# 12855-100
POWRUP SYBR MASTER MIX	Life Technologies	Cat# A25777
MiSeq Reagent Kit v3 (600-cycle) • 16S sequencing	illumina	Cat# MS-102-3003
Nextera XT Index Kit v2 Set A • 16S sequencing	illumina	Cat# FC-131-2001
NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles)	illumina	Cat# 20024905
Illumina® DNA Prep, (M) Tagmentation (24 Samples)	illumina	Cat# 20018704
Nextera™ DNA CD Indexes (96 Indexes, 96 Samples)	illumina	Cat# 20018708
Nextera XT DNA Library Preparation Kit (96 samples)	illumina	Ca# FC-131-1096

Continued

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Fut2$^{+/+}$ C57BL/6JSah (Males and females 6 weeks of age)	SAHMRI, Australia	N/A
Fut2$^{-/-}$ C57BL/6JSah (Males and females 6 weeks of age)	SAHMRI, Australia	N/A
Oligonucleotides		
Primers for genotyping m*Fut2*_KO_F: 5'- GGCACAATGCAGATGATTAG -3'	Designed in this study	N/A
Primers for genotyping m*Fut2*_KO_R1: 5'- GCTTCACTATTCGTTGCTGG -3'	Designed in this study	N/A
Primers for genotyping m*Fut2*_KO_R2: 5'- AGTTTTCAGGAACAGGAGCC -3'	Designed in this study	N/A
16S V4 Amplicon PCR Forward Primer	Choo *et al.* 2015[182]	N/A
16S V4 Amplicon PCR Reverse Primer	Choo *et al.* 2015[182]	N/A
Miseq Illumina sequencing Index primer	Illumina, California, USA	Cat# FC-131-2001 Cat# FC-131-2002 Cat# FC-131-2003 Cat# FC-131-2004
Deposited Data		
Sequence data	https://github.com/Yiming-Wang-1992/PhD_FUT2	PhD_FUT2
Software and Algorithms		
QIIME 2 version 2021.11.0	Caporaso *et al.* 2010[183]	N/A
R studio 4.1.0	N/A	N/A
GraphPad Prism 9.0.0	GraphPad Software, California, USA	N/A
PRIMER 6.1.16 & PERMANOVA+ 1.0.6	PRIMER-e, Auckland, NZ	N/A

2.3 Data availability

- All 16S sequencing data have been deposited at PRJNA1011386 (NCBI) and are available under accession number
- Additional supplemental tables and figures are available at Appendix section
- The computational codes used in this study are available in the GitHub repository (https://github.com/Yiming-Wang-1992/PhD_FUT2). Codes for main results are available at Appendix section

2.4 Method details

2.4.1 Mice

2.4.1.1 Establishment of Fut2 knock-out (KO) mouse line

CRISPR-Cas9 gene editing technology was employed to establish a *Fut2* knock-out mouse line. This was performed by the SA Genome Editing Facility at SAHMRI, with the overview and primer sequences described in Figure 2.1 and Table 2.2 (SAGE report in Appendix 2.1). Specifically, two single-stranded guided RNA strains (sgRNA) and one cas9 mRNA (Figure 2.1A) were designed to excise a 1230 bp region of the only coding exon of *Fut2* gene (Figure 2.1B). Synthesised sgRNA and cas9 mRNA were injected into freshly fertilized (prepared 24 hours earlier) wild-type mouse zygotes, using micro-injection, on a petri dish. The fertilized eggs were then transplanted into the oviducts of pseudo-pregnant female mouse and the genetic mosaic mice given birth were subjected to genotyping using both PCR and Sanger sequencing methods. Offspring with a confirmed gene fragment knockout were used for the first filial generation (F1) of heterozygous mice. All animals had health assessments to check for non-specific gene modifications and came back as healthy. All breeding subsequently was kept as heterozygous x heterozygous breeding and genotypes assessed by PCR from ear notches.

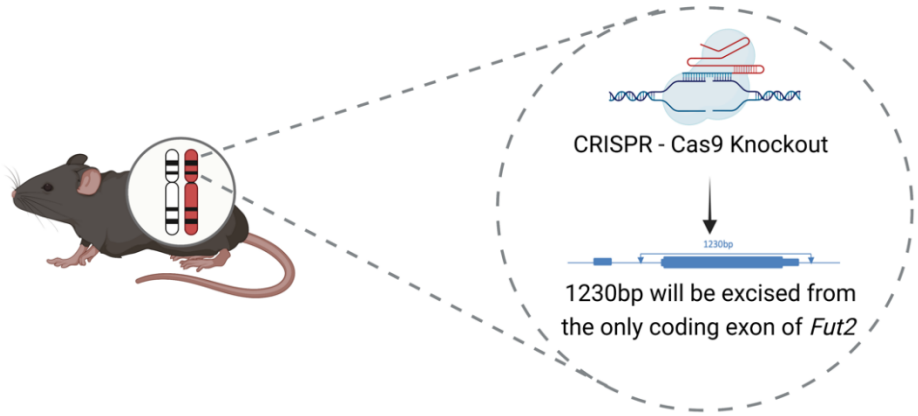
2.4.1.2 Determination of Fut2 genotypes

To distinguish between homozygous wildtype (WT), heterozygous (HET) and homozygous mutant alleles (KO), mouse DNA was isolated from ear notches using crude DNA extraction method (Section 2.5.1) and was then subject to genotyping PCR. Specifically, one forward primer and two reverse primers were designed to amplify a 216 bp region on WT and a 154 bp region on KO separately. Each primer combination was assessed alone at first by qPCR which confirmed the primer design. An assessment was then conducted on mouse DNA by qPCR using both primer pairs to see whether the melt curve can be used as a surrogate for amplicon size of the 2 amplicons. Results confirmed the successful distinction on genotypes (Appendix 2.2).

Each PCR reaction consisted of: 1 µl of DNA template, 12.5 µL of 2×PowerUp SYBR Green Master Mix, 10 µL of Nuclease-Free water, 0.5 µL of 10 µM forward primer, 0.5 µl of 10 µM reverse 1 primer and 0.5 µl of 10 µM reverse 2 primer. Cycling conditions and PCR primer sequences are listed in Table 2.2.

The genotyping PCR was such that samples with a single peak at 84.4°C in the PCR melt curve were wildtype (WT) mice (homozygous wildtype); samples with a single peak at 76.99°C were Knock-out (KO) mice (homozygous mutant alleles); and samples with peaks at both 76.99°C and 84.4°C were heterozygous (HET) mice. This design allowed for analysis of the melt curve to distinguish between homozygous wildtype, heterozygous and homozygous mutant alleles by virtue of the dissociation patterns produced.

A.



B.

```
GCTCACTCTGGATTCCTACACCAAAG
CAGAAAGATTGGGCCACACAGTTTCCAGCTAGACTAAGCTATGGCATGAAATCAGATATCAAACAAACTC
AAAAGAAAACAAAAAATATCCAAGAGGCACAATGCAGATGATTAGCCCAACGGATGTCAGCTATTTATGC
ATACATGATTGCAAGCGTGCACCTAGCTGCTCTCCAGGTAACCTGCCATGCTTTCTTTCCTGGCAGCCAT
GGCGAGTGCCCAGGTACCTTTCTCCTTTCCTCTGGCCCACTTCCTCATCTTTGTCTTTGTGACTTCCACC
ATCATCCACCTCCAGCAACGAATAGTGAAGCTCCAAACCCTGTCAGAGAAGGAATTACAGGCGGTTCAAA
TGTCCTCACCAAACGCGGCAAGAACAGACATGCAGCAGAGTGCCAAGCTGCAGGGCATATTCACGATCAA
TTCCATCGGCCGCCTGGGGAACCAGATGGGCGAATATGCTACATTGTTTGCACTGGCCAGGATGAACGGT
CGGCTTGCCTTCATCCCTGAATCCATGCACAACGCTCTAGCGCCCATCTTCAGGATCAGTCTCCCGGTGT
TACACAGCGACACAGCCAGAAGGATCCCGTGGCAGAATTACCACCTCAACGACTGGATGGAGGAGCGTTA
CCGCCACATCCCGGGGCAGTATGTGCGTTTCACGGGATACCCGTGCTCCTGGACCTTCTACCACCACCTG
CGCCCAGAGATCCTGAAGGAGTTCACCCTGCACGACCATGTGCGTGAGGAGGCCCAGGCTTTCCTGCGTG
GCCTGCGGGTGAATGGGAGCCAGCCGAGTACCTTTGTGGGTGTCCATGTGCGCCGAGGGGACTATGTGCA
TGTCATGCCCAAGGTGTGGAAGGGCGTGGTGGCTGACCGGGGTTACCTAGAAAAGGCCCTGGACAGGTTC
CGGGCACGCTATTCATCTCCAGTCTTCGTGGTTACAAGCAACGGTATGGCCTGGTGCCGGGAGAACATCA
ACACCTCCCTAGGAGACGTGGTGTTTGCGGGCAATGGTATTGAGGGCTCACCAGCCAAGGACTTCGCGCT
CCTCACCCAGTGCAACCACACCATCATGACCATTGGAACCTTTGGGATTTGGGCTGCCTACCTGGCAGGT
GGTGATACCATCTACCTAGCCAACTACACCCTTCCGGATTCTCCGTTCCTCAAAATCTTTAAGCCAGCAG
CAGCCTTCCTCCCCGAGTGGATGGGCATCCCCGCAGACCTGTCCCCACTCCTTAAGCACTAACAACCAGC
CTGTCCTTGGTCCCATTTCCATCTCAAGGCAGAAGGAGTCACTTGGTACATCCCAGGAAGGGCCTTCTAC
TGGGAAGATGTGTGCCTTGGACTGCCAACGAGAACTAATATGTGCAGAAAACGTATTACATGTTAGATAC
AAGTTCCAACATTCATTAACAGCTCAGACATTATCAATGGCTCCTGTTCCTGAAAACTAACAGGCCTTCA
CCTTTTTTCAGCACAGCTGTCTCATGGTGCCAGCCGGGCTGCCACCCACAGACAGTCTCTTCCAGAGGCA
GCAAGAAAAGGTGTGTTATCGATTTTACTTTAGGTTCCTTTTGAAGGGTAAGGCAAACTCTTGTTTATAC
ACTTTTTGTCATTAGCATTTCCATGGCGGGGCAGCATGCGACAGGCAATAACCATGTAAGGAAGCCTGAG
AAAGGAGAGGAGGCTGGCTTGCTCTTTTTATAGCAACTGCTTTTCACCAACTGACAGGAGTCTGCTGAGA
ACTGCTAACCCCTTTGAAAGTGGTGCCTTCAGTGACCTCAGAACGTCTTATCATCTGTTAAGATTCTTTT
TTTTTTTTAATTATGGATATATATGTGGGTGTGTGCAGTACCTTC
```

Coding exon of *Fut2* gene

C.

sgRNA sequence

1) 5′ CRISPR Guide Sequence:

- 5′ ATAAATAGCTGACATCCGTT 3′ (reverse strand)
- Off-target Score: 87.6

2) 3′ CRISPR Guide Sequence:

- 5′ CTGCACATATTAGTTCTCGT 3′ (reverse strand)
- Off-target Score: 86.0

Notes:
sgRNA sequences were designed based on coding exon of *Fut2* gene, targeting the sequences in the area that highlighted in purple

Figure 2.1 *Fut2 KO mouse line establishment and genotype determination*

A. Overview of how the *Fut2* KO mouse line was generated.
B. Sequence of coding exon of *Fut2* gene which was removed by CRISPR-Cas9
C. Details of the single-stranded guide RNA sequences (sgRNA)

Table 2.2 *Primers sequences and cycling conditions*

Target	Standard DNA	Primer sequences	Cycling conditions	Reference
Fut2	N/A	mFut2KO_F: GGCACAATGCAGATGATTAG mFut2KO_R1: CTGCACATATTAGTTCTCGT mFut2KO_R2: AGTTTTCAGGAACAGGAGCC	50°C for 2 min, 95°C for 10 min Repeat below for 45 cycles: 95°C for 15 secs 60°C for 1 min	Designed in this study
16S	*E. coli* ATCC25922	qB331F: TCCTACGGGAGGCAGCAGT qB797R: GGACTACCAGGGTATCTAATCCTGTT	50°C for 2 min, 95°C for 10 min Repeat below for 40 cycles: 95°C for 15 secs 60°C for 1 min	Nadkarni *et al.* 2002[184]
B. longum sp. infantis	*B. infantis* JCM1222 (7.04 ng/uL)	BIN-F: CGCGAGCAAAACAATGGTT BIN-R: AACGATCGAAACGAACAATAGAGTT	50°C for 2 min, 95°C for 10 min Repeat below for 40 cycles: 95°C for 15 secs 60°C for 1 min	Haarman *et al.* 2005 and Zmora *et al.* 2018 and Suez *et al.* 2018[161,185,186]
B. bifidum	*B. bifidum* JCM1255 (11.0 ng/uL)	B_bif-f: CTCCGCAGCCGACCCCGAGGTT B_bif-r: TGGAAACCTTGCCGGAGGTCAGG	50°C for 2 min, 95°C for 10 min Repeat below for 40 cycles: 94°C for 15 secs 64°C for 15 s 72°C for 15 s 83°C for 15 s	Junick *et al.* 2012[187]
B. breve	*B. breve* JCM1192 (20.1 ng/uL)	BBR-F: GTGGTGGCTTGAGAACTGGATAG BBR-R: CAAAACGATCGAAACAAACACTAAA	50°C for 2 min, 95°C for 10 min Repeat below for 40 cycles: 95°C for 15 secs 60°C for 1 min	Haarman *et al.* 2005 and Zmora *et al.* 2018 and Suez *et al.* 2018[161,185,186]

2.4.1.3 Confirmation of Fut2 knock-out by tissue immunohistochemistry

To confirm whether the *Fut2* knockout genotype resulted in a loss of α(1,2)-fucosylated glycan secretion in the intestine, immunohistochemistry was performed on intestinal tissue. This involved small and large intestines from PCR-determined *Fut2*$^{-/-}$ (KO) and *Fut2*$^{+/+}$ (WT) mice stained using horseradish peroxidase conjugated Ulex Europaeus lectin 1 (HRP-UEA-1) (which binds to α(1,2)-fucosylated glycans).

Tissue samples were embedded with paraffin prior to immunostaining according to the instruction by Matsuo *et al*.[188]. In detail, proximal small intestine, distal small intestine, and large intestine were removed from 3 genotype-confirmed *Fut2*$^{+/+}$ (WT) and 3 genotype-confirmed *Fut2*$^{-/-}$ (KO) mice. Intestine segments were flushed by passing 2 mL of sterile PBS through using a syringe barrel. Flushed tissues were then pinned to a piece of absorbent tissue paper and submerged in a specimen jar containing ~20mL of methanol Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid) for 24 hours at 4°C. Tissues were then removed, trimmed and placed into cassettes and then immersed in 100% methanol for 24 hour at room temperature. Tissues were then placed in cassettes and processed using an Excelsior ES tissue processor according to standard operating procedure (Details of SOP see Appendix 2.3A) where gradient ethanol to xylene processing and wax replacement occurred. Tissues were then embedded into wax using HistoStar embedding station according to standard operating procedure (Details of SOP see Appendix 2.3B) and sectioned for future use.

During immunostaining, paraffin was firstly removed using xylene and ethanol. Biopsies were then rehydrated in de-ionised water. To minimize the background noise caused by reaction between endogenous peroxidase and substrate, endogenous peroxidase was inactivated by 20 min incubation in 3% H_2O_2. Tissues were then rinsed in 0.3% Tween-20 and incubated with 1% BSA for 30 min at room temperature. Sections were then incubated with 200 µL of 5 µg/mL Ulex europaeus Agglutinin 1 (HRP-UEA1, a lectin protein derived from the gorse plant that can bind to glycoproteins and glycolipids containing α(1,2)-linked fucose residues[175]) an

overnight and then washed by 0.3% Tween-20 three times. AEC substrates were added on the tissue slides for 15 min in dark before addition of haematoxylin counterstain. One drop of mounting solution was added on the tissue slide together with a clean cover slip for results visualisation using an Olympus BX53 microscope. Representative pictures (Figure 2.2) confirm a loss of α(1,2)-fucosylated glycans in *Fut2* KO mice.

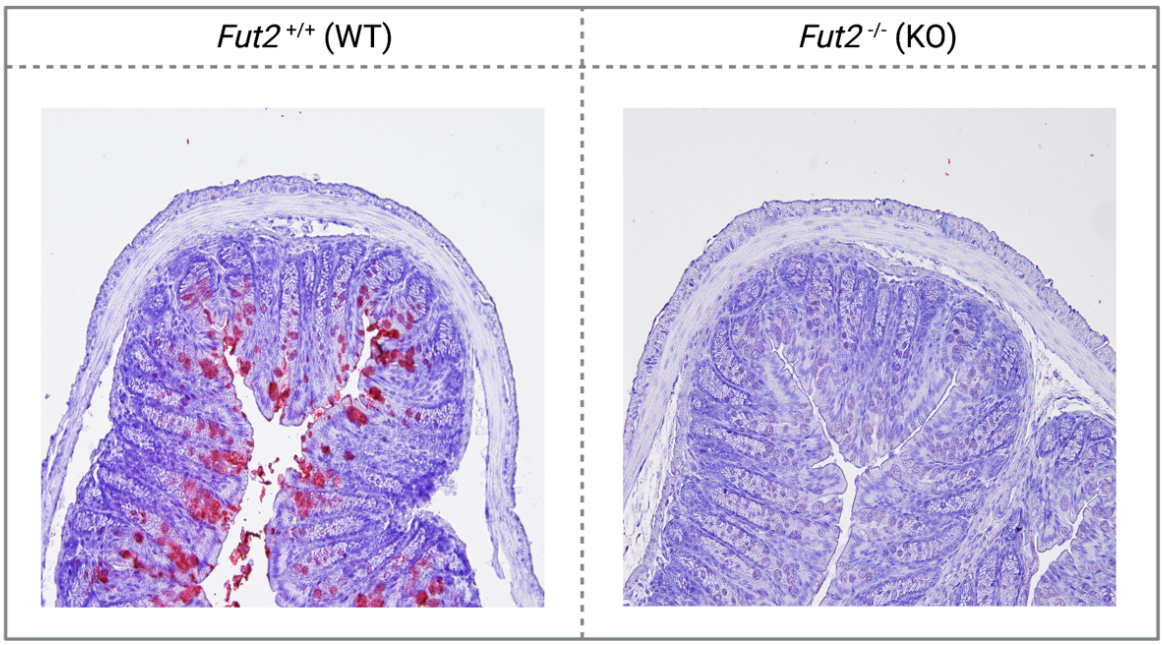


Figure 2.2 *Fut2 phenotype confirms genotype.*

Mouse large intestine tissue stained with HRP-UEA-1 (n=3/genotype). Left: *Fut2*$^{+/+}$ showing secreted α(1,2)-fucosylated glycans; Right: *Fut2*$^{-/-}$ showing no secreted α(1,2)-fucosylated glycan. This result indicated the successful development of *Fut2* Knockout mice model

2.4.2 Bacterial strains

To assess the influence of α(1,2)-fucosylated glycans on gut bacterial colonisation, three *Bifidobacterium* strains were carefully selected and employed in the experiments (selection criteria in Chapter 3). These three *Bifidobacterium* strains are *B. bifidum* JCM 1255 (=ATCC 29521), *B. longum subspecies infantis* JCM 1222 (ATCC 15697), *B. breve* JCM 1192 (=ATCC 15700). All three strains were obtained from the Japan Collection of Microorganisms (JCM; RIKEN, Saitama, Japan).

Differential utilisation of α(1,2)-fucosylated glycans has been described in the literature previously.[189] Specifically, *B. bifidum* JCM 1255 (ATCC 29521) has been shown to produce 1,2-α-L-fucosidase extracellularly, and therefore is able to degrade and utilize α(1,2)-fucosylated glycans. In contrast, *B. infantis* JCM 1222 (ATCC 15697) produces 1,2-α-L-fucosidase intracellularly and therefore is able to degrade and utilize α(1,2)-fucosylated glycans. Finally, *B. breve* JCM 1192 does not produce a 1,2-α-L-fucosidase, and is therefore unable to degrade α(1,2)-fucosylated glycans.

To validate previously reported observations, the α(1,2)-fucosylated glycan utilization of each of these three strains was assessed through *in vitro* growth assays (see Figure 4.2 in Chapter 4). Briefly, the strains were initially cultured in MRS-CS media and subsequently diluted to an optical density of 1 at 600 nm (OD_{600}). Subsequently, 100 µL of each bacterial suspension was inoculated into 9.5 mL of minimal media (mBasal broth, detailed ingredients provided in Section 2.4.10), with or without supplementation of α(1,2)-fucosylated glycans, specifically 2'-Fucosyllactose (5% w/v, purity: 98%, Layer Origin, Ithaca, New York, USA). The cultures were then incubated for 72 hours. The bacterial biomass of each strain under each culturing condition was quantified by measuring the optical density at 600 nm using a multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA).

2.4.2.1 Culture of Bifidobacterium strains

Growth conditions and kinetics for each *Bifidobacterium* strain were optimised (Figure 2.4). All *Bifidobacterium* strains used in this study were cultured in De Man, Rogosa and Sharpe (Becton Dickinson, Franklin Lakes, NJ, USA) broth or agar supplemented with 0.34% (w/v) sodium ascorbate and 0.02% (w/v) cysteine-HCl (MRS-CS) and were grown under anaerobic conditions (75% N2, 20% CO2, 5% H2, Coy Laboratory Products, Grass Lake, Michigan, USA) at 37 °C, as suggested by ATCC and confirmed by Sakanaka *et al*.[190]. The growth of *Bifidobacterium* was measured by optical density (600 nm) using multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA).

2.4.2.2 Survival of Bifidobacterium strains after 2-hour transportation on ice

Freshly cultured *Bifidobacterium* strains were required for the gavage experiments in the Chapter 4. However, the anaerobic chamber was located at Flinders Medical Centre, while the mouse facility was located at SAHMRI. Therefore, the cultures needed to be transported for up to 2-hours after leaving the incubator. To assess bacterial viability during the transport survival rates of each *Bifidobacterium* strain were assessed. To test this, each of the three *Bifidobacterium* strains were freshly cultured overnight, collected in Eppendorf tubes with MRS-CS media and stored on ice for 2 hours. After 2 hours, the viability of all three *Bifidobacterium* strains were tested by counting the colony forming unit (CFU) on MRS-CS agar at pre-determined OD_{600} (Section 3.32) (Figure 2.3, Table 2.3). As shown, the survival rates varied among species, with the survival rate of *B. infantis* being 142%, *B. bifidum* being 34% and *B. breve* being 80%. This survival rate was considered during gavaging experiments in Chapter 4.

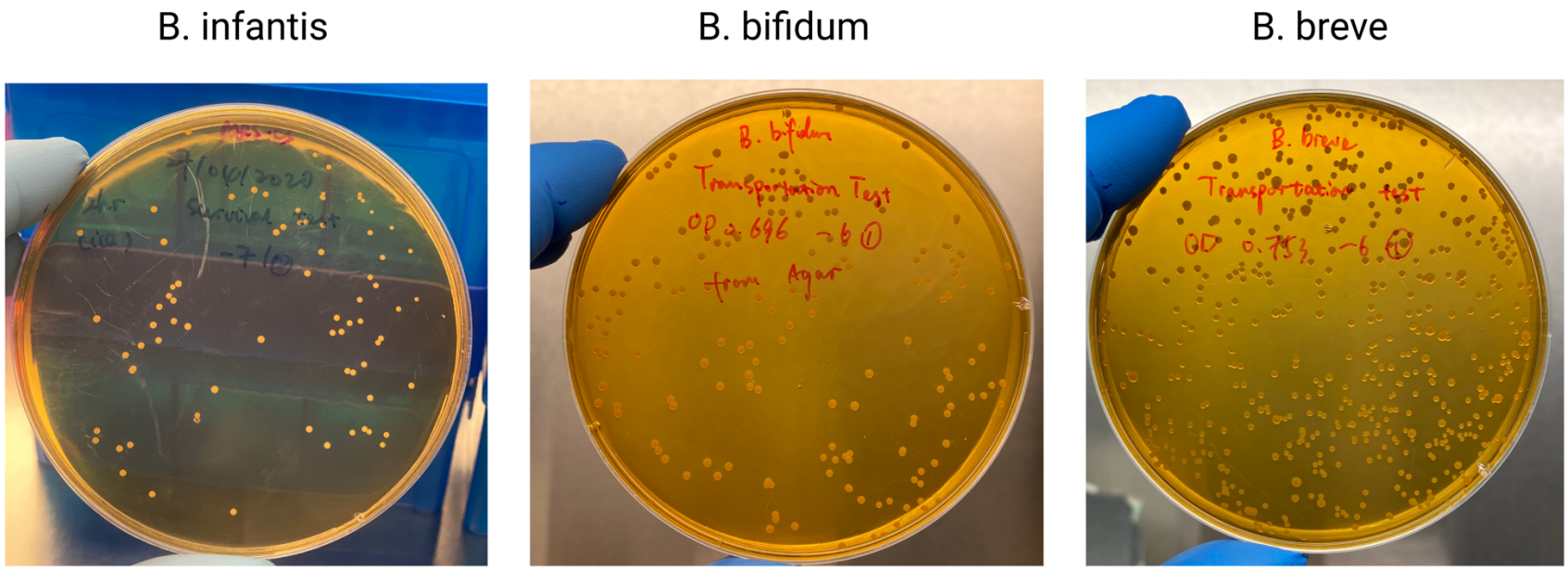


Figure 2.3 *Viability check of three Bifidobacterium strains by visualization of colony*

All these *Bifidobacterium* strains are cultured on De Man, Rogosa and Sharpe agar supplemented with 0.34% (w/v) sodium ascorbate and 0.02% (w/v) cysteine-HCl (MRS-CS). Colonies were checked after 24 hours.

Table 2.3 *Viability check of three Bifidobacterium strains by CFU count*

Bifidobacterium strain	Survival test after 2-hour transportation on ice						
	OD_{600}	Dilution ratios	CFU Count 1	CFU Count 2	Average CFU (CFU/mL)	Supposed CFU (CFU/mL)	Survival rate
B. infantis	1.150 (Target: 1.19)	1.00E+07	62	73	6.75E+09	4.75E+09	142.11%
B. bifidum	0.805 (Target: 0.81)	1.00E+06	164	142	1.53E+09	4.44E+09	34.46%
B. breve	0.753 (Target: 0.75)	1.00E+06	414	399	4.07E+09	5.08E+09	80.04%

2.4.3 Oral gavage

2.4.3.1 Optimisation of overnight culture for oral gavage

In total, 5 days of oral gavage were performed throughout this thesis. To standardise the CFU that were administered between oral gavage days, as well as streamline the process, accurate growth curves comparing optical density (OD) to CFU/mL were calculated for each *Bifidobacterium* strain. For this, a multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA) machine was used to determine the OD_{600} across a series of time points throughout multiple repeats of *Bifidobacterium* culture. Alongside this, the -CFU/mL of cultured broth was calculated by plating out serial dilutions of *Bifidobacterium* culture. As shown in Figure 2.4, a linear curve was generated for each *Bifidobacterium* strain. It is also important to note that as each curve is linear, it is in the bacterial growth phase, not the stationary phase. Annotated to each curve in red is the concentration required for oral gavage. Therefore, overnight cultures were recorded and diluted to each respective OD_{600}. Specifically, for *B. infantis*, cultures were standardised to OD_{600} 1.19, *B. bifidum* to OD_{600} = 0.81, and *B. breve* to OD_{600} = 0.75, (Figure 2.4D). After adjustment, these bacterial strains were collected in Eppendorf tubes and were ready for oral gavage. Confirmation test was conducted and verified the OD_{600}-CFU curve (Table 2.1).

2.4.3.2 Oral gavage process and dosage

The dosage of oral gavage for each mouse was 5×10^7 bacteria/g/24h based on previous report[191]. The volume of oral gavage for each mouse/day was 10 µL/g according to ethic requirement and probiotic bacteria were prepared in a PBS gavaging solution at the concentration of 5 10^6 bacteria/µL (5 10^9 CFU/mL). The probiotic administration by oral gavage will be performed according to the SAHMRI Standard Operating Procedure (SOP) #0475 (Appendix 2.3C) and all procedures were entered into the SAHMRI digital mouse recording and monitoring system (Emus).

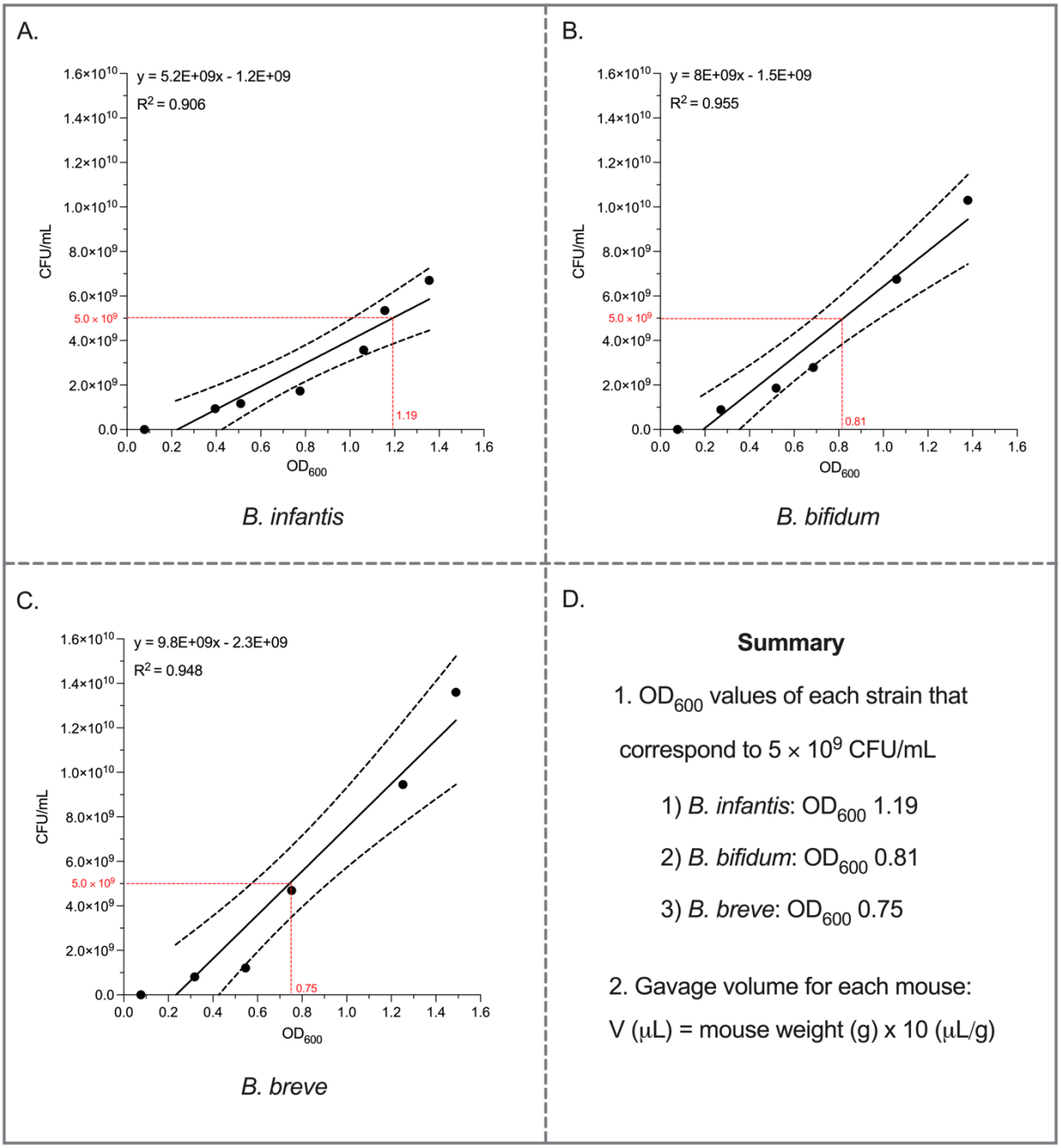


Figure 2.4 *Establishment of OD_{600}-CFU standard curves for oral gavage*

Three *Bifidobacterium* strains were cultured in MRS-CS broth. The OD_{600} values were measured at different timepoints and the corresponding colony forming unit (CFU) per mL were calculated based on the colony counts at those timepoints and dilution ratios. The growth curve of each *Bifidobacterium* strain was then established (2 replicates/timepoints, the experiment was only performed once). The established standard curves were used for oral gavage purpose in mouse study.

A. OD_{600}-CFU curve of *B. infantis*
B. OD_{600}-CFU curve of *B. bifidum*
C. OD_{600}-CFU curve of *B. breve*
D. Pre-determined OD_{600} values for oral gavage

2.4.4 Sample collection

2.4.4.1 Faecal sampling

Prior to faecal sampling, mice were placed on a sterile surface until at least two faecal pellets were produced. Fresh faecal pellets were collected into 1.5 mL tubes on pre-determined days at the beginning of the light phase, and immediately placed in dry ice and stored at -80 °C until further processing.

2.4.4.2 Mucosal tissue sampling

Mice were sacrificed by CO_2 asphyxiation and laparotomy was immediately performed using a vertical midline incision. Once the digestive tract was exposed, separate dissection tools were used to dissect tissue into four parts: the proximal small intestine; distal small intestine; caecum and large intestine. For small and large intestine tissue segments, the luminal content was collected by instilling sterile PBS using a syringe barrel and the flushed mucosal tissue was collected into separate tubes. The collected organs, and luminal contents were immediately frozen on dry ice and stored at -80 °C until further processing.

2.4.5 DNA extraction

2.4.5.1 DNA extraction on faecal samples

Faecal pellets were weighed, and 25 mg samples (±10 mg) were resuspended in 300 µL of cold phosphate buffered saline (PBS) (pH 7.2) by vortexing and pelleted by centrifugation at 10,000 × g for 10 min at 4 °C. Microbial DNA was extracted from faecal samples using the PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as described previously[192] (Manufacturer's instruction see Appendix 2.4).

2.4.5.2 DNA extraction on mucosal tissue samples

Mucosal tissue from the proximal small intestine, distal small intestine, and large intestine were semi defrosted and 3 cm was removed from the tissue centre using sterile scalpel. The dissected tissues were cut open longitudinally and mixed with 750 µL PowerSoil® bead solution and 60 µL solution C1 in a PowerSoil® bead tube. The bead tube was then incubated at 65 °C for 10 mins prior to bead beating. The subsequent DNA isolation was performed using the PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as described previously[192].

2.4.6 16S rRNA gene amplicon sequencing

Amplicon libraries of the V4 hypervariable region for 16S rRNA gene were prepared from DNA extracts using modified universal bacterial primer pairs 515F and 806R[182] (Sequencing see below).

- 515F:5′′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3′
- 806R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3′

Amplicon libraries were indexed, cleaned, and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol on a 2 × 300 bp Miseq reagent kit v3 at the South Australian Genomics Centre (SAGC), South Australian Health and Medical Research Institute.

2.4.7 Bioinformatic processing on 16S sequencing results

Paired-end 16S rRNA gene sequence reads were analysed using QIIME2 version 2021.11.0 [193]. Briefly, de-noising was performed on de-multiplexed sequences using the Dada2 plugin [194]. Taxonomic classification of amplicon sequence variants was performed based on the V4 hypervariable region of the SILVA 16S rRNA reference database (version 138) at 99% similarity [195]. Sufficient coverage at this depth is confirmed by the rarefaction curve, which reached an asymptote. Sequence data has been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJNA1011386. Codes used for bioinformatic processing on 16S sequencing results were included in Appendix 2.5.

2.4.8 Microbiome characterisation

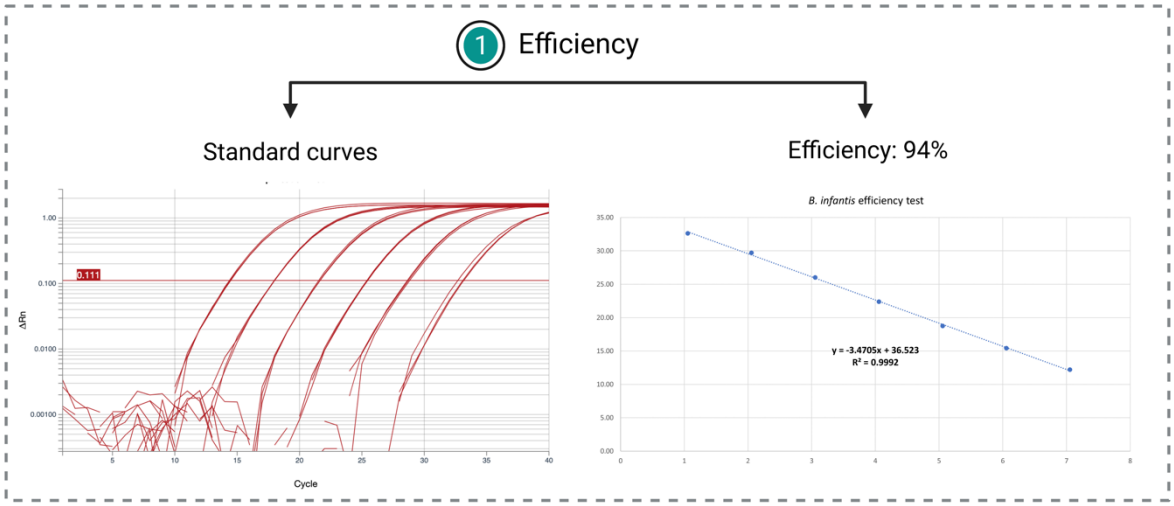
The taxonomic relative abundance at the species level was used to generate alpha diversity (within-group) and beta diversity (between-group) measures. Alpha diversity matrix were obtained from QIIME2 at sampling depth of 9,883 reads (Faecal samples) and 662 reads (Mucosal tissue samples). Rarefaction curves were included in Appendix 2.6, indicating a sufficient capture of diversity with high counts. Alpha diversity measures included Chao's richness (*S'*: total number of unique species identified per participant), Pielou's evenness (*J'*: a score between 0-1 where scores are influenced more by the evenness of abundant species), the Shannon-Wiener diversity (*H'*): a score of the number and equal representation of different types of species, and the Faith's Phylogenetic diversity (*PD'*): a score that represent the phylogenetic analogue of taxon richness. Four dissimilarity distance matrices (Jaccard distance, Bray-Curtis distance, Weighted Unifrac distance and Unweighted Unifrac distance) were calculated to compare microbiome similarity between groups (beta diversity), using square-root transformed species relative abundance data using the 'vegan' package in R. Non-metric multidimensional scaling (nMDS) for all beta diversity measures were generated using the 'vegan' package in R. Core taxa were defined as those present in more than 95% of samples, with a mean relative abundance of >0.01%.

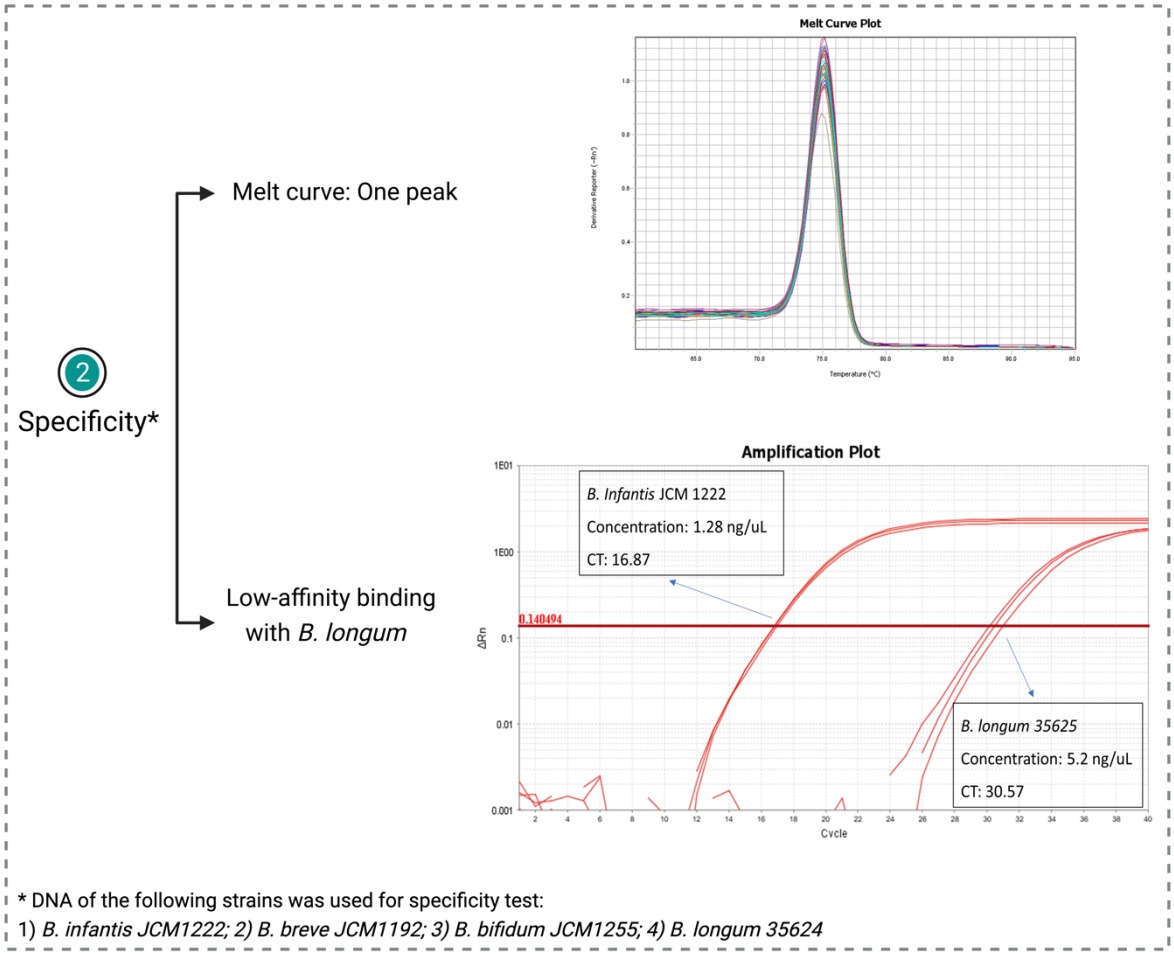
2.4.9 Quantification of *Bifidobacterium* and total bacterial load

Quantification of total bacterial load, *B. breve*, *B. infantis* and *B. bifidum* was performed by SYBR Green-based qPCR assays. Details of primer sequences, cycling conditions and sensitivity threshold used are listed in Table 2.2. For all qPCR assays, 1 µL of DNA template was combined with 0.7 µL of 10 µM forward primer, 0.7 µL of 10 µM reverse primer, 17.5 µL of 2×SYBR Green (Applied Biosystems, Waltham, Massachusetts, USA) and 15.1 µL nuclease-free water. Amplification efficiency and specificity check of each set of primer was conducted by NCBI primer blast and qPCR test with other *Bifidobacterium* strains (*Figure 2.5*).

All samples were run in triplicate (10 µL each replicate). Gene copy quantification was performed using a standard curve generated from a known concentration of a pure colony control. Any sample with a cycle threshold (CT) ≥40 cycles was defined as 40 (threshold of detection).

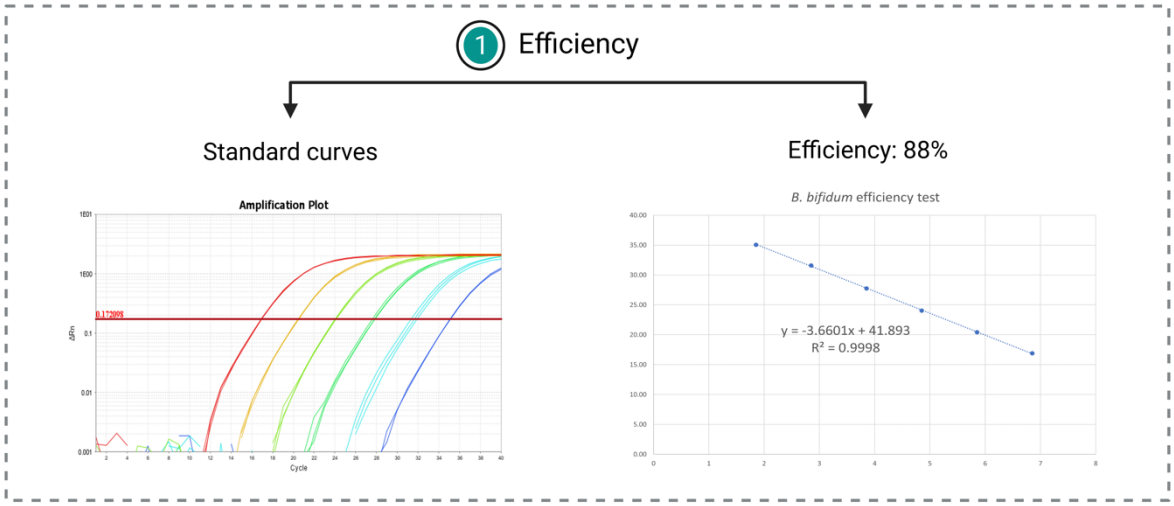
A. Efficiency and Specificity test of *B. infantis*

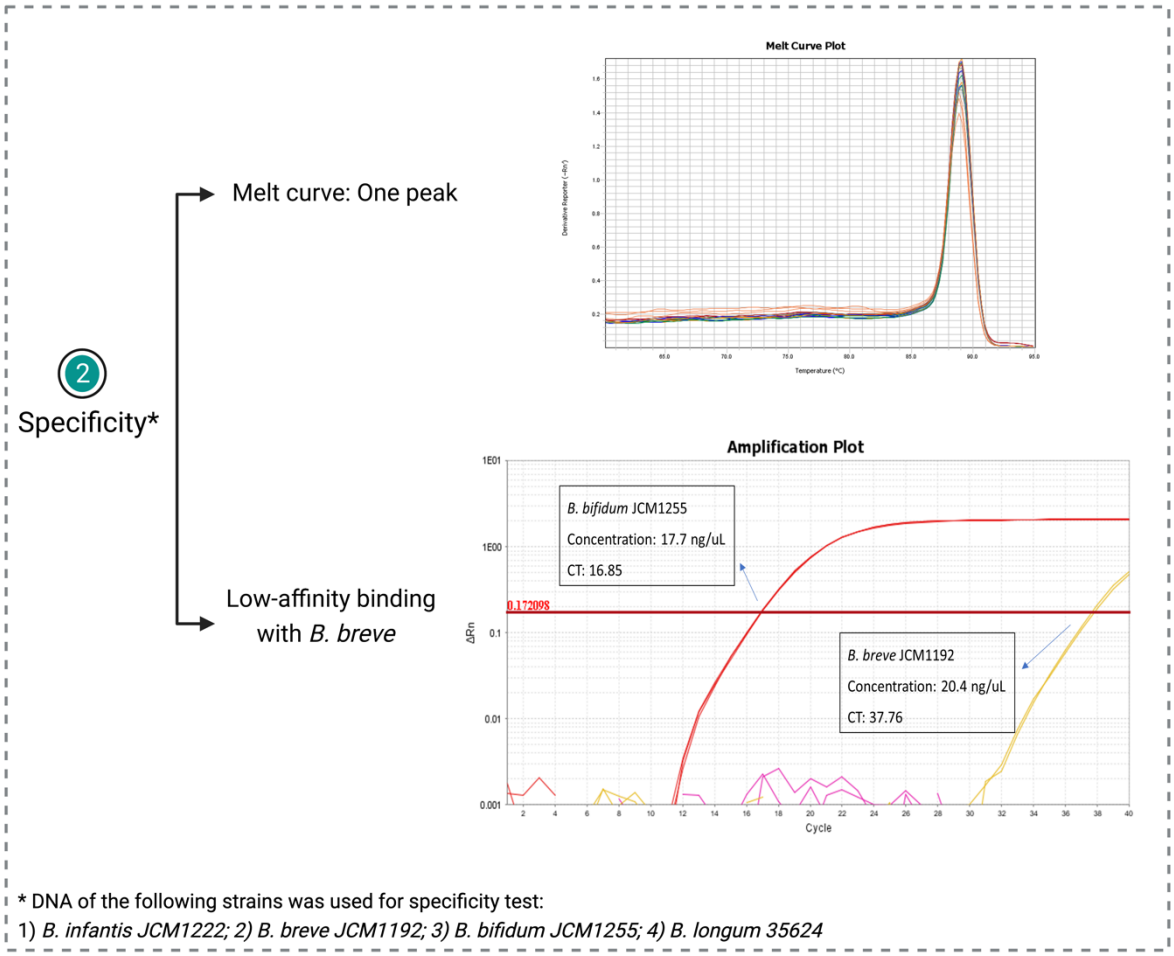




* DNA of the following strains was used for specificity test:
1) *B. infantis JCM1222; 2) B. breve JCM1192; 3) B. bifidum JCM1255; 4) B. longum 35624*

B. Efficiency and Specificity test of *B. bifidum*





* DNA of the following strains was used for specificity test:
1) *B. infantis JCM1222; 2) B. breve JCM1192; 3) B. bifidum JCM1255; 4) B. longum 35624*

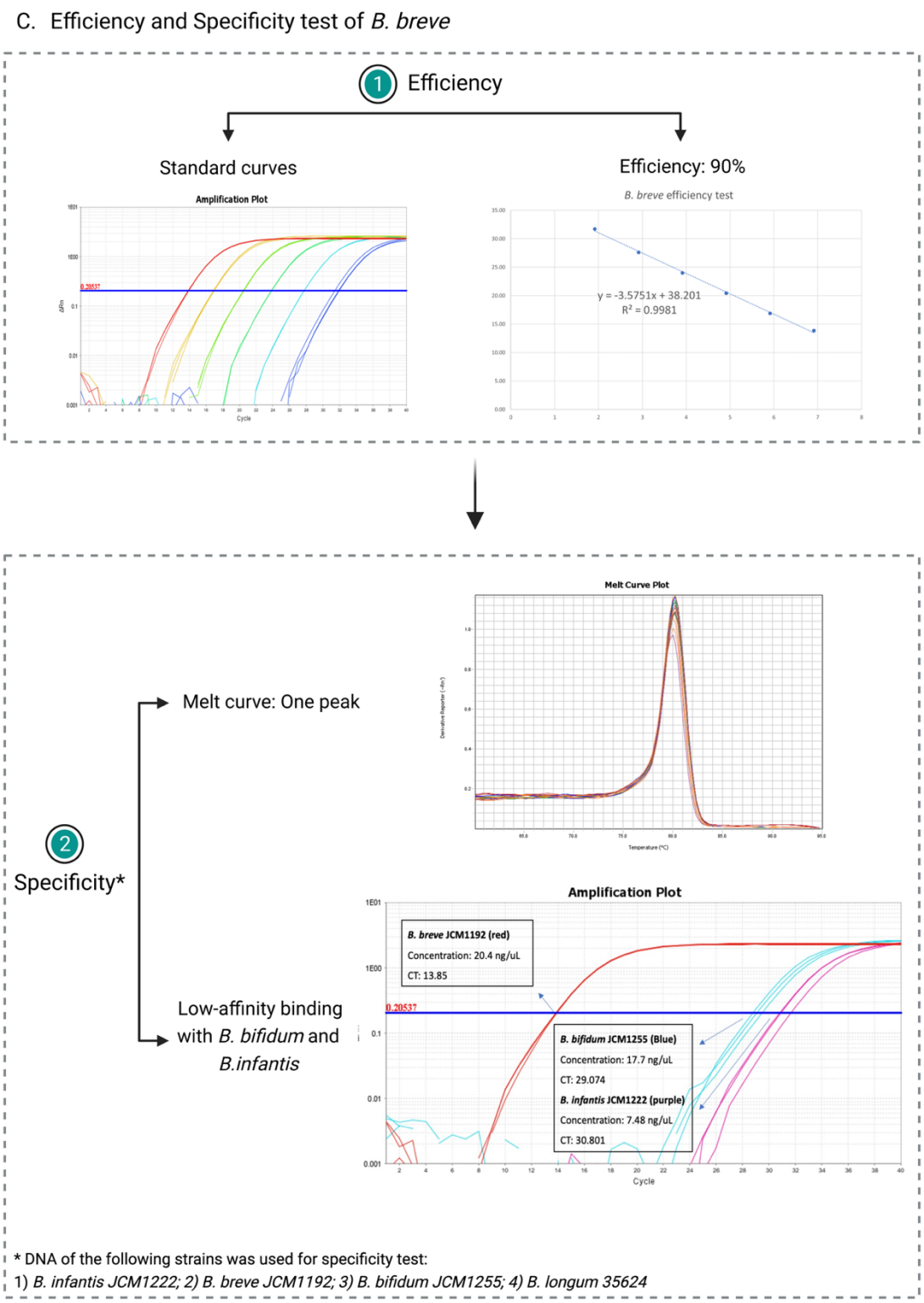


Figure 2.5 *Amplification efficiency and specificity test of three Bifidobacterium species primer*

All three primers have acceptable amplification efficiency (>85%) and are specific enough (low-affinity with other *Bifidobacterium* strains) for quantification purposes in Chapter 4; A. Efficiency and specificity test of *B. infantis*; B. Efficiency and specificity test of *B. bifidum*; C. Efficiency and specificity test of *B. breve*; * DNA of the following strains was used for specificity test: 1) *B. infantis JCM1222; 2) B. breve JCM1192; 3) B. bifidum JCM1255; 4) B. longum 35624*

2.4.10 In vitro glycan utilisation assay

Faecal pellets from untreated WT mice were incubated in minimal media (mBasal; 10 g/L Trypton, 2 g/L yeast extract, 5 g/L NaCl, 0.2 g/L magnesium sulfate, 2 g/L dipotassium hydrogen phosphate, pH 6.4, the formula was modified according to Zabel *et al*.[196]) with/without supplementation of 5% w/v of 2′-Fucosyllactose (Layer Origin, Ithaca, New York, USA) at 37 °C under strict anaerobic conditions (75% N_2, 20% CO_2, 5% H_2, Coy Laboratory Products, Grass Lake, Michigan, USA). Bacterial biomass was measured by OD_{600} using multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA). Bacterial colonies cultured from faecal samples were identified by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) (Billerica, Massachusetts, United States), as described in Section 3.11[197].

2.4.11 MALDI-TOF mass spectrometry

2.4.11.1 Colony preparation

After 48 hours of culturing in minimal broth, bacterial suspensions from cultured samples were spread on mBasal agar plates and incubated under anaerobic condition at 37 °C for 48 hours. A total of 10 colonies (2 big colonies, 4 medium colonies and 4 small colonies) were randomly collected from each agar plate to sterile tubes with 400 µL PBS using sterile loops. These colonies were purified by streaking on agar plates and cultured under anaerobic condition for 48 hours prior to MALDI-TOF MS analysis.

2.4.11.2 MALDI-TOF MS analysis

One purified colony from each agar plate was picked by a sterile toothpick. A thin layer was applied on a target spot that sitting on MALDI plate using the toothpick that carried the colony. After that, 1 µL of formalin was dropped to each spot to fix the samples and the MALDI plate was incubated at room temperature until formalin became dry. Another 1 µL matrix material was applied on each spot. When the MALDI plate was completely dry, it was then placed into the MALDI Biotyper (Billerica, Massachusetts, United States) and the mass spectra obtained from each isolate were imported into software and were mapping with bacterial databases for species identification. Score values from the output that were smaller than 1.7 were considered as unreliable identification. Score values were between 1.7 to 2.0 were considered genus-level identification. Score values were larger than 2.0 were considered species-level identification.

2.5 Quantification and statistical analysis

Experimental mice were randomly assigned to different treatment groups. The investigators were not blinded to the experimental groups. No outliers have been removed from any of the data presented. All data analyses were performed using R (R Foundation for Statistical Computing; version 4.1.0).

For parametric data, unpaired Student's t-test was used to compare data between two unpaired groups; One-way ANOVA was used to compare data among three or more unpaired groups. For non-parametric data, Mann-Whitney U test was used to compare data between two unpaired groups; Kruskal-Wallis test was used to compared data among three or more unpaired groups. Differences in Bray-Curtis dissimilarity between groups was performed by permutational multivariate ANOVA (PERMANOVA) and pairwise PERMANOVA, using the 'adonis' package in R, with 9,999 permutations. Similarity Percentage (SIMPER) analysis was used to assess the relative contribution of each taxon to the dissimilarity between each two groups. Linear discriminant analysis Effect Size (LEfSe) was used to identify the abundant taxa in each site[198]. Log-rank test was employed to compare survival time differences based on bacterial qPCR detection. One-tailed tests were used where differences between groups were hypothesised to be in a single direction. Statistical outcomes with p-value <0.05 were considered statistically significant. Statistically significant findings were noted according to the following cut-offs: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Core taxa plot was generated using GraphPad Prism (GraphPad Software, Inc.; version 9.00), other data were visualised using R. Details of statistical methods used were summarised in Table 2.4.

Table 2.4 *Summary of statistical methods used*

AIMS	PARAMETRIC DATA	NON-PARAMETRIC DATA
Compare scale data between two unpaired groups	Unpaired t test	Mann-Whitney test
Compare longitudinal scale data between two paired groups	Paired t test	Wilcoxon test
Compare scale data among three or more groups	One-way ANOVA	Kruskal-Wallis test
Assess correlations of scale data between two groups	Pearson correlation	Spearman correlation
Assess correlations of nominal data between two groups	Fisher's exact test	
Compare survival time difference	Log-rank test	
Compare the composition difference between groups	PERMANOVA / Pairwise PERMANOVA	
Assess within-group and between-group variance - The equality of sample compositional variance within all groups - Differences between within-group variance and between-group variance	- Levene's test - Analysis of similarities (ANOISM)	
Assess taxa difference between groups	Linear discriminant analysis Effect Size	

CHAPTER 3. THE IMPACT OF α(1,2)-FUCOSYLATED GLYCANS ON THE GUT MICROBIOTA

3.1 Introduction

The *Fut2* gene encodes an α(1,2)-fucosyltransferase enzyme in mammals that, when expressed in mucosal secretory cells, facilitates the attachment of the L-fucose monosaccharide to specific O-linked glycan chains [127,128]. As detailed in Chapter 1, the resulting α(1,2)-fucosylated glycans play a notable role in modulating host-microbe interactions, by acting as a carbon source or anchor point for common gut bacteria [199]. Important to this chapter, not all bacterial species commonly found in the gut utilise α(1,2)-fucosylated glycans meaning that the presence or absence of these glycans have a disproportionate effect on particular species. While more than 50 gut bacterial species have been shown to carry at least one gene copy number of glycosyl hydrolases (GHs) that are responsible for degrading α(1,2)-fucosylated glycans[200], only a small number make up the gut microbiota. Those that have been well-characterised (tested in *in vitro* study) include those belonging to the genera *Bacteriodes*, *Bifidobacterium*, and *Streptococcus* (Carbohydrate Active Enzymes database: http://www.cazy.org/). Species able to use α(1,2)-fucosylated glycans have specialised genes able to cleave the L-fucose from the terminal saccharide, which are called α(1,2)-L-fucosidases. Apart from the glycosyl hydrolase that specifically only cleaves α(1,2)-L-fucosidases (GH95), other glycosyl hydrolases (GHs) harboured by gut bacteria, including N-acetyl-glucosaminidases (GH84, GH85, G89, GH20), N-acetyl-galactosaminidases (GH101, GH129), and galactosidases (GH2, GH35, GH42, GH98), are also capable of degrading mucin glycans but on different linkages[200,201]. Similar to α(1,2)-L-fucosidases, many gut bacterial species carry the genes for other GHs by analysis genome sequence[200], but they were not fully characterised and validated by *in vitro* cultivation.

Beyond the direct impact α(1,2)-fucosylated glycans have on the fitness of individual species within the gut microbiome, their presence can have secondary impacts for bacterial fitness. For example, *Blautia* (an anaerobic gut bacterial genus) was found to be able to grow better in broth supplemented with α(1,2)-fucosylated glycans when it was co-cultured with presence of *Bifidobacterium bifidum* (glycan degrader)[202]. Similar cases also happen to other non-glycan degrading bacteria, such as *Anaerostipes*, *Enterococcus* and *Lactobacillus* [202-207]

In this chapter, the impact of secreted α(1,2)-fucosylated glycans on the gut microbiome was assessed. As presented in the methods, the gut microbiota from mice with and without a functional *Fut2* gene have been compared. Given the confounding effects of sex and cage effects on the gut microbiota, these variables are also explored in relation to *Fut2* in this chapter.

3.2 Methods and materials

Full details of all experimental methods and materials, including optimisation and validation are presented in Chapter 2.

3.2.1 Experimental settings

A *Fut2* knock-out mouse line was established using CRISPR-Cas9 gene editing technology, which was performed by the SA Genome Editing Facility at South Australian Health and Medical Research Institute (SAHMRI). All mice were derived, bred, mated and maintained under specific and opportunistic pathogen free conditions in Specific Pathogen-Free (SPF) and PC2 rodent facility (Bioresource) at SAHMRI. Details of mouse model establishment were described in Chapter 2 (Section 3.1.1).

A total of 64 (6 weeks of age, age and sex matched) experimental WT ($Fut2^{+/+}$, n = 32) and KO mice ($Fut2^{-/-}$, n = 32) were employed in this study. These mice were obtained by mating heterozygous breeding male and heterozygous breeding female mice originated from F1 heterozygous mice. All mice given birth for breeding or experimental purposes were subjected to PCR genotyping. To minimize and evaluate the impact of other unwanted covariates (such as cage effects) on microbial community, a total number of 19 cages were employed in this study to ensure at least 9 cages for each genotype.

To analyse the impact of α(1,2)-fucosylated glycans on gut microbial community, baseline faecal samples were collected and were subjected to stool DNA isolation, 16S rRNA gene sequencing and microbiome analysis. To minimize and evaluate the impact of other unwanted covariates (such as cage effects) on microbial community, a total number of 19 cages were employed in this study to ensure at least 4 cages for each genotype x sex type (Figure 3.1). On top of that, all experimental mice with different genotypes were homogenized 3-4 weeks prior to the experiment.

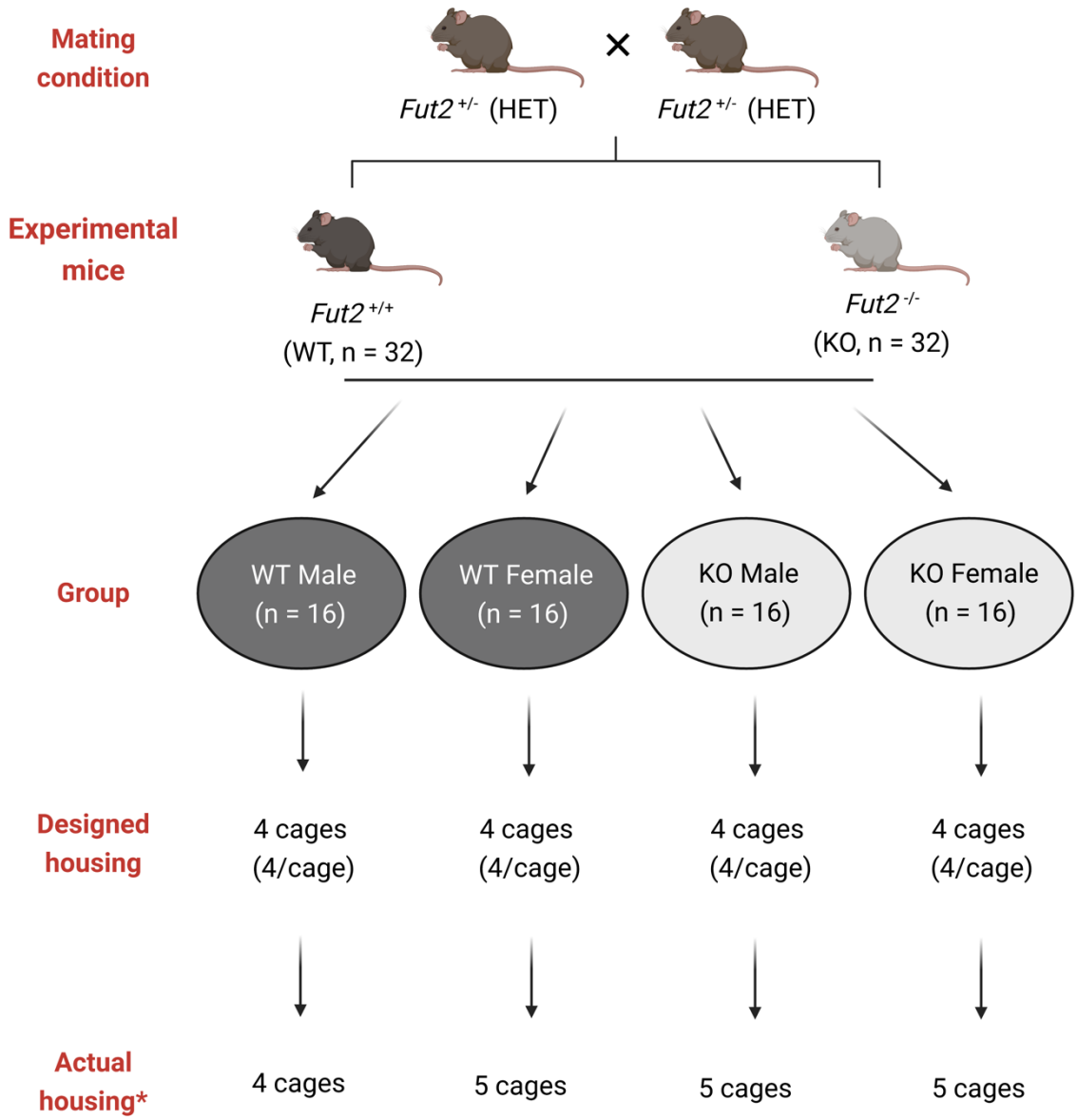


Figure 3.1. *Mating and housing condition*

All experimental mice were obtained from Het × Het breeding and they were gender and age matched. For each group (genotype × sex), mice were randomly assigned to at least 4 cages to minimize the impact of cage effects on experiment. *Certain mice enjoy fighting with their housemates and cause damage to their housemates and they were housed individually

3.2.2 *Fut2* Genotyping

Mice ear punch tissue were collected and DNA extracted as detailed in Chapter 2 (Section 3.1.2). *Fut2* genotype was determined by assessing the melt curve of the qPCR as detailed in Chapter 2 (Section 3.1.2). Detailed information of primer usage or cycling conditions for genotyping can be found in Chapter 2 (Table 2.2).

3.2.3 Sample collection

Mice were separated into sterile, empty cages. Faecal pellets were collected into sterile 1.5 mL Eppendorf tubes using sterile toothpicks. Between each collection, new gloves and toothpicks were used and cages were cleaned using 80% ethanol and F10 solution. All faecal samples were immediately frozen on dry ice prior to long term storage at -80°C.

3.2.4 DNA extraction, 16S rDNA sequencing and analysis

Full details DNA extraction, amplicon sequencing, and bioinformatic processing are included in Chapter 2 (Section 3.52, 3.6, 3.7 respectively). Briefly, faecal pellets were weighed and approximately 25 mg was subjected to DNA extraction using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany). The isolated DNA were then amplified and prepared for 16S sequencing. Reads were demultiplexed and cleaned using QIIME2. α-diversity was evaluated using two diversity indices: Faith's phylogenetic diversity and Chao's Richness. These α-diversity measures capture variability among all types of taxa in communities and the number of different species in communities, respectively. β-diversity was measured using four dissimilarity distance matrixes: Jaccard distance matrix, Bray-Curtis distance matrix, unweighted Unifrac distance matrix and weighted Unifrac distance matrix. These β-diversity measures capture microbiome composition in different perspectives (taxa presence, abundance and phylogeny respectively).

3.2.5 Statistical analysis

All data analyses were performed using R (R Foundation for Statistical Computing; version 4.1.0). D'Agostino-Pearson normality test (*PowerR* package) were employed to assess if data are normally distributed. For parametric data, unpaired t test was used to compare data between two unpaired groups; One-way ANOVA was used to compare data among three or more unpaired groups. For non-parametric data, Mann-Whitney test was used to compare data between two unpaired groups; Kruskal-Wallis test was used to compared data among three or more unpaired groups. PERMANOVA (*vegan* package) was employed to assess microbiome composition differences between two groups. Pairwise PERMANOVA was employed to analyse the composition difference among three or more groups. Three statistical approaches were employed to determine taxa that differ between groups. SIMPER was used to assess the relative contribution of each taxon to the overall average Bray-Curtis dissimilarities by pairwise comparison of WT and KO group. LEfSe (Galaxy web application) was used to compare the abundance difference between WT and KO group and identify the outstanding taxa. Fisher exact test with FDR correction were used to assess detection differences in detection of organisms. *P* values less than 0.05 were considered statistical significance.

3.3 Results

In this chapter, I hypothesised that α(1,2)-fucosylated glycans that either secreted into the GI lumen or anchored to the gut epithelial surface provide either carbon source or niche space for gut microbes, impacting the gut microbiome. On top of that, I also hypothesised that sex, another host genetic factor, also played a role in shaping the gut microbiome. To test these hypotheses, analyses were conducted in the following sections to:

1) Characterise the gut microbial community in the murine gut
2) Compare microbial communities between *Fut2*$^{+/+}$ (WT) and *Fut2*$^{-/-}$ (KO) mice
3) Compare microbial communities between male and female mice
4) Explore the synergistic effect of genotype and sex on gut microbial community

To capture the microbial community differences, different aspects of microbial communities, including α-diversity, β-diversity and taxa, were calculated and compared based on 16S sequencing results.

3.3.1 Characteristics of gut microbial community

Across both WT and KO mice, a total of 8 phyla, consisting of 111 genera, were detected in faeces. Within mice, the median number of genera was 47 (IQR=39, 53) (Figure 3.2). Of the 111 genera detected, 17 were in at least 90% of mice (57/64) with a mean relative abundance of at least 0.1% and were defined as core taxa. Nine of the 17 core taxa had a mean relative abundance >1%, including *Lactobacillus, Muribaculaceae, Faecalibaculum, Lachnospiraceae_NK4A136_group, Lachnospiraceae_unassigned, Alistipes, Bacteroides, Clostridia_UCG014, Enterorhabdus* and *Bifidobacterium*. The genera detected across 100% (64/64) samples included *Lactobacillus*, *Muribaculaceae*, *Bacteroides*, *Alistipes*, *Lachnospiraceae_NK4A136_group*, *Lachnospiraceae_unassigned* and *Enterorhabdus*. Interestingly, of these genera that detected across all samples, at least two taxa (lactobacillus and Bacteroides) are capable of degrading and utilizing *Fut2* related α(1,2)-fucosylated glycans.

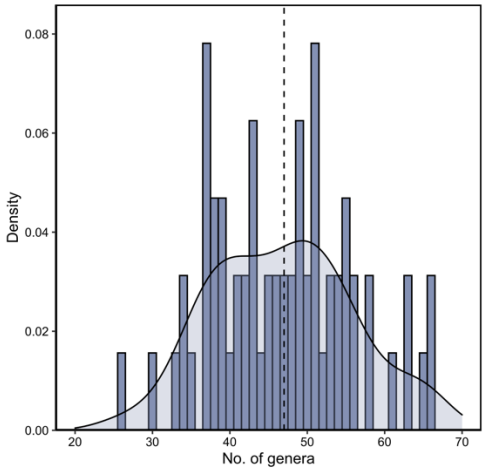


Figure 3.2 *Histogram of number of taxa found in murine gut*

Baseline faeces (n = 64) were subjected to DNA extraction and 16S sequencing. Of 64 mice being analysed, a median number of 47 genera were found in murine gut

3.3.2 *Fut2* contributes to an altered gut microbial community

In this section, gut microbial communities were compared between WT mice (n=32) and KO mice (n=32) by assessing differences in α-diversity, β-diversity, and relative abundance of identified taxa.

3.3.2.1 Microbiome structure (α-diversity)

Samples collected from WT mice had slightly higher Faith's phylogenetic diversity score and Chao's richness score than those collected from KO mice (Chao's richness: 150.8 vs 139.0; Faith's phylogenetic diversity: 14.06 vs 12.05). However, the differences did not achieve any statistical significance (p[Chao]=0.16; p[Faith]=0.076; Figure 3.3A and 3.3B). This result suggests that the availability of α(1,2)-fucosylated glycans may not play a role in shaping microbial diversity.

3.3.2.2 Microbiome composition (β-diversity)

Non-Metric Multidimensional Scaling (NMDS) analysis was applied to visualise the dispersion and similarity of microbial communities by using four dissimilarity distance matrices (Jaccard, Bray-Curtis, unweighted Unifrac and weighted Unifrac). Each distance matrix was used to assess different diversity traits. In short, Jaccard matrix and Bray-Curtis matrix were employed to interpret microbiome difference in the aspect of taxa presence/absence and abundance individually without considering taxa phylogeny while unweighted Unifrac (taxa presence/absence) and weighted Unifrac (taxa abundance) also take the phylogenetic relatedness into consideration.

As shown in Figure 3.3C-1 and Figure 3.3C-3, there were visible differences in microbial dissimilarity between WT and KO groups. To quantify these differences, PERMANOVA tests were conducted based on both Jaccard and Bray-Curtis dissimilarity distance matrices first. Results show that the carriage of *Fut2* gene significantly contributed to microbial composition differences of the samples in both aspects of taxa presence/absence and taxa abundance

(p[Bray-Curtis] = 0.028, pseudo-F[Bray-Curtis] = 2.74; p[Jaccard] = 0.035, pseudo-F[Jaccard] = 1.31).

To further assess whether taxa that contribute to microbiome composition are closely related in phylogeny, compositional analysis was then conducted using weighted Unifrac distance and unweighted Unifrac distances respectively. NMDS analysis and PERMANOVA test showed that samples in WT group were significantly different to samples in KO group when analysed using unweighted Unifrac distances (p = 0.015, pseudo-F = 1.53, Figure 3.3C-2), but not significantly different using weighted Unifrac distance (p = 0.076, pseudo-F = 2.16, Figure 3.3C-4). These results suggest that the taxa that are not shared between WT and KO mice are likely from different phylogeny; however, most shared taxa whose abundance are different between WT and KO mice are likely from similar phylogeny.

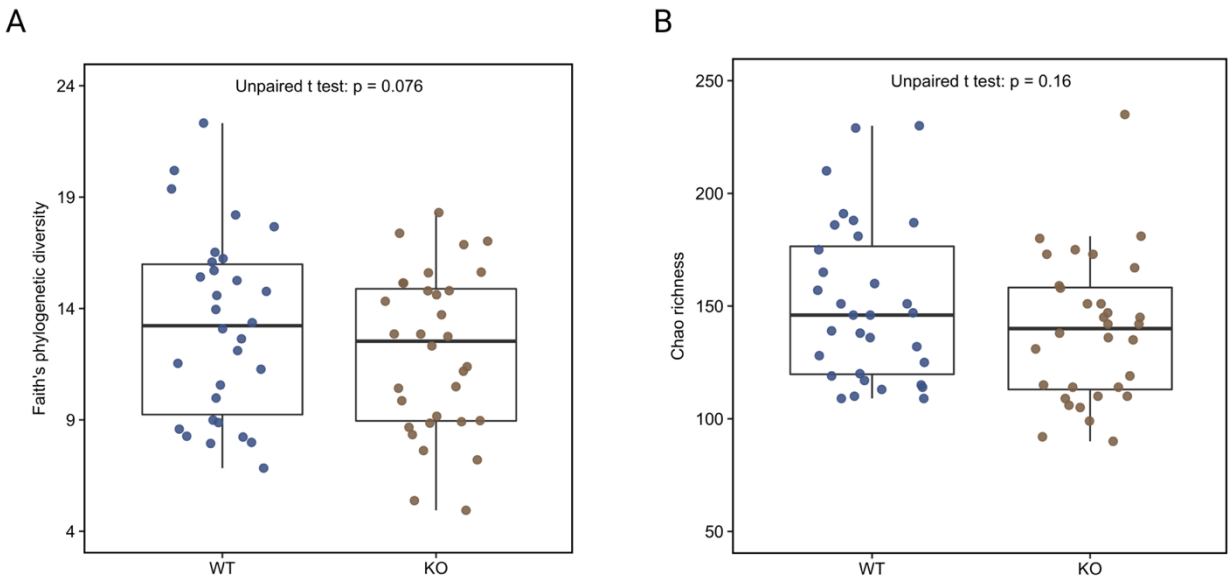


Figure 3.3A and Figure 3.3B *Fut2 does not impact the structure of gut microbial community*

A total of 64 baseline faeces were subjected to DNA extraction and 16S sequencing. The differences of α-diversity between WT and KO groups were assessed. Both Faith's phylogenetic diversity scores and Chao richness scores were comparable between WT and KO groups ($p>0.05$), indicating *Fut2* does not impact the structure of gut microbial community.

A. Faith's phylogenetic diversity comparison between WT and KO mice.

B. Chao richness comparison between WT and KO mice.

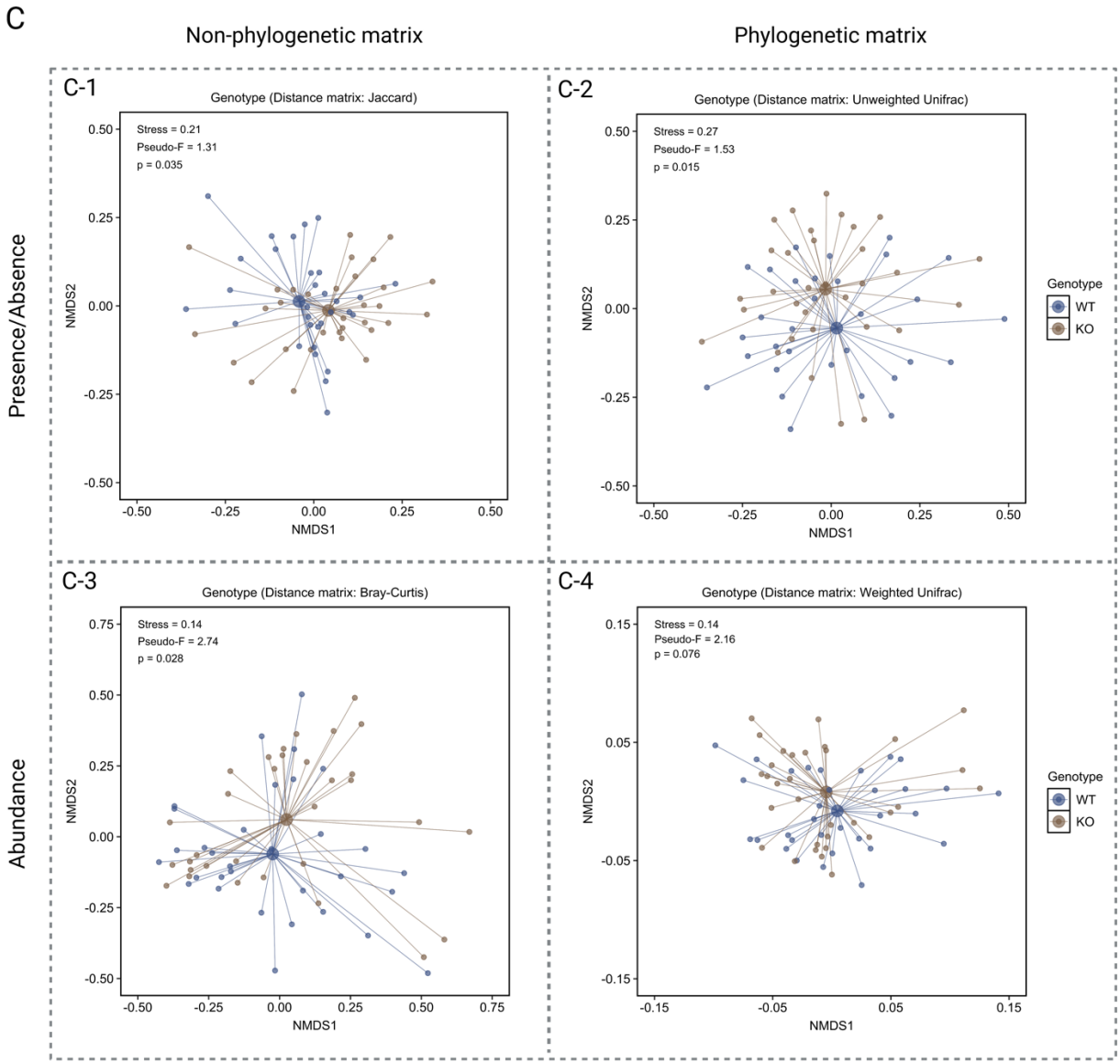


Figure 3.3C *Fut2 impact the composition of gut microbial community*

A total of 64 baseline faeces were subjected to DNA extraction and 16S sequencing. The differences of β-diversity between WT and KO groups were assessed in different aspects using four dissimilarity distance matrices (Jaccard: C-1, Bray-Curtis: C-3, unweighted Unifrac: C-2 and weighted Unifrac: C-4) and were visualised through Non-Metric Multidimensional Scaling (NMDS) plots.

Each measure assesses microbiome difference in different traits. Jaccard: Non-phylogenetic matrix that focus on taxa presence/absence difference; Bray-Curtis: Non-phylogenetic matrix that focus on taxa abundance difference; unweighted Unifrac: Phylogenetic matrix that focus on taxa presence/absence difference; weighted Unifrac: Phylogenetic matrix that focus on taxa abundance difference

3.3.2.3 Taxa

Of 111 genera that were found in faecal samples collected from experimental mice, 35 genera together contributed to more than 90% of total variance between WT and KO mice (Appendix 3.2). Within these 35 genera, *Lactobacillus* and *Faecalibaculum* contribute most, with 25% and 14% of variance can be explained by these two genera.

Apart from contribution of each analysed microbial taxon to the variance of microbiome composition between WT and KO mice, it is also interesting to identify taxa that could potentially benefit from the presence of α(1,2)-fucosylated glycan. LEfSe results showed that five genera including *Blautia* (p=0.041, LDA score=3.09), *Lachnospiraceae_GCA900066575* (p = 0.043, LDA score = 2.89), *Acetatifactor* (p = 0.021, LDA score = 2.84), *Ruminococcaceae_UBA1819* (p=0.011, LDA score=2.75) and *Tyzzerella* (p=0.019, LDA score=2.47) were significantly more abundant in WT mice (Figure 3.4). In line with the abundance difference, four of these five genera (*Blautia, Lachnospiraceae_GCA900066575, Tyzzerella* and *Ruminococcaceae_UBA1819*) had significantly higher detection rate in WT than KO mice after FDR correction (Table 3.1), indicating that these genera were better adapted to murine gut with the presence of α(1,2)-fucosylated glycans.

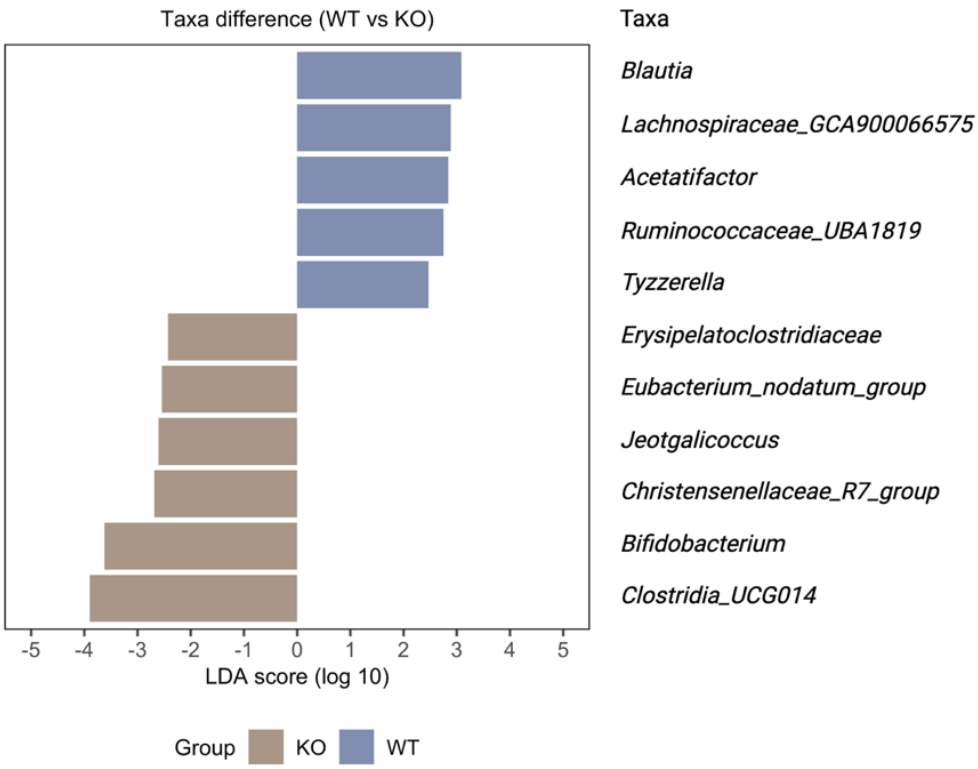


Figure 3.4 *Taxa difference between WT and KO mice*

LEfSe analysis were conducted on the microbial communities of 64 faecal samples to assess the taxa difference in gut microbial community between WT mice and KO mice. Taxa that were significantly abundant in one group than in the other were listed. Bars in blue: taxa are more abundant in WT group; Bars in brown: taxa are more abundant in KO group

Table 3.1 *Detection rate of taxa in WT and KO mice*

Group	Genus	*Fut2*$^{+/+}$ (%)	*Fut2*$^{-/-}$ (%)	P value
WT	*Blautia*	72 (23/32)	41 (13/32)	0.022
	Lachnospiraceae_GCA900066575	84 (27/32)	56 (18/32)	0.027
	Tyzzerella	28 (9/32)	6 (2/32)	0.043
	Ruminococcaceae_UBA1819	44 (14/32)	16 (5/32)	0.027
KO	*Erysipelatoclostridiaceae*	16 (5/32)	50 (16/32)	0.007

3.3.3 Interaction between sex and *Fut2* contributes to an altered gut microbiota

An increasing number of studies reported a significant impact of sex on the gut microbiome [208-211]. It is therefore possible that the sex of the mice might influence the relationship between *Fut2* and the gut microbiota. The association between sex and the gut microbiome was therefore explored, as well as the interaction between sex and *Fut2* genotype on the gut microbiome.

3.3.3.1 Effect of sex on microbiome structure (α-diversity) and composition (β-diversity)

Both Faith's phylogenetic diversity score (male vs female: 13.23 vs 12.01, p = 0.25, Appendix 3.4A) and Chao's richness score (male vs female: 143.5 vs 140.0, p = 0.76, Appendix 3.4B) were comparable between male and female mice. While no significant β-diversity differences were observed between male and female mice using Bray-curtis (p=0.072), unweighted Unifrac (p=0.22) and weighted Unifrac distance matrices (p=0.15), β-diversity in male group were significantly different with that in female group when analysed using Jaccard distance matrix (p[Jaccard] = 0.039, pseudo-F [Jaccard] = 1.29). Details can be found in Appendix 3.3. These results suggest that sex also contribute to microbial composition differences of the samples, but only in the aspects of taxa presence/absence.

3.3.3.2 Impact of Fut2 within individual sex types

Considering both sex and genotype impact gut microbial community, it is likely that the genotype effect is mainly driven by one single sex type. To further assess whether the impact of α(1,2)-fucosylated glycan on gut microbiome is mainly found within single sex type, all mice were re-classified into four groups: *Fut2*$^{+/+}$ male (WT male), *Fut2*$^{+/+}$ female (WT female), *Fut2*$^{-/-}$ male (KO male) and *Fut2*$^{-/-}$ female (KO female). The impact of α-1,2-fucosylated glycan on gut microbiome were then re-assessed within each sex type by evaluating the α-diversity, β-diversity and taxa differences. For α-diversity, Faith's and Chao's metrics were used, while for

β-diversity, Bray-Curtis was used as the primary metric and Jaccard, weighted and unweighted Unifrac distances were used as secondary metrics.

Although WT mice and KO mice achieved comparable Faith's phylogenetic diversity (p[WT male vs KO male] = 0.34; p[WT female vs KO female] = 0.12) and Chao's richness scores (p[WT male vs KO male] = 0.72; p[WT female vs KO female] = 0.11) within single sex type, the microbial compositions (Bray-Curtis distance matrix) were significantly different between WT male and KO male groups (p = 0.021, pseudo-F = 2.38, Figure 3.5A); however, such significant variation was not seen in female group (p = 0.38, pseudo-F = 2.38, Figure 3.5A), suggesting the microbiome composition difference between WT and KO was only found within male mice. In line with results using Bray-Curtis distance matrix, the composition variations between WT and KO group within female mice were small when using other distance matrixes (p > 0.05). The composition variance between WT and KO in male mice, on the other hand, are also small when assessed using Jaccard and Weighted Unifrac distance matrixes (p[Jaccard]= 0.30, pseudo-F[Jaccard] = 1.22; p[Weighted Unifrac]= 0.19, pseudo-F[Weighted Unifrac] = 1.62), suggesting that the composition differences between WT male and KO male mice were only seen in aspects of abundance of shared taxa but not presence/absence of unshared taxa. Given that the Weighted Unifrac distance matrix take phylogeny into consideration, the small variation indicates that the taxa that shared between WT male and KO male mice are likely from similar phylogeny.

Given the composition difference between WT and KO group is more pronounced in male mice, LEfSe analysis was employed to identify the taxa of which abundance are significantly different between WT male and KO male. It was shown that three genera were significantly more abundant in WT male mice while 13 genera were significantly more abundant in KO male mice (Figure 3.5B). More interestingly, of those three genera that were more abundant in WT male mice, *Lactobacillus* was the one which contribute most (25%) to the total variance between WT and KO mice based on SIMPER analysis, which has been described in previous

Chapter (Section 3.3.2.3). This result suggests that genus *Lactobacillus* is also likely to benefit from the presence of α(1,2)-fucosylated glycans in the gut environment.

Taken together, the α-diversity of faecal microbiome between WT and KO mice within each sex type were comparable; however, the composition difference between WT and KO mice was only found in male group but not female group, suggesting the covariate sex type also impact on gut microbiome. The altered microbiome composition difference between WT male and KO male was attributed to the abundance of shared taxa and these shared taxa were phylogenetically related.

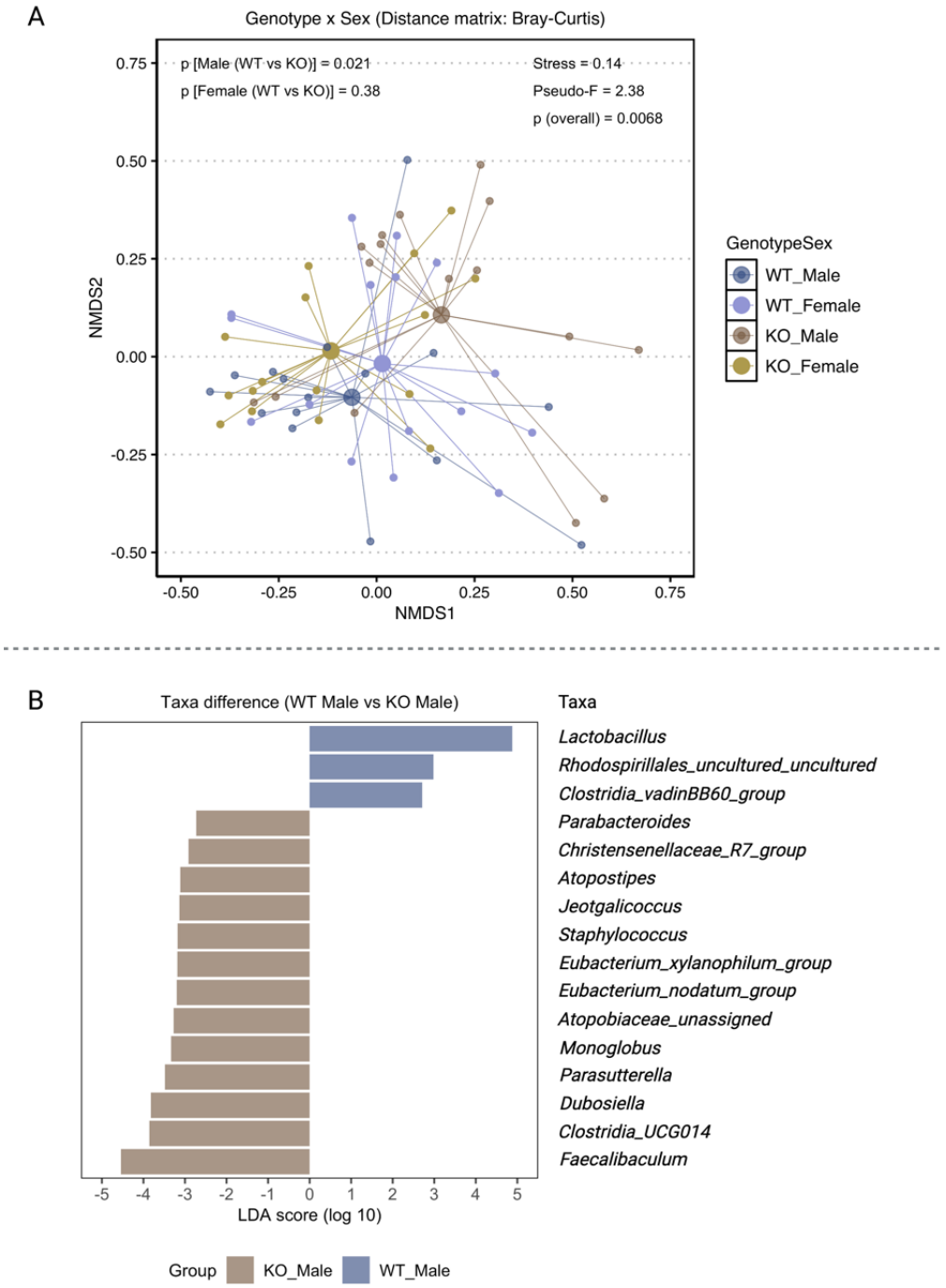


Figure 3.5 *Gut microbiome difference and taxa difference between WT and KO in male mice*

To assess the impact of *Fut2* on microbiome composition and taxa difference within individual sex types, the microbial communities were compared by conducting PERMANOVA test and LEfSe analysis according to genotype and sex.

A. NMDS plot on microbiome composition
B. Comparison of taxa abundance

3.4 Discussion

While many murine studies have reported the impact of *Fut2* gene on gut microbiome [94,96,212], no studies have taken both sex (mouse and human study) and cage effect (mouse study) into consideration while analysing the results, which might lead to biased conclusion. This chapter serves as a confirmation study, aiming to validate the previous findings and assess the possibility of synergistic effects of genotype and sex types by taking cage effect into considerations.

I firstly characterised the taxa in murine gut microbial community. Of the seven genera detected across all faecal samples, *Bacteroides* is known to be capable of degrading and utilizing *Fut2* related α(1,2)-fucosylated glycan via the production of α-1,2-L-fucosidase. However, different species displayed different capability of glycan utilization, not all species within one genus produce the α-1,2-L-fucosidase. The identity of taxa at species level in faecal samples (especially *Bacteroides*) deserved to be explored (Chapter 5).

The assessment of faecal microbiota between *Fut2*$^{+/+}$ (WT) and *Fut2*$^{-/-}$ (KO) mice displayed the influence of *Fut2* on microbiome structure and composition. Although the Faith's phylogenetic diversity score and Chao's richness scores representing alpha diversity are comparable between WT and KO mice, the composition are significantly different regardless of taxa abundance or presence/absence. Besides, gut microbes that are not shared between WT and KO mice were phylogenetically different, contributing to composition difference in aspect of presence/absence. On the other hand, most gut microbes that are shared between WT and KO mice are phylogenetically related and their abundance differences contribute to overall microbiome composition difference in respect of taxa abundance. Other than *Fut2*, sex effect on gut microbiome was also assessed despite its insignificant impact. However, stratification according to sex identified a greater genotype divergence in male mice ($p=0.021$) compared to female mice ($p=0.38$). Exploration of this sex effect identified a significant interaction between sex and *Fut2* genotype ($p=0.0068$). These results together indicate a

relatively strong impact of *Fut2* on microbiome composition, and the impact is more pronounced in male mice. The observed differences in microbiome composition between male and female mice could potentially be attributed to the influence of sex hormones on the gut microbiota. Sex hormones, such as estrogen and testosterone, have been shown to modulate the gut environment and microbial composition[209,211,213,214] To explore this further, future studies could involve hormone manipulation experiments, where male and female mice are subjected to hormonal treatments to alter their hormone levels. This could help elucidate the direct effects of sex hormones on the gut microbiome. Additionally, investigating the role of specific microbial metabolites, such as those produced by gut bacteria in response to sex hormone fluctuations, may provide insights into the mechanisms underlying sex differences in microbiome composition. Moreover, longitudinal studies tracking changes in microbiome composition throughout different stages of the reproductive cycle could shed light on the dynamic interplay between sex hormones and the gut microbiome. By employing these approaches, a deeper understanding of the underlying mechanisms driving sex-specific differences in microbiome composition can be attained. Taken together, these results have confirmed my first hypothesis and are line with other studies [94,96,212].

On top of that, taxa within the gut microbial community of each genotype were also assessed. The SIMPER analysis highlighted that *Lactobacillus* and *Faecalibaculum* contribute most to the inter-group variance in microbiome composition. Of all taxa detected in mice, five taxa (*Blautia*, *Lachnospiraceae* GCA900066575, *Acetatifactor*, *Ruminococcaceae* UBA1819 and *Tyzzerella*) are significantly more abundant in WT mice and four of them (Blautia, *Lachnospiraceae* GCA900066575, *Tyzzerella* and *Ruminococcaceae* UBA1819) also had significantly higher detection rate in WT. Given that the influence of *Fut2* is more pronounced in male mice, taxa comparison between WT and KO group in male mice were also conducted and results identified that three taxa are more abundant in WT male than KO male mice. These results together indicate that these genera were better adapted to murine gut with the presence of α(1,2)-fucosylated glycans. More interestingly, of taxa that are found more

abundant in WT mice or in WT male mice, *Lactobacillus* and *Blautia* have the glycan utilization potentials, which have been reported in other studies [202,215].

In summary, we found *Fut2* not only impact the microbiome composition but also impact the abundance of certain gut microbes in murine gut. Despite the indirect evidence, this is an interesting finding that can provide preliminary support for the impact of *Fut2* on the colonization of gut microbes. Thus, in the next chapter, an intervention mouse model was developed to validate the findings here.

CHAPTER 4. THE IMPACT OF α(1,2)-FUCOSYLATED GLYCANS ON BACTERIAL COLONISATION

4.1 Introduction

In the previous chapter, differences in the abundance and detection of certain gut microbes were observed between *Fut2*$^{+/+}$ (WT) and *Fut2*$^{-/-}$ (KO) mice. These microbes consisted of species known to directly utilise α(1,2)-fucosylated glycans (e.g., *Lactobacillus*) as well as species without known α(1,2)-fucosylated glycan utilisation capability (e.g. *Rhodospirillales spp.*). The combination of both glycan utilisers and not utilisers highlight the complex dynamics *Fut2* plays across multiple ecological niches, with some being a result of direct glycan utilisation and some being an indirect result.

In this chapter, I will further explore the direct role *Fut2* plays in creating a bacterial niche in the gut. Specifically, I will assess how *Fut2* impacts the ability of bacteria to colonise and persist within the GI tract. To do this, I have selected bacterial strains that can directly utilise α(1,2)-fucosylated glycans. Direct utilisation of α(1,2)-fucosylated glycans is tested using the *Bifidobacterium bifidum* strain JCM1255 and *Bifidobacterium infantis* strain JCM1222, two strains known to encode the α(1,2)-L-fucosidase genes AfcA and Blon_2335 respectively. These fucosidases catalyses the α-1,2 linkage between L-fucose and β-D-galactose (Gal), allowing the cleaved L-fucose (a six-carbon sugar) to be utilised as a carbon source.

In addition to enabling the assessment of *Fut2* on bacterial colonisation capability, *Bifidobacterium* species are widely marketed as beneficial commensal species. As detailed in Chapter 1, numerous diseases, including obesity, diabetes and atopy are positively associated with a decrease in the abundance of *Bifidobacterium* at different stages of life [216-223]. For example, obesity individuals have lower counts of *Bifidobacterium* than lean people [216-218]. Studies that have investigated the capacity for *Bifidobacterium* spp. to engraft the GI tract

have identified variability in the population [224], however have not determined the contribution of *Fut2* to *Bifidobacterium* persistence. Thus, in this chapter, I hypothesized a non-functional *Fut2* gene limits the ability of *Bifidobacterium* to colonise and aimed to model this in mice. Besides, microbiome features have been found to influence bacterial colonisation persistence[161]. To minimize the influence of indigenous microbiome on *Bifidobacterium* persistence, mice were pre-exposed to antibiotics. The results of this study can confirm the influence of host genetics on gut bacterial colonization and also can inform how probiotics persist in people and whether some people have reduced ability for probiotic colonisation in the clinical perspective.

4.2 Methods and materials

4.2.1 Selection of antibiotics

Ampicillin and neomycin were selected as the antibiotic to administer to the mice as they have broad spectrum activity and are clinically relevant antibiotics often prescribed in the clinical setting for treatment of many common bacterial infections.

Previous studies (including ethic no. SAM151 and SAM218) have shown that the combination of ampicillin and neomycin affects nearly all bacterial species [192]. Preliminary data in this study showed that the total bacterial load dropped to undetected level after 7 days of antibiotics (1g/L ampicillin and 0.5 g/L neomycin in drinking water) treatment. Thus, for the purpose of studying post-antibiotic alterations to probiotic colonization and bacterial composition, ampicillin and neomycin is therefore an optimal antibiotic of choice. Ampicillin and neomycin were administered at a dose used in previous studies (1g/L ampicillin and 0.5 g/L neomycin in drinking water), as per standard operating procedure at SAHMRI (SOP-0296), which was expected to alter the gut microbiome composition of the mice within the first few days of administration. Mice received drinking water containing antibiotics for 7 days and were then switched to regular water for the remaining duration of the experiment. Water bottles were weighed at the beginning of the experiment to ensure water is being consumed.

4.2.2 *In vitro* glycan utilisation assay by probiotic strains

To assess the influence of α(1,2)-fucosylated glycans on gut bacterial colonisation, three *Bifidobacterium* strains were carefully selected and employed in the experiments (selection criteria in Chapter 3). These three *Bifidobacterium* strains are *B. bifidum* JCM 1255 (=ATCC 29521), *B. longum subspecies infantis* JCM 1222 (ATCC 15697), *B. breve* JCM 1192 (=ATCC 15700). All three strains were obtained from the Japan Collection of Microorganisms (JCM; RIKEN, Saitama, Japan).

To assess the glycan utilization properties of these three *Bifidobacterium* strains, the α(1,2)-fucosylated glycan utilization of each of these three strains was assessed through *in vitro* growth assays (see Figure 4.2 in Chapter 4). Briefly, the strains were initially cultured in MRS-CS media and subsequently diluted to an optical density of 1 at 600 nm (OD_{600}). Subsequently, 100 µL of each bacterial suspension was inoculated into 9.5 mL of minimal media (mBasal broth, detailed ingredients provided in Section 2.4.10), with or without supplementation of α(1,2)-fucosylated glycans, specifically 2'-Fucosyllactose (5% w/v, purity: 98%, Layer Origin, Ithaca, New York, USA). The cultures were then incubated for 72 hours. The bacterial biomass of each strain under each culturing condition was quantified by measuring the optical density at 600 nm using a multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA).

4.2.3 Experimental mice preparation

To avoid the microbiome drift due to vertical transmission, heterozygote breeding was performed by SAHMRI Bioresources staff as per normal breeding procedures. This led to approximately ¼ *Fut2* $^{+/+}$ and ¼ *Fut2* $^{-/-}$ offspring. The remaining ½ *Fut2* $^{+/-}$ mice were either humanely killed or used for future breeding. These mice cannot be used experimentally because *Fut2*$^{+/-}$ mice have been shown to have an intermediate phenotype between *Fut2* $^{+/+}$ and *Fut2* $^{-/-}$ which will confound any results.

All mice were kept at a strict 24 hr light-dark cycle, with lights on from 7 am to 7 pm and had access to food and water ad libitum were housed in individually ventilated cages containing nesting material and with absorbent bedding as per standard SAHMRI bioresources husbandry practices. At the conclusion of the experiment, all mice will be humanely killed as per SOP-0491 and SOP-0501 and dissected for tissue collection for further analyses.

4.2.4 Experimental design

Mice were genotyped at weaning as per standard breeding practices. Experimental mice with different genotypes were homogenized around 4 weeks before the start of the experiment. At 6 weeks of age, they were randomly allocated to 64 cages containing ~ 2 mice each cage for each genotype and sex type. A total of 128 experimental mice (64 *Fut2* $^{+/+}$ and 64 *Fut2*$^{-/-}$ mice with equal male and female numbers) were used in this study based on power calculation performed using data from three references[161,225,226].

These mice were randomly assigned to four treatment groups (n = 32/treatment group) as followed (Figure 4.1 and Table 4.1):

1) Antibiotic + Infantis group: *B. infantis* after antibiotic treatment
2) Antibiotic + Bifidum group: *B. bifidum* after antibiotic treatment
3) Antibiotic + Breve group: *B. breve* after antibiotic treatment
4) Infantis group: *B. infantis*

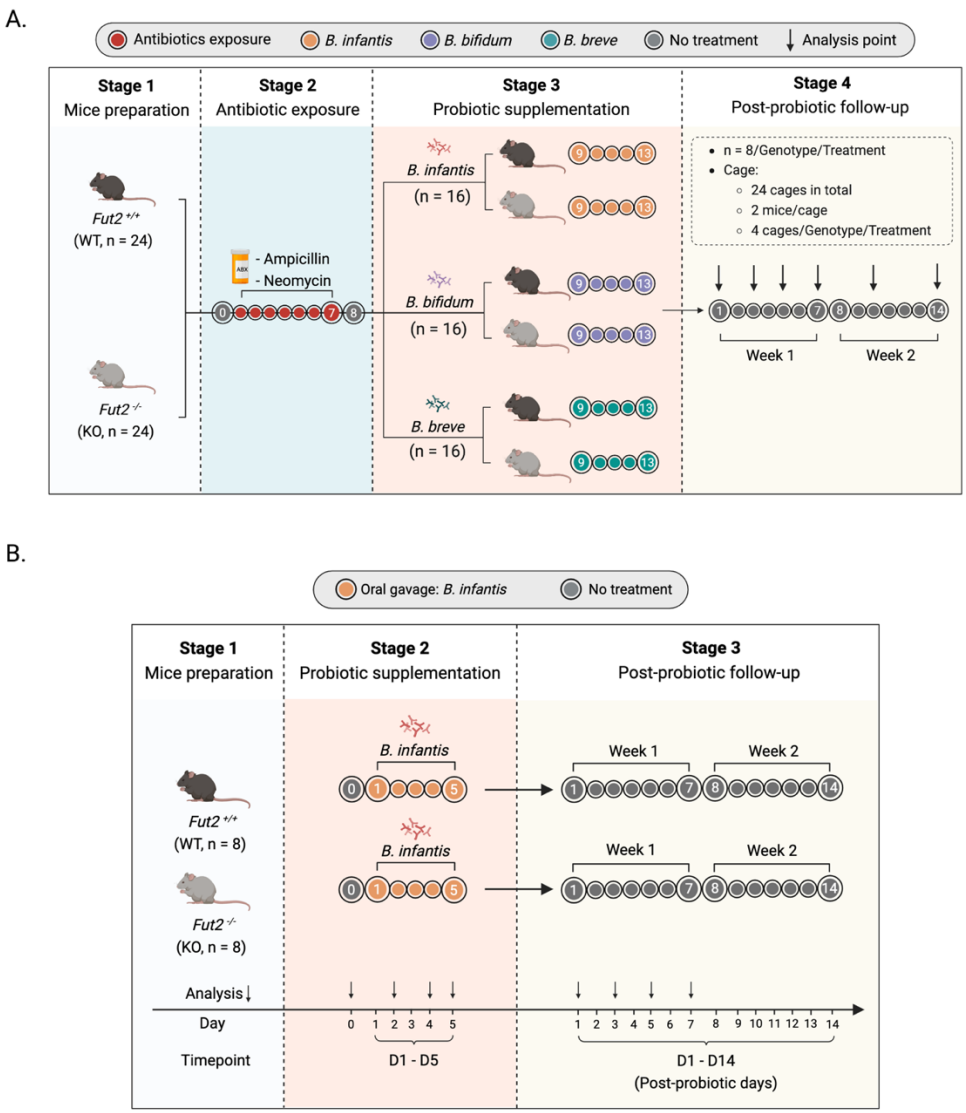


Figure 4.1 *Experiment design in Chapter 4*

The summary of experiment design was visualized on single sex type

A. Antibiotic + probiotic supplementation groups: Mice received the supplementation of three different probiotic strains for 5 days (three groups, each received one strain) after 7 days of antibiotic exposure
B. Probiotic supplementation group: Mice received the supplementation of *B. infantis* only for 5 days

Table 4.1 *Group arrangement in mice model*

Experimental group	Group information
Group 1. *B. infantis* after antibiotic treatment (Abx + Infantis, n = 32)	1) The ratio of male : female is 1:1 2) The ratio of *Fut2* $^{+/+}$: *Fut2* $^{-/-}$ is 1:1 3) 2 mice/genotype x sex type 4) Mice will receive ampicillin and neomycin dissolved at 1g/L and 0.5g/L respectively in their drinking water (according to SOP-0296) and will receive this water ad libitum for 7 days. This dose has been calculated to equal 200 mg/kg per 24 hours per mouse which is a low to average antibiotic dose based off previous ampicillin and neomycin mice experiments. This method is routinely performed in mouse husbandry to maintain the sterility of the water and does not affect water consumption. Water bottles will be weighed at the beginning of the experiment to ensure water is being consumed. After 7 days, ABX water will be swapped for normal autoclaved water which will be supplied ad libitum throughout the remaining duration of the experiment. Mice will then receive this probiotic at 5 X 10^{10} bacteria per kg (around 1 X 10^{9} bacteria per mouse as suggested in the literature) by oral gavage daily for 5 days. This dosage was used based on previous literature, and is a similar dose recommended for humans.
Group 2. *B. bifidium* after antibiotic treatment (Abx + Bifidum, n = 32)	1) The ratio of male : female is 1:1 2) The ratio of *Fut2* $^{+/+}$: *Fut2* $^{-/-}$ is 1:1 3) ~2 mice/genotype x sex type 4) Mice will receive the same treatment as Group 1 however with different probiotic bacteria.
Group 3. *B. breve* after antibiotic treatment (Abx + Breve, n = 32)	1) The ratio of male : female is 1:1 2) The ratio of *Fut2* $^{+/+}$: *Fut2* $^{-/-}$ is 1:1 3) 2 mice/genotype x sex type 4) Mice will receive the same treatment as Group 1 however with different probiotic bacteria.
Group 4. *B. infantis* (Infantis, n = 32)	1) The ratio of male : female is 1:1 2) The ratio of *Fut2* $^{+/+}$: *Fut2* $^{-/-}$ is 1:1 3) 2 mice/genotype x sex type 4) Mice will then receive this probiotic at 5 X 10^10 bacteria per kg (around 1 X 10^9 bacteria per mouse as suggested in the literature) by oral gavage daily for 5 days. This dosage was used based on previous literature, and is a similar dose recommended for humans.

4.2.5 Sample collection

Faecal samples were collected in 1.5 mL sterile tubes at pre-determined timepoints (Figure 4.1) using sterile toothpicks. After collection, these faecal samples were immediately frozen using dry ice and and stored at -80°C for future analysis. A total of 3328 faecal samples were collected. Specifically, faeces from three groups of mice (n = 32/group/collection timepoint, Antibiotics + Probiotics groups) were collected for 28 days and faeces from one group of mice (n = 32/collection timepoint, Probiotics groups) were collected for 22 days. Only one faecal sample was collected per mice per day. Of all faeces collected, 1376 faecal samples were analysed according to preliminary analysis.

4.2.6 DNA extraction

Faecal pellets (25 ± 10 mg/sample) were weighted individually and were subjected to DNA isolation using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. Faecal supernatant was kept and stored at -80°C. The concentration of DNA was quantified using a Qubit 2.0 (Invitrogen, Carlsbad, CA, USA).

4.2.7 16S qPCR and *Bifidobacterium* species specific qPCR

Faecal DNA was subjected to SYBR Green-based qPCR assays for quantification of total bacterial load and each Bifidobacterial species (*B. breve*, *B. infantis* and *B. bifidum*). Details of primer sequences, master mix formula and cycling condition for each primer can be found in Chapter 2 (Table 2.2). The specificity and efficiency of primers for quantification of Bifidobacterial species have been validated in this study, results can be found in Chapter 2 (Figure 2.5). All qPCRs were conducted by QuantStudio 6 Real-Time PCR Systems (Thermo Fisher Scientific, Waltham, Massachusetts, US) and qPCR results were analysed by QuantStudio 6 Flex Real-Time PCR System Software v1.7.2.

4.2.8 Statistical analysis

All data analyses were performed using R (R Foundation for Statistical Computing; version 4.1.0). Parametric data was analysed using unpaired t test (for two unpaired groups); One-way ANOVA (for >2 unpaired groups); Paired t test (for longitudinal, paired data); Pearson correlation (for assessing correlations of scale data between two groups). Non-parametric data was analysed using Mann-Whitney test (for two unpaired groups); Kruskal-Wallis test (for >2 unpaired groups), Wilcoxon test (for longitudinal, paired data); Spearman correlation (for assessing correlations of scale data between two groups). Other than that, Fisher's exact test was used to assess the correlations of nominal data between two groups. Log-rank test was employed to compare survival time differences between groups.

4.2.9 Ethics

This animal study and all experimental procedures involved in this chapter were approved by the SAHMRI Animal Ethics Committee. The project number in this study is SAM-21-036.

4.3 Results

4.3.1 *In vitro* assessment of α(1,2)-fucosylated glycans by probiotic bacteria

As previously mentioned, the experimental murine model in this chapter was designed to assess the influence of secreted α(1,2)-fucosylated glycans on gut bacterial colonization. To achieve this, it is crucial to identify gut bacteria that were not harmful to mice and were both glycan degraders and glycan utilizers. To identify the strain that displayed the capability of degrading and utilising α(1,2)-fucosylated glycans, three common *Bifidobacterium* species were compared (Table 4.2) and were then subjected to glycan degradation and utilization test *in vitro* (Figure 4.2). In addition, a further two *Lactobacillus* species were assessed.

Across the 48 hours of growth in minimal broth (mBasal) supplemented with 2′-fucosyllactose (2'-FL: a type of α(1,2)-fucosylated glycans), there was substantial variation between the five assessed strains. Both *B. infantis* and *B. bifidum* showed rapid growth (Figure 4.2A and C). In contrast, *B. breve*, *L. casei* and *L. rhamnosus* group showed lower growth in 2'-FL-supplemented media (Figure 4.2B, D, E). This confirms the α-1,2-fucosylated glycan utilisation capability of *B. infantis* and *B. bifidum,* that is absent in the other three strains. As seen in Figure 4.2, there is not much growth of *B. bifidum* during the first 24 hours of incubation. However, *B. infantis* grew fast with the help of 2'-FL within the first 12 hours of incubation.

Based on the results from glycan degradation and utilization test, *B. infantis* was selected to be included in this experimental model. Although *B. bifidum* was reported not be able to successfully colonize in murine gut after 2 weeks of antibiotic treatment [186], *B. bifidum* is the only probiotic species that produced extracellular 1,2-α-L-fucosidase, which could impact the colonization of other gut microbes via cross-feeding. Given that, it was also included as a treatment intervention in this model. Finally, given that *B. breve* was not found to utilise 2'-FL, it was included in the experimental model as a negative control.

Table 4.2 *Characterization of α(1,2)-fucosylated glycan utilization capabilities by five common probiotic species on market*

Criteria	Probiotic information				
Genus	*Bifidobacterium*			*Lactobacillus*	
Species	*B. bifidum*	*B. infantis*	*B. breve*	*L.casei*	*L. rhamnosus*
GH95: 1,2-α-L-fucosidase (Glycan degrading gene)	AfcA	Blon_2335	×	×	×
Enzyme type (1,2-α-L-fucosidase)	-Extracellular	-Cell surface-bound - Intracellular	×	×	×
Probiotic colonization in murine GIT tract (post-abx treatment)	×	✓	✓	×	×
Carbohydrate transporter	✓	✓	✓	✓	✓
References	131,227-231	131,135,190,228,232-234	186,235,236	190,215,234	190,215,234,237,238

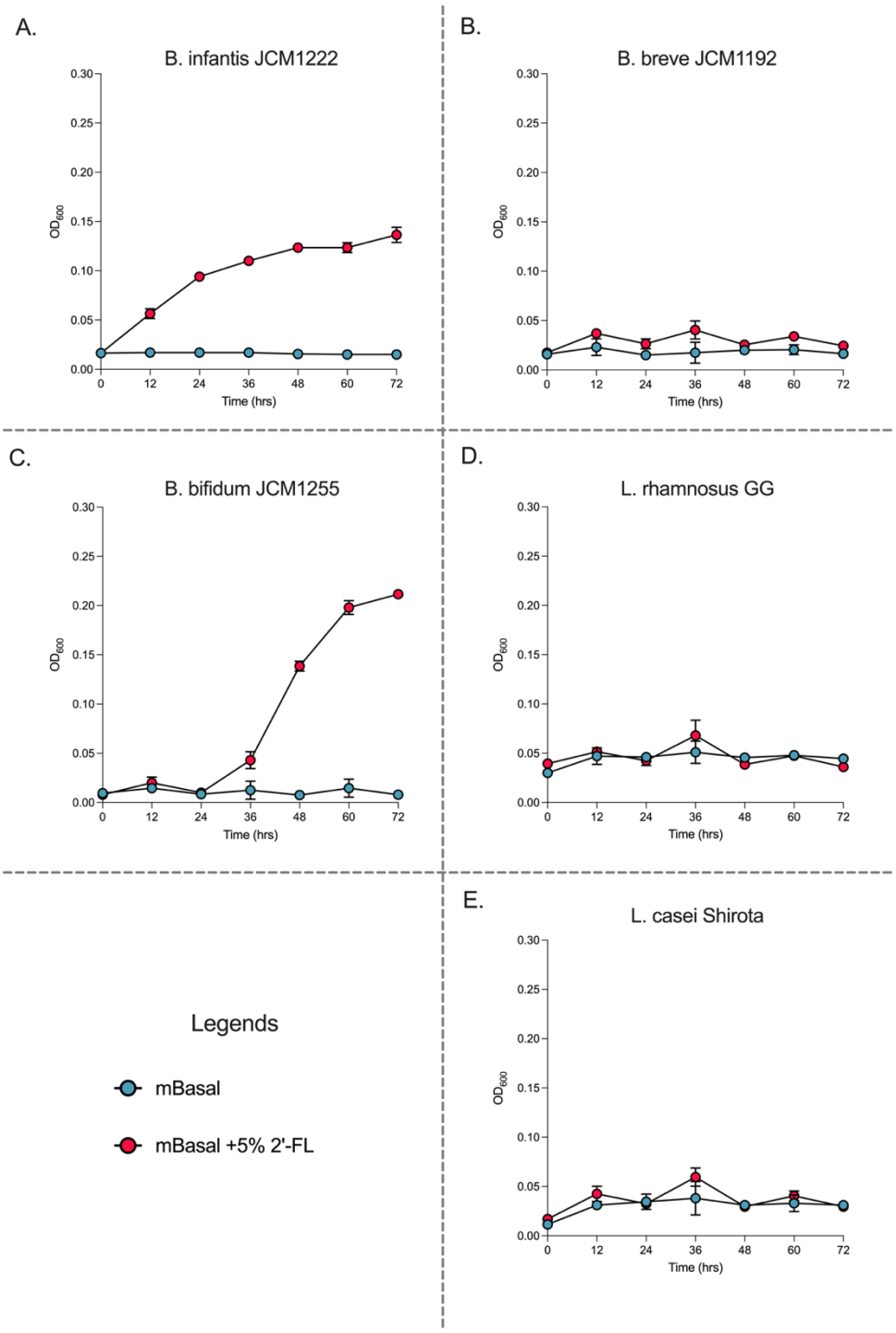


Figure 4.2 *In vitro test of α(1,2)-fucosylated glycan utilization by five probiotic strains that are commonly available on market*

The *in vitro* assessment of glycan utilization by probiotic strains were conducted with two replicates for each timepoint per treatment group. To ensure robustness and reliability, two independent experiments were conducted using different broths (mBasal broth and tryptone soy broth), yet yielded consistent results. Detailed methods see Section 2.4.2 in Chapter 2.

4.3.2 Exogenously introduced probiotic bacteria exhibit transient survival in the murine gut

To ensure the successful inoculation of these probiotic strains to mice via oral gavage, *Bifidobacterium* were freshly cultured overnight and were supplemented to mice via oral gavage and their abundance were monitored in a preliminary study where 10 mice was given *B. infantis* daily for 5 days and faeces were collected from these mice twice a day during gavage period (10 timepoints) and once per day for the follow-up period (5 days).

The temporal dynamics of *B. infantis* in stool over the course of this 5-day gavage period is presented in Figure 4.3A. This shows that, following *B. infantis* gavage into mice, it could be quantified in stool within 6 hours (Figure 4.3A). Specifically, it was detected across all mice at a similar copy number, with an average of 5.10 (SD=0.33) $\log_{10}$ copies/ng of stool DNA. However, the following collection point (24 hours after gavage) showed a rapid decline in *B. infantis* levels, dropping to 2.48 (SD=0.60) $\log_{10}$ copies/ng DNA. This trend repeated for each of the 5 consecutive gavage days, with minimal accumulation of *B. infantis*.

During the follow-up days (days post-gavage), the abundance of *B. infantis* significantly dropped as compared with the last gavage day (5.21 $\log_{10}$ copies/ng DNA vs 0.47 $\log_{10}$ copies/ng DNA, $p < 0.0001$, Figure 4.3B) while *B. infantis* can be still detected in majority of mice (9/10) at D5 post-gavage.

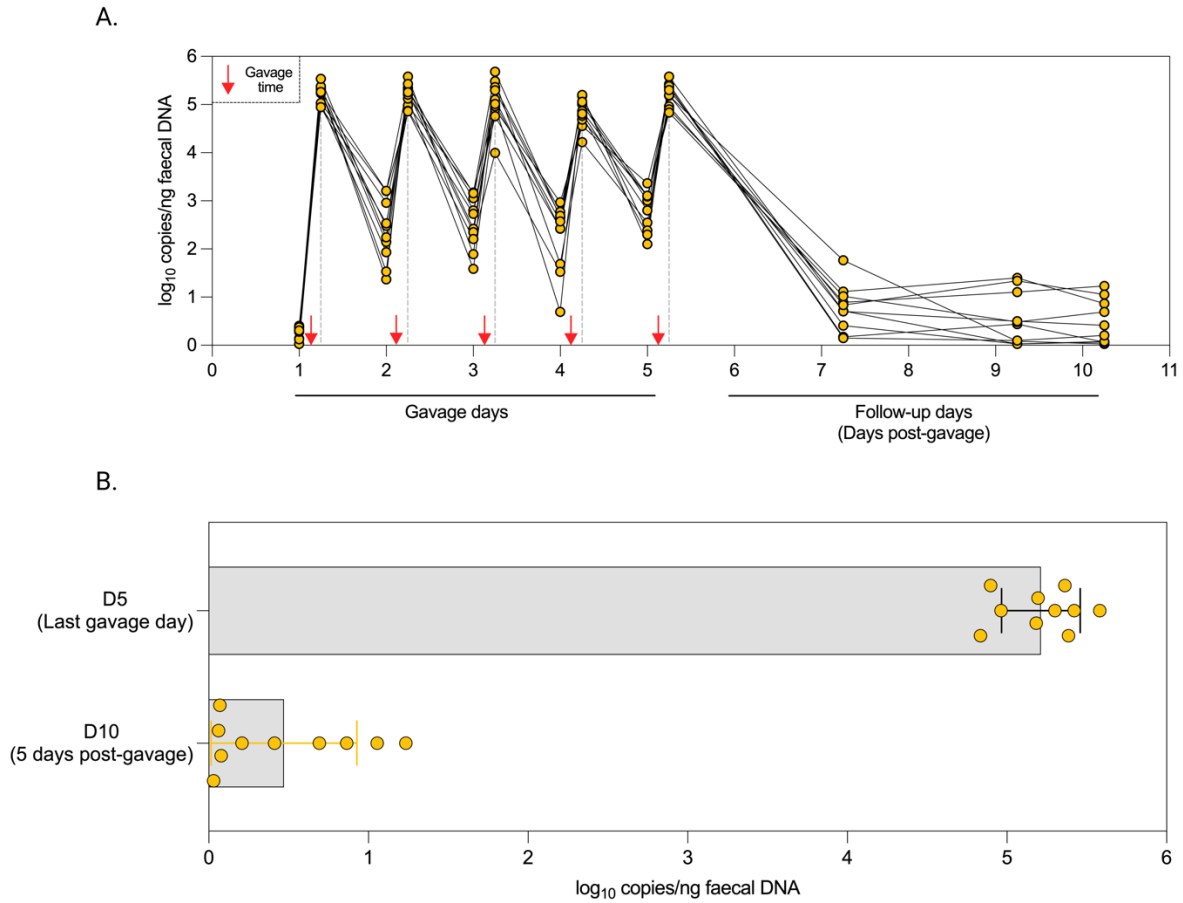


Figure 4.3 *B. infantis colonization in murine gut without antibiotics pre-treatment*

A preliminary study (n=10) on persistence and abundance of *B. infantis* in murine gut: *B. infantis* was supplemented to mice for 5 days via oral gavage and its abundance was monitored during gavage and the follow-up period (5 days after gavage). The experiment here was conducted by qPCR with three replicates for each timepoint per sample. Only independent experiment was conducted.

A. The abundance of *B. infantis* during gavage days
B. Comparison of *B. infantis* abundance in murine gut between D5 (last gavage day) and D10 (5 days post-gavage)

4.3.3 Antibiotic pre-treatment enhances probiotic colonization in the murine gut

Previous studies have reported that the gut microbiome prevents probiotic colonization, and that this colonisation resistance is lost with antibiotic pre-exposure [186]. To validate this, I assessed probiotic colonization with and without prior antibiotic exposure. To control for any effect that loss of *Fut2* has on this, WT mice were used.

After 7 days of antibiotic treatment, 72% (23/32) of mice did not carry any detectable bacteria in their faeces (Figure 4.4A). While the remaining mice (9/32) still had detectable levels of bacteria, the total bacterial load at D8 is significantly lower than that at D0 (medium abundance: 5.5 $\log_{10}$ copies/ng DNA [IQR=$\log_{10}$ 5.4, $\log_{10}$ 5.6] at D0 vs 3.65 $\log_{10}$ copies/ng DNA [IQR=$\log_{10}$ 3.3, $\log_{10}$ 3.8] at D8, p=0.0039, Figure 4.4B). These results indicate that 7 days of antibiotic treatment prior to probiotic supplement depleted the gut microbiome. These mice were then supplemented with *B. infantis* for 5 days and it was observed that the pre-exposure of antibiotics significantly improved the colonization of *B. infantis*. Specifically, the overall survival time of *B. infantis* within 7 days post-gavage in the Antibiotics + Infantis group was significantly longer than that in the Infantis group (Log-rank test, $p < 0.0001$, Figure 4.5A). This effect was most pronounced at D7 post-gavage where *B. infantis* was detected in 31/32 mice (97%) in the Antibiotics + Infantis group compared to 0/32 in the Infantis alone group. This result suggests that prior antibiotic exposure significantly enhances *B. infantis* persistence.

The abundance of *B. infantis* in the Antibiotic + Infantis group was assessed next. While detection was substantially different between the Antibitic + Infantis group and the Infantis group, both groups showed a decline in the absolute abundance of *B. infantis* (Figure 4.5). The abundance of *B. infantis* dropped quicker in Infantis group than Antibiotic + Infantis group. By D7, the mice in the Antibiotic + Infantis group still has a detectable level of *B. infantis* (median: 1.5 $\log_{10}$ copies/ng DNA [IQR=$\log_{10}$ 0.9, $\log_{10}$ 2.5]). To assess the impact of antibiotic exposure on overall *B. infantis* abundance across 7 follow-up days after gavage, areas under

the curve (AUC) were calculated for each group and were compared. AUC measurements reflected the visual trends, with the total area of Antibiotic + Infantis group being significantly greater than that of Infantis group (median area: 25.5 [IQR=24.5, 26.9] vs 3.1 [IQR=2.7, 3.5]; $p<0.0001$, Figure 4.5B).

Taken together, while probiotics only survive in murine gut for a short period of time (Section 4.3.2 in this chapter), antibiotic pre-treatment, largely enhanced both the persistence and abundance of probiotics, promoting a better adaptation of exogenous probiotics in murine gut.

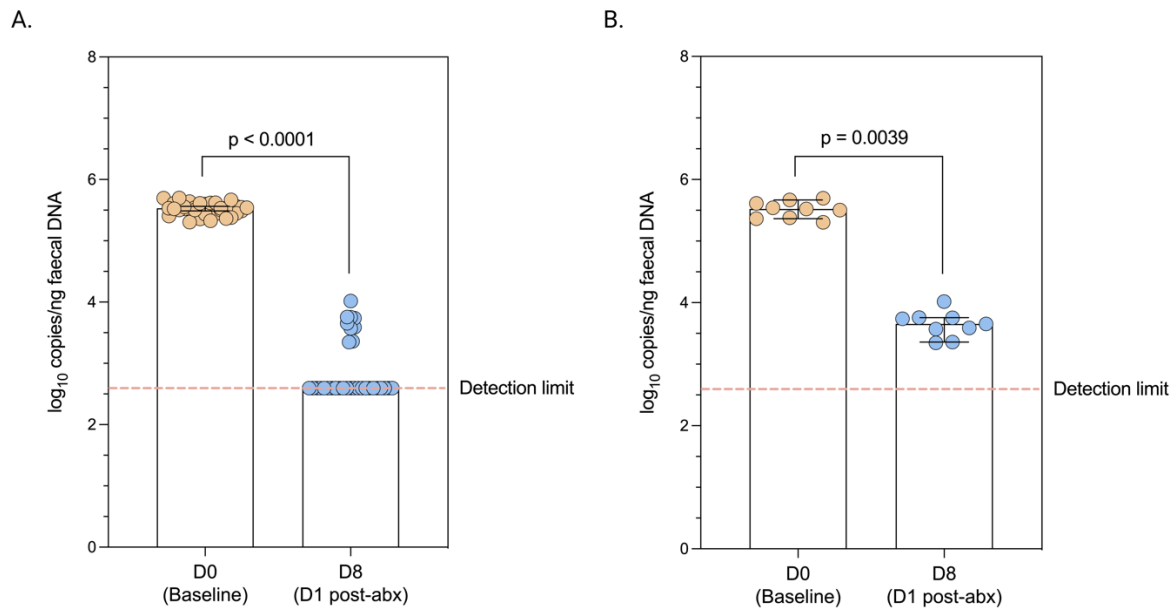


Figure 4.4 *The impact of antibiotics on total bacterial load*

Antibiotics were given to mice for 7 days .Total bacterial loads were determined by 16S qPCR and were compared between Day 0 and Day 8 (the first day after 7 days of antibiotic treatment). This figure aimed to assess the impact of antibiotics exposure on gut microbial community. The experiment here was conducted by 16S qPCR with three replicates for each timepoint per sample. Only independent experiment was conducted.

A. Total bacterial comparison between D0 and D8: all mice were included
B. Total bacterial comparison between D0 and D8: mice who still carried detectable bacteria in their faeces after antibiotic pre-exposure

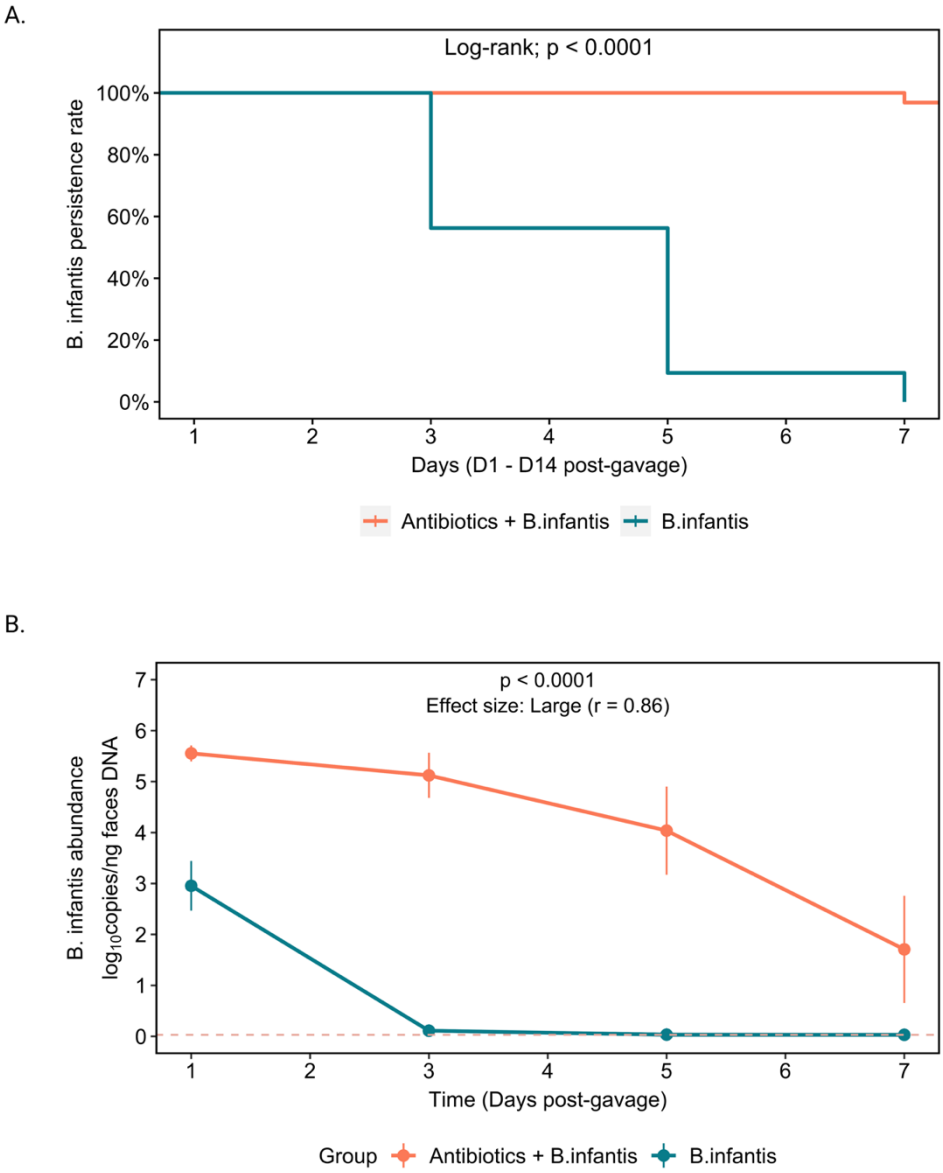


Figure 4.5 *The impact of antibiotics pre-treatment on probiotic colonization*

To assess the impact of antibiotics pre-treatment colonization, the persistence and abundance of *B. infantis* were compared between *B. infantis* group (n=32) and *antibiotic* + *B. infantis* group (n=32). The persistence and abundance of *B. infantis* here was obtained through *B. infantis* specific qPCR with three replicates for each timepoint per sample. Only independent experiment was conducted.

A. Persistence of *B. infantis* between B. infantis group and antibiotics + B. infantis group: log-rank test on persistence rate

B. Abundance of *B. infantis* between B. infantis group and antibiotics + B. infantis group: Unpaired t-test on area under the curve (AUC)

4.3.4 Post-antibiotic *Bifidobacterium* colonization is impacted by the availability of α-1,2-fucosylated glycan

In the previous section, the pre-exposure of antibiotics was found to be able to enhance the *B. infantis* colonization in mice gut, which related to the depletion of their indigenous microbiome. Given that three strains that we employed in this study all belong to *Bifidobacterium* genus, it is likely that this impact could also happen to other two *Bifidobacterium* strains, which have been tested by Suez *et al*. [186]. To evaluate the impact of *Fut2* on *Bifidobacterium* colonization without being impacted by indigenous microbiome as Zmora *et al.* reported [161], mice were supplemented with antibiotics in their drinking water for 7 days prior to *Bifidobacterium* supplementation.

Following 5 days of gavage with 5×10^7 CFU/g/24h of either *B. infantis*, *B. bifidum* and *B. breve*, faecal samples were collected daily for 14 days to assess persistence. The survival time of each *Bifidobacterium* species were firstly compared between *Fut2* $^{+/+}$ (WT, n=16/group) and *Fut2* $^{-/-}$ (KO, n=16/group) mice. For *B. bifidum*, the detection rate across the 2 weeks following gavage was comparable between WT and KO (Log-rank test, p[*B. bifidum*: WT vs KO]=0.62, Figure 4.6A). By day 7 post-gavage, detection had dropped to under 50% (40% for WT and 20% for KO) for both genotypes. Similar with *B. bifidum*, the survival of *B. breve* across the 2 weeks following gavage was also comparable between WT and KO (Log-rank test, p[*B. breve*: WT vs KO]=0.70, Figure 4.6B). However, as compared with *B. bifidum*, *B. breve* survived longer in murine gut for both genotypes. At day 7 post-gavage, *B. breve* was still detected in all mice (32/32). As for *B. infantis*, the overall survival of *B. infantis* was significantly higher in WT mice than KO mice (p [*B. infantis*: WT vs KO] = 0.00013, Figure 4.6C) as reflected in Kaplan-Meier survival analysis. This survival time difference occurred between D7 post-gavage to D10 post-gavage with a comparable survival rate (100%, 16/16 vs 94%, 15/16, p > 0.99) found at D7 post-gavage but a different survival rate found at D10 post-gavage (88%, 14/16 vs 19%, 3/16, p = 0.0002). At the end of the follow-up period, the survival rate of *B. infantis* in both WT mice and KO mice dropped to a low level (WT mice: 6%,

1/16 vs KO mice: 0%, 0/16). Given that the α(1,2)-fucosylated glycans was only available in WT mice, the above results suggested the survival time of post-antibiotics *B. infantis* in murine gut were significantly influenced by the availability of α(1,2)-fucosylated glycans.

Overall abundance of each *Bifidobacterium* species across 2 weeks of follow-up day were assessed by calculating areas under the curves and were compared within group between WT and KO mice (Figure 4.7). Similar to survival results, while both *B. bifidum* (median area: 13.3 [IQR=9.9,17.4] vs 13.6 [IQR=11.7,14.6]; p=0.75, Figure 4.7A) and *B. breve* (median area: 26.8 [IQR=25.0,32.8] vs 29.0 [IQR=23.3,36.0]; p=0.64, Figure 4.7B) abundance were comparable between WT mice and KO mice across 2 weeks of follow-up period, the overall abundance of *B. infantis* were significantly higher in WT mice than KO mice (median area: 31.7 [IQR=29.0,32.9] vs 28.4 [IQR=25.7,30.1]; p=0.036, Figure 4.7C). However, the significant abundance difference between WT mice and KO mice was not seen in all timepoints of follow-up period. Only at D5 and D10 post-gavage, WT mice carried significantly higher level of *B. infantis* than KO mice (median abundance at D5 post-gavage: 32338 copies/ng DNA vs 9536 copies/ng DNA, p [D5: WT vs KO] = 0.047; median abundance at D10 post-gavage: 2.91 copies/ng DNA vs 0.07 copies/ng DNA, p [D10: WT vs KO] < 0.0001; Figure 4.8). Given that all experimental mice were exposed to an environment with the access to non-pathogenic bacteria, it is likely that *B. infantis* gradually lost competition advantage over colonization niches and nutrition with other gut microbes during microbiome re-establishment after antibiotic exposure, leading to the comparable *B. infantis* abundance at other timepoints after 5 days post-gavage.

Taken together, WT mice had significantly longer survival time and higher abundance of *B. infantis*; however, the colonization capability of *B. bifidum* and *B. breve* are comparable between WT mice and KO mice. These results suggest that *Fut2* impacted post-antibiotic *Bifidobacterium* colonization in a species-specific pattern.

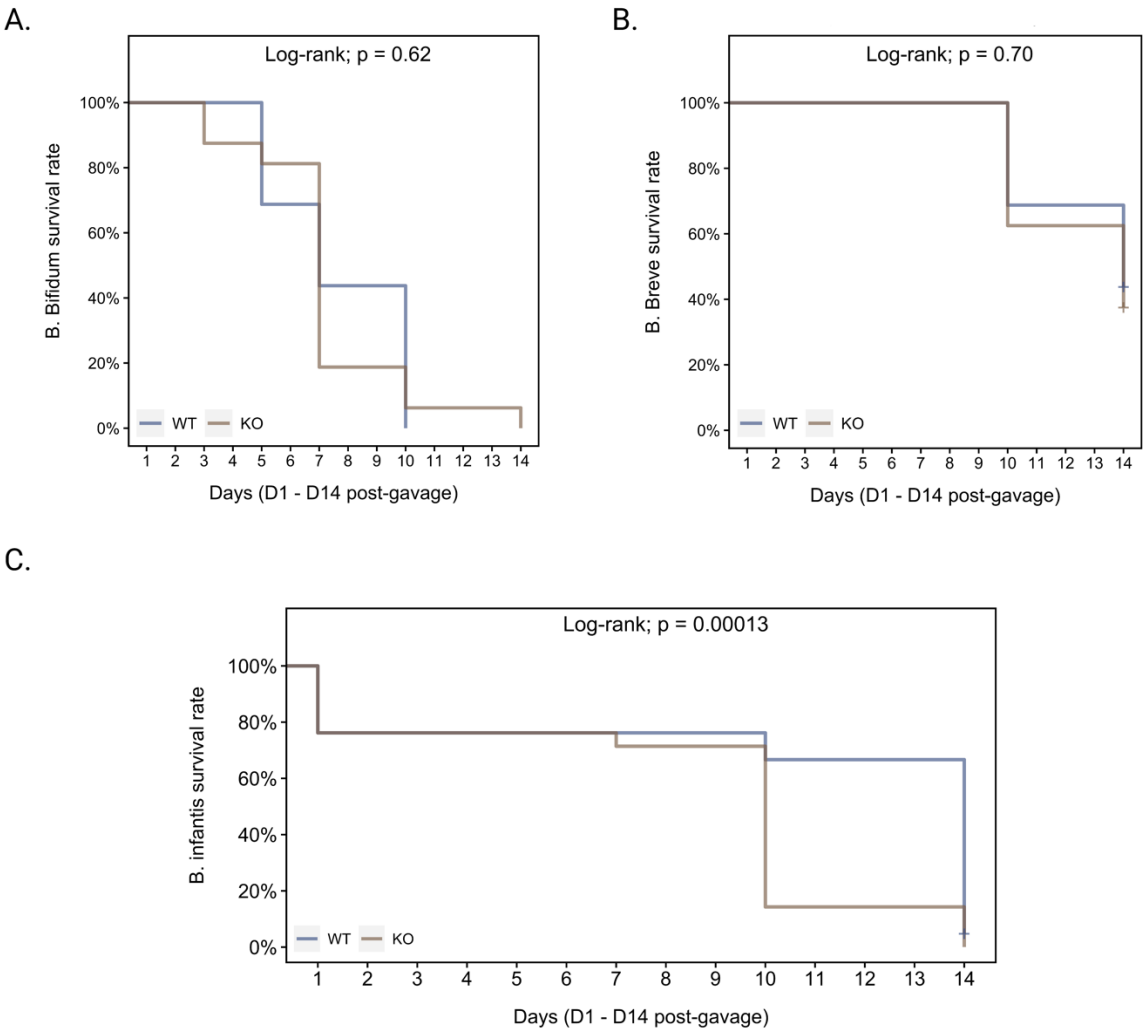


Figure 4.6 *Persistence of Bifidobacterium species in murine gut between WT mice and KO mice*

To assess the impact of availability of α-1,2-fucosylated glycan on the persistence of three *Bifidobacterium* species, WT mice (n=16/treatment) and KO mice (n=16/treatment) were supplemented with either *B. bifidum* (4.6A), or *B. breve* (4.6B) or *B. infantis* (4.6C) after being exposed to antibiotics. The survivals of three *Bifidobacterium* species were determined by species-specific qPCR with three replicates for each timepoint per sample. Only independent experiment was conducted. The survival rates of each *Bifidobacterium* species post supplementation were assessed by log-rank tests. Survival of each *Bifidobacterium* species was visualised by Kaplan-Meier curve.

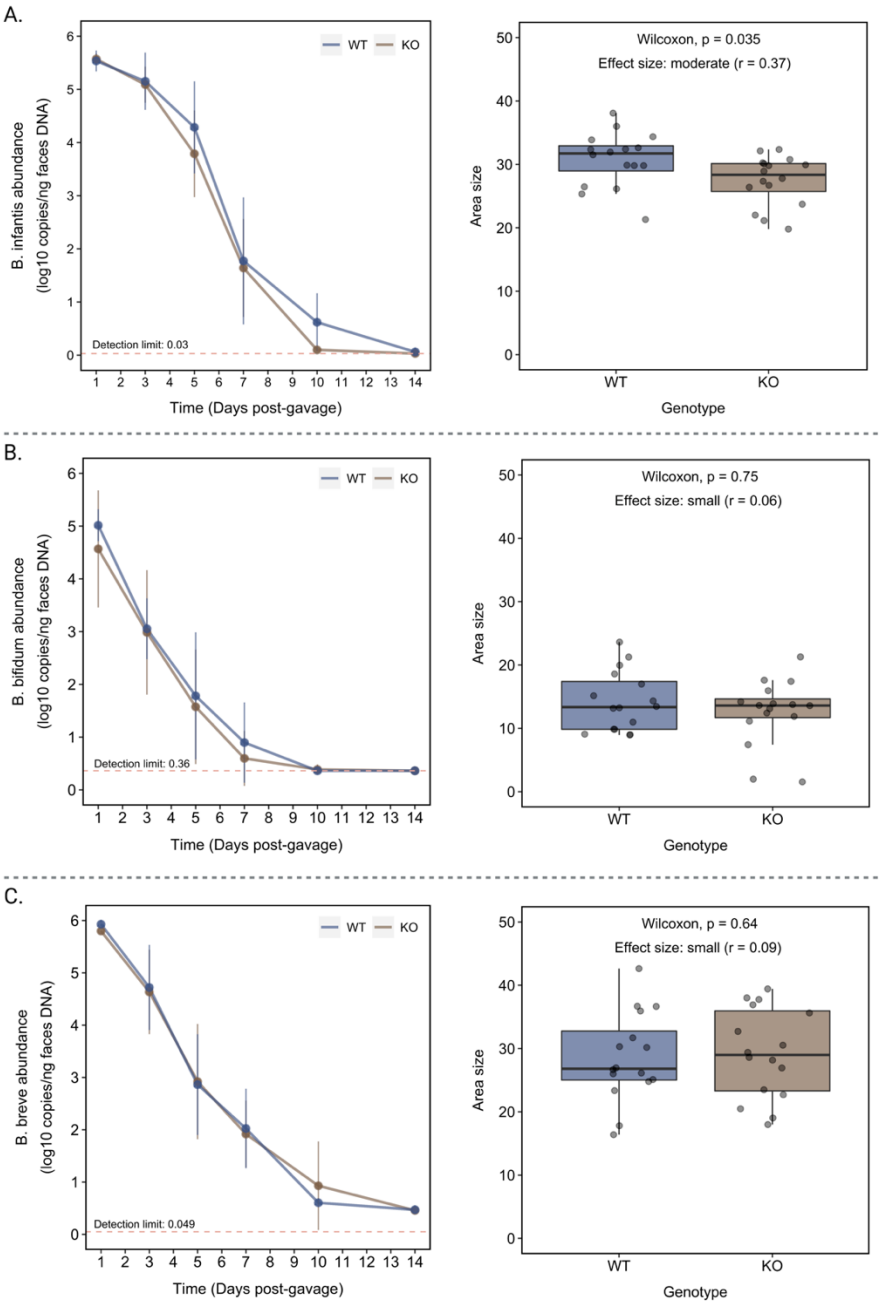


Figure 4.7 *Abundance comparison of Bifidobacterium species between WT mice and KO mice during follow-up period*

To assess the impact of availability of α-1,2-fucosylated glycan on the abundance of three *Bifidobacterium* species, WT mice (n=16/treatment) and KO mice (n=16/treatment) were supplemented with either *B. infantis* (4.7A), or *B. bifidum* (4.7B), or *B. breve* (4.7C) after being exposed to antibiotics. The abundance of three *Bifidobacterium* species were determined by species-specific qPCR with three replicates for each timepoint per sample. Only independent experiment was conducted. The abundance of each *Bifidobacterium* species post supplementation was assessed by either t-test or Mann-Whitney U test depending on the results from normality test.

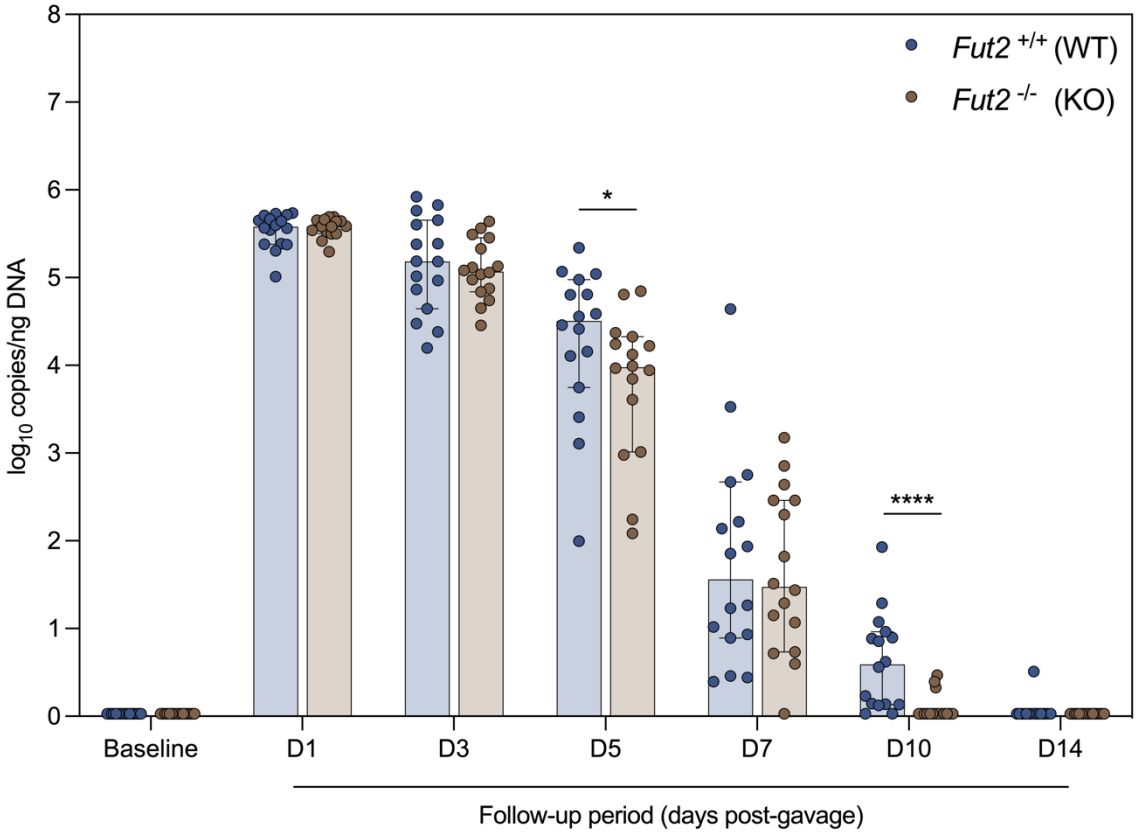


Figure 4.8 *B. infantis abundance comparison between WT mice and KO mice during follow-up period*

To assess the impact of availability of α-1,2-fucosylated glycan on the abundance of *B. infantis* during each follow-up day. The abundance of *B. infantis* species were determined by *B. infantis*-specific qPCR with three replicates for each timepoint per sample. Only independent experiment was conducted. The abundance of *B. infantis* post supplementation were compared between WT (n=16) and KO (n=16) group by either t-test or Mann-Whitney U test depending on the results from normality test.

4.3.5 Impact of other covariates on post-antibiotics probiotic colonization

4.3.5.1 Sex

As sex was found to affect the gut microbiome in Chapter 3, its effect on *Bifidobacterium spp.* persistence was also investigated. As reviewed by Francesco Valeri and Kristina Endres, links between sex and *Bifidobacterium* abundance has been previously identified [239], however not in the context of administration as a probiotic. Given that, it is possible that sex, on top of glycan variation, might also contribute to post-antibiotic *Bifidobacterium* colonization.

To assess this, the overall impact of sex on probiotic colonization was firstly explored. The colonization of three *Bifidobacterium* species were compared within each treatment group between male mice and female mice. Although the overall survival time of *B. infantis* and *B. bifidum* across all follow-up periods were comparable between male and female mice (Log-rank test, p [B. infantis: Male vs Female] = 0.47, p [B. bifidum: Male vs Female] = 0.78, Figure 4.9A and 4.9B), it is interesting and unexpected that *B. breve* had significant longer survival time in female mice than male mice (Log-rank test, p [*B. breve*: Male vs Female] = 0.011, Figure 4.9C). The survival difference between male and female occurs from D10 post-gavage with 81% (13/16) of *B. breve* found in female mice as compared with 50% (8/16) of *B. breve* found in male mice (p [D10 post-gavage: Male vs Female] = 0.14). The difference became larger at the end of follow-up days (D14 post-gavage) with 63% (10/16) of *B. breve* found in female mice as compared with 19% (3/16) of *B. breve* found in male mice (p [D14 post-gavage: Male vs Female] = 0.029), indicating a longer survival time of *B. breve* in female mice.

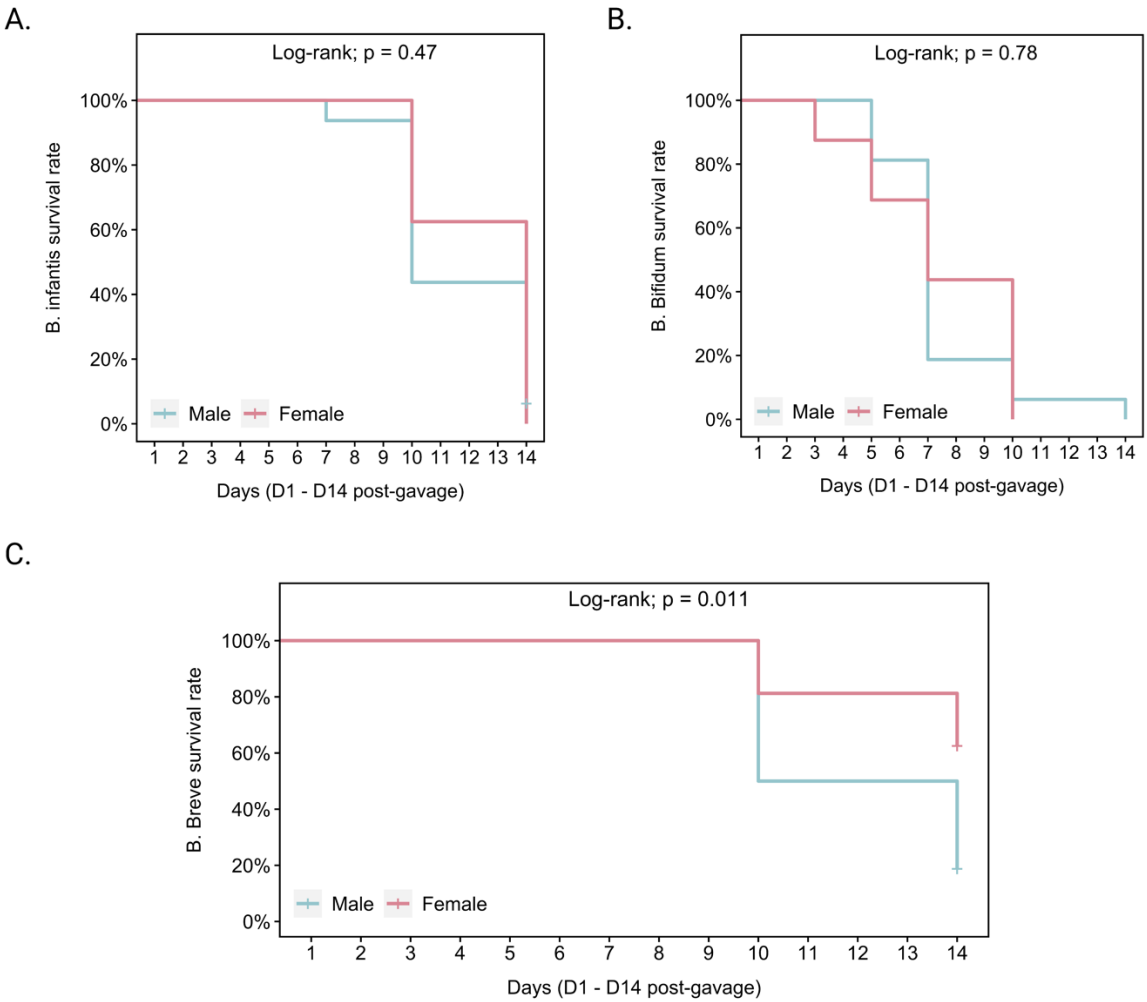


Figure 4.9 *Persistence of Bifidobacterium species between male mice and female mice*

To assess the impact of sex on the persistence of three *Bifidobacterium* species, Male mice (n=16/treatment) and Female mice (n=16/treatment) were supplemented with either *B. bifidum* (4.9A), or *B. breve* (4.9C) or *B. infantis* (4.9B) after being exposed to antibiotics. The survivals of three *Bifidobacterium* species were determined by species-specific qPCR with three replicates for each timepoint per sample. Only independent experiment was conducted. The survival rates of each *Bifidobacterium* species post supplementation were assessed by log-rank test. Survival of each *Bifidobacterium* species was visualised by Kaplan-Meier curve.

In addition to the detection of *Bifidobacterium* species, the abundance of these species over time was also compared between male and female mice. The overall abundance of *B. breve* was significantly higher in female mice than male mice (median area: 30.3 [IQR=27.7,36.7] vs 26.3 [IQR=20.1,30.9]; p=0.043, Figure 4.10C). Taking a close look at the *B. breve* abundance of each analysis day, female mice carried significantly higher levels of *B. breve* at D3 post-gavage (median abundance [Male vs Female]: 1.29 x 10^4 copies/ng DNA vs 1.16 x 10^5 copies/ng DNA, p = 0.012, Figure 4.11) and D14 post-gavage (median abundance [Male vs Female]: 0.12 copies/ng DNA vs 2.84 copies/ng DNA, p = 0.0068, Figure 4.11) than male mice. As for other two *Bifidobacterium* species, although male mice carried higher *B. bifidum* at D3 post-gavage (median: 4136 copies/ng DNA vs 896 copies/ng DNA, p = 0.012), the overall abundance of the other two *Bifidobacterium* species across the whole follow-up period were comparable between male mice and female mice (p [infantis: Male vs Female] = 0.78, p [bifidum: Male vs Female] = 0.29, Figure 4.10A and 4.10B).

Taken together, female mice had significantly longer persistence and abundance of *B. breve* than male mice; however, this colonization capability difference between male and female mice were not observed in other two *Bifidobacterium* species (*B. infantis* and *B. bifidum*). These results suggest that other than α(1,2)-fucosylated glycans, sex, as an important host genetic factor, also impacted post-antibiotic *Bifidobacterium* colonization in a species-specific pattern. Given that both survival rates and abundance of *B. infantis* are comparable between male and female, it is unlikely that the impact of α(1,2)-fucosylated glycans on *B. infantis* colonization in previous section relate to sex type.

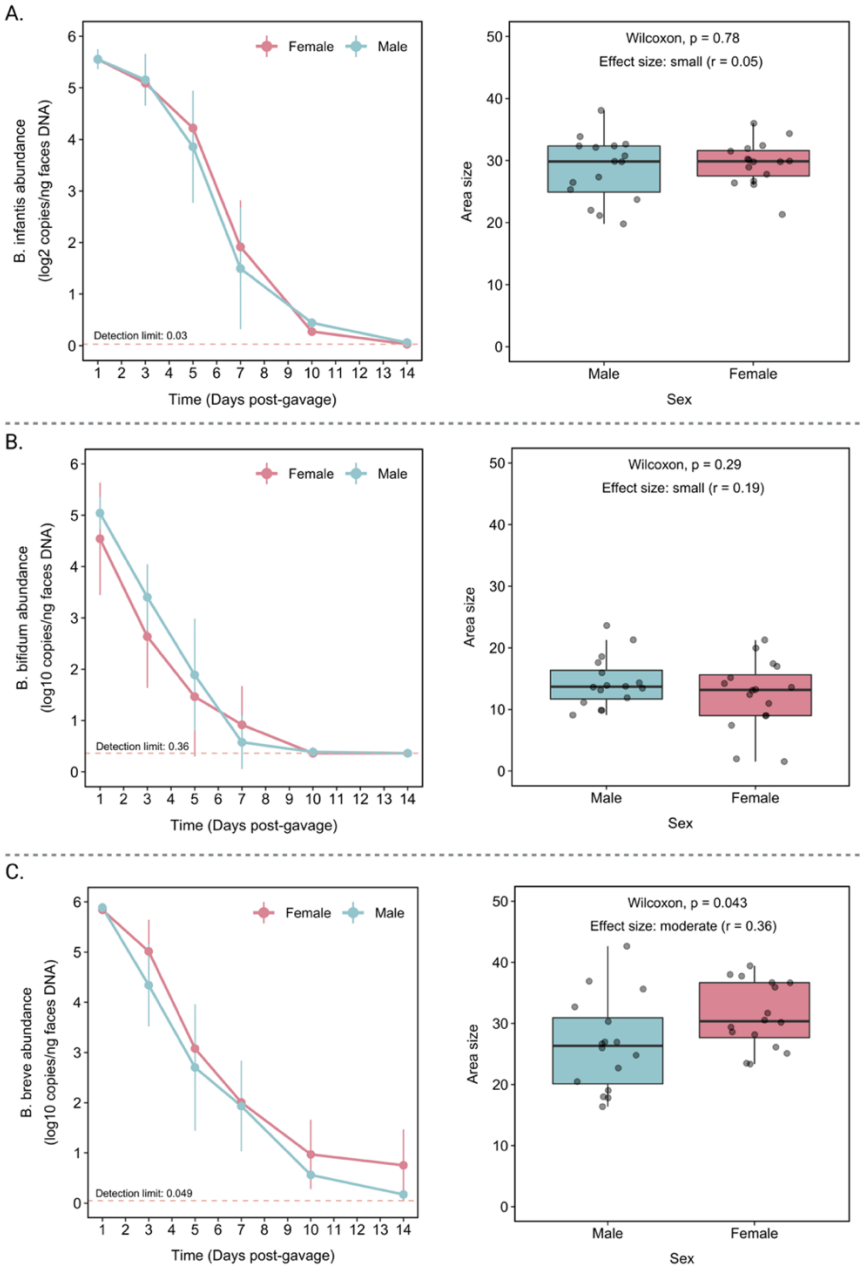


Figure 4.10 *Abundance comparison of Bifidobacterium species between male mice and female mice during follow-up period*

To assess the impact of sex on the abundance of three *Bifidobacterium* species, Male mice (n=16/treatment) and Female mice (n=16/treatment) were supplemented with either *B. infantis* (4.10A), or *B. bifidum* (4.10B), or *B. breve* (4.10C) after being exposed to antibiotics. The abundance of three *Bifidobacterium* species were determined by species-specific qPCR with three replicates for each timepoint per sample. Only independent experiment was conducted. The abundance of each *Bifidobacterium* species post supplementation was assessed by either t-test or Mann-Whitney U test depending on the results from normality test.

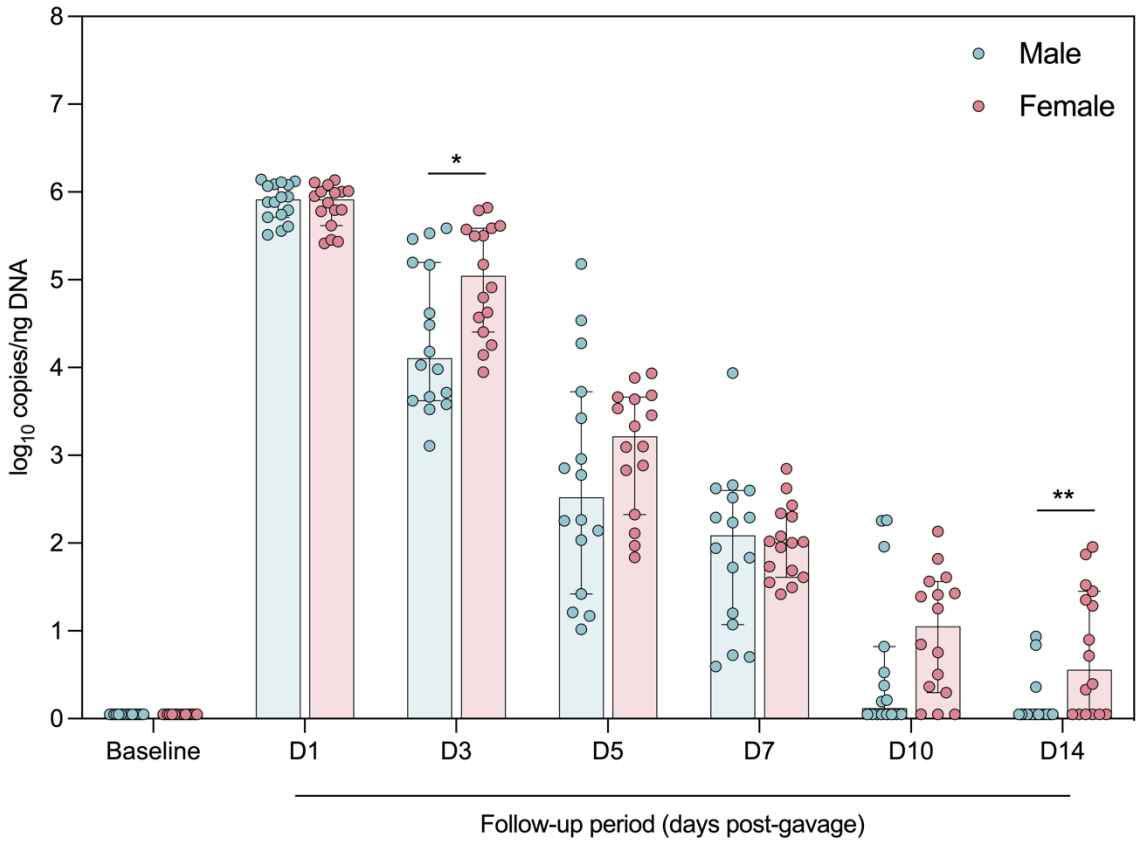


Figure 4.11 *B. breve abundance comparison between male mice and female mice during follow-up period*

To assess the impact of sex on the abundance of *B. breve* during each follow-up day. The abundance of *B. breve* was determined by *B. breve*-specific qPCR with three replicates for each timepoint per sample. Only independent experiment was conducted. The abundance of *B. breve* post supplementation was compared at each timepoint between Male (n=16) and Female (n=16) group by either t-test or Mann-Whitney U test depending on the results from normality test.

4.3.6 Cage effect

As previously discussed in Chapter 2, cage effects were reported in many studies. Due to the application of the 3Rs principle ("Replacement", "Reduction" and "Refinement"), the sample size in many studies usually is not powerful enough to assess the impact of cage effects on their results. In the previous section (Chapter 4, section 3.4), a significant contribution of α(1,2)-fucosylated glycans to post-antibiotic *B. infantis* colonization was observed. Due to the coprophagy behaviour of small animals, it is possible that one mouse was re-exposed to live *B. infantis* in the faeces from the other one in the same cage, which could impact the measurement on persistence and abundance. To minimize the impact of cage effect prior to experiment, mice in each treatment group were housed in 16 cages (8 cages/genotype/group). On top of that, cage effect on post-antibiotics *B. infantis* colonization was evaluated and adjusted to enhance the conclusion validity and repeatability of the animal study (Figure 4.12A).

The impact of cage effect on the survival of post-antibiotics *B. infantis* was firstly assessed within each cage using Fisher's exact test. In section 4.3.3, the survival difference of *B. infantis* between WT mice and KO mice occurred at D10 post-gavage (Figure 4.6C) and *B. infantis* abundance at this timepoint were closed to detection limit (Figure 4.7A), which could contribute to a biased finding. Therefore, D10 post-gavage was chosen as the key timepoint to analyse cage effect. The odds of *B. infantis* being co-carried by two mice in the same cage and the odds of *B. infantis* being carried by only one mouse in the same cage were compared at D10 post-gavage using Fisher's exact test. Results displayed a significant correlation of *B. infantis* detection between one mouse and the other mouse in the same cage ($p = 0.041$, odds ratio = 16.20, $r_{\varphi} = 0.63$, Table 4.3). Specifically, 43.8% (7/16) cages could potentially be impacted by cage effects as *B. infantis* were detected in both mice at the same cage whereas only 18.8% (3/16) of cages were not likely to be impacted by cage effects with only one mouse carried *B. infantis* in that cage. This result provided information on the likelihood of *B. infantis* co-carriage by two mice in the same cage, indicating a potential cage effect on *B. infantis* survival.

To assess whether the potential cage effect on *B. infantis* survival affect our previous conclusion, the overall survival times between WT mice and KO mice across 2 weeks of follow-up period were re-evaluated based on cage size (for each timepoint, those cages where at least one mouse carried *B. infantis* were identified as *B. infantis*-positive cages while those cages with *B. infantis* that were not detected in both mice were identified as *B. infantis*-negative cages) rather than sample size. Despite the potential cage effect on *B. infantis* survival, the overall survival time of *B. infantis* calculated based on cage size was still significantly different between WT mice and KO mice (Log-rank test, $p = 0.003$, Figure 4.12B). While a significant association between genotype and *B. infantis* survival was found in the previous section (section 3.4 of Chapter 4), the potential for cage effects confounded the strength of this association. However, this finding that *B. infantis* persistence calculated based on cage was different between WT and KO group indicates that the genotype effect that we found previously is true independent of cage effect.

Other than the survival time of post-antibiotics *B. infantis*, the impact of cage effect on *B. infantis* abundance was also assessed. Specifically, all mice were randomly assigned to two groups (Group A and Group B) using R with Group A including one mouse from each cage and Group B including the other one from the same cage (Figure 4.12A). The abundance of *B. infantis* in each mouse across all timepoints was calculated based on areas under the curves. Pearson correlation analysis was employed to assess the correlation of *B. infantis* abundance between Group A and Group B. It was found that samples in Group A and samples in Group B were not correlated regarding the abundance of *B. infantis* (p [Group A vs Group B = 0.89, $r = 0.039$, Figure 4.12C). This result indicated that no significant impact of cage effect was observed. However, this is not enough to fully validate our previous finding, a strong cage effect might only be observed in certain cages within one genotype, which contributes to our previous results. To address this, the correlation of *B. infantis* abundance between Group A and Group B was assessed within each genotype. Similarly, it was found that samples in Group A and samples in Group B within each genotype were also not correlated regarding the

abundance of *B. infantis* (p [WT: Group A vs Group B] = 0.40, r = -0.35; p [KO: Group A vs Group B] = 0.89, r = 0.039, Figure 4.12C), suggesting no obvious cage effects was observed within each genotype.

Taken together, cage effect was observed. However, the cage effect was not strong enough to impact our previous finding and conclusions.

Table 4.3 *Correlation analysis of B. infantis detection between one mouse and the other mouse in the same cage*

One mouse	The other mouse in the same cage		Odds ratio	Phi coefficient (rφ)	p value
	***B. infantis* negative**	***B. infantis* positive**			
***B. infantis* negative**	6	1	16.2	0.63	0.041
***B. infantis* positive**	2	7			

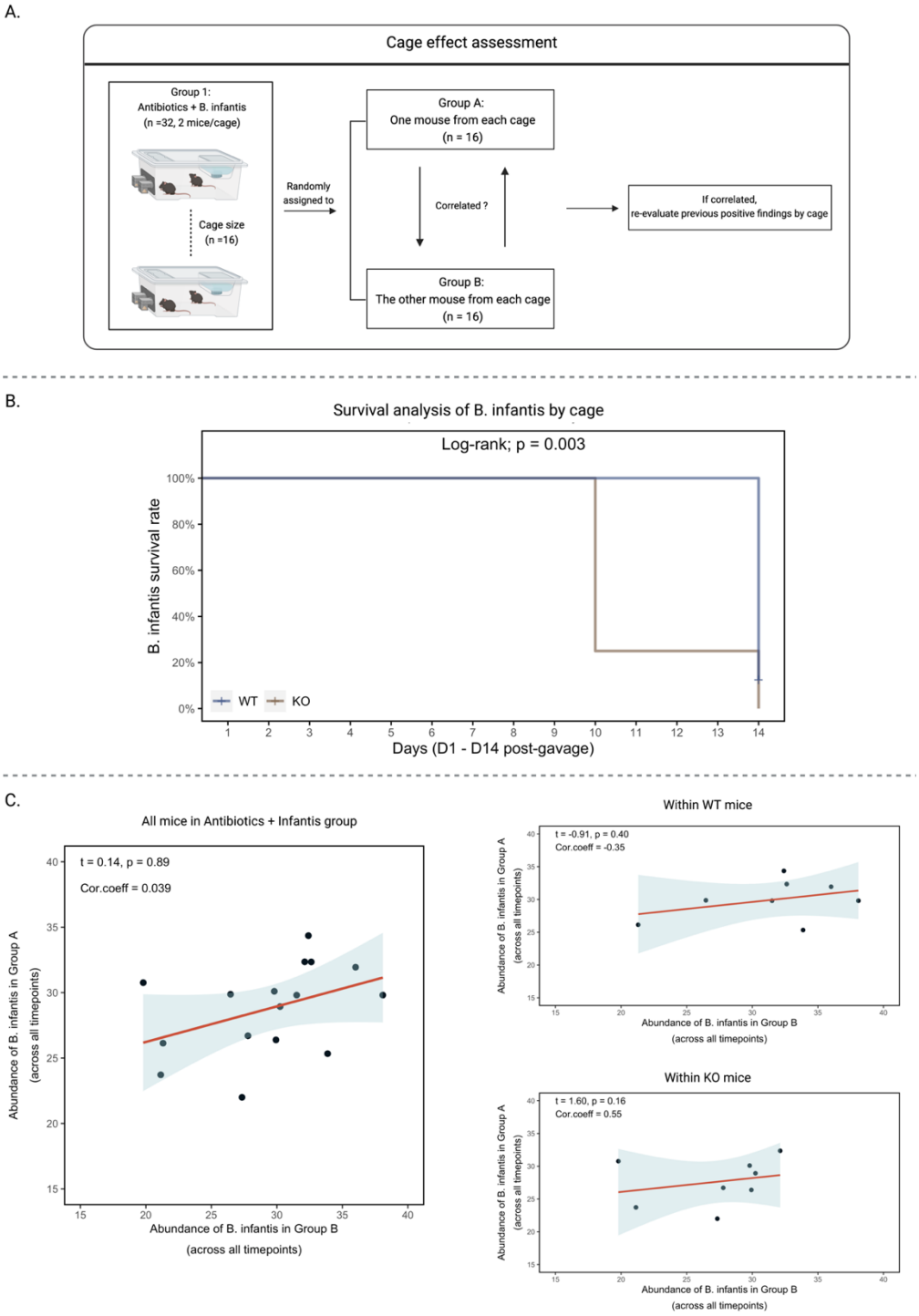


Figure 4.12 *Assessment of cage effect on previous findings*

A. Design of cage effect assessment;

B. Survival analysis of *B. infantis* by cage;

C. Association analysis of *B. infantis* abundance between one mouse from each group and the other mouse in the same group

4.4 Discussion

In the previous chapter (Chapter 3), the gut microbiome differed between *Fut2*$^{+/+}$ (WT) and *Fut2*$^{-/-}$ (KO). Studies have shown that many bacterial species that reside in the gut can utilise mucus sugars for adherence or as a source of energy[240-243]. Take *Fut2* related mucus sugar as an example, these bacterial species are capable of producing a specialized enzyme called α(1,2)-L-fucosidase, catalysing the α-1,2 linkage between L-fucose and β-D-galactose (Gal). The free L-fucose (a six-carbon sugar) are then further absorbed as energy source by these glycan degraders. Considering the glycan utilization capabilities possessed by glycan degraders, it is likely that the persistence and abundance of these gut microbes are also impacted by *Fut2*, which have been neglected by other studies. Other than *Fut2*, sex, as another important host genetic factor, also impacts the gut microbiome. Given the correlations between sex and *Bifidobacterium* abundance in human gut reported, it is also possible that sex contributes to variation of post-antibiotic *Bifidobacterium* colonization. Thus, in this chapter, I aimed to explore the influence of host genetics such as *Fut2* gene (primary focus) and sex on gut bacterial colonization. *Bifidobacterium* was employed in this mouse model given its health-related benefits and glycan utilization properties. Antibiotic was used to assess the importance of indigenous gut microbiome on *Bifidobacterium* colonization.

The persistence and abundance of *Bifidobacterium* in murine gut was firstly assessed in a preliminary study using *B. infantis*, it has been found that *B. infantis* that were supplemented to mice only survived in murine gut for a short period of time and the transient colonization of exogenous *B. infantis* only last for up to 5 days. This result suggested the persistence of exogenously introduced probiotic bacteria in the murine gut is temporary. This situation was altered when antibiotics were introduced to mice prior to *B. infantis* supplementation. Both survival rates and abundance across entire follow-up days were compared between Infantis group and Antibiotics + Infantis group, and a significantly longer survival time and higher *B. infantis* abundance were observed in Antibiotics + Infantis group. These results are in line with previous findings[161,186], indicating that exposure to antibiotics prior to probiotic

supplementation largely enhanced both the persistence and abundance of probiotics, promoting a better adaptation of exogenous probiotics in murine gut. Given this, antibiotic cocktails were given to mice in other *Bifidobacterium* species groups to dilute the indigenous-microbiome driven colonization resistance to probiotics.

The findings from this chapter highlight that *Fut2* impacts *Bifidobacterium* colonisation. Specifically, by comparing the survival time, I found the survival time of *B. infantis* in WT mice was significantly longer than that in KO mice as reflected by Kaplan-Meier survival curve and Log-rank test. Similar to survival time, the abundance of *B. infantis* across entire follow-up period was statistically higher in WT mice than in KO mice. However, these differences were not observed in Antibiotics + Bifidum group and Antibiotics + Breve group, with comparable survival rates and abundance values found between WT mice and KO mice. These results suggest that *Fut2* impacted post-antibiotic *Bifidobacterium* colonization in a species-specific pattern.

Other than *Fut2*, the impact of sex on *Bifidobacterium* colonisation was also assessed. Previous human studies have reported an association between sex and *Bifidobacterium* abundance in the gut [239]. *B. breve* was found to be better adapted in female murine gut with significantly longer persistence and higher abundance observed. However, the difference was not observed in other two treatment groups (Antibiotics + Infantis group and Antibiotics + Bifidum group), suggesting the sex impacts on colonization of gut microbes is species dependent.

To confirm the findings in this chapter are not largely impacted by the cage effect, analyses were conducted to explore the probiotic abundance and persistence correlations between two mice in the same cages. Results showed that the abundance of *B. infantis* was not correlated between mice in the same cages. However, a significant correlation of *B. infantis* detection was observed within mice in the same cages, the persistence difference was still observed between WT mice and KO mice when the survival analysis was conducted based on cage.

These results indicate that cage effect was observed. However, the cage effect was not strong enough to impact our previous conclusions.

This study provides evidence to support our assumption, in which *Fut2* gene impacts *B. infantis* colonisation. These results are likely due to glycan utilization given that *B. infantis* carried the α(1,2)-L-fucosidase gene Blon_2235 (Glycan degrading gene). Unexpectedly, the colonisation of another glycan degrader *B. bifidum* was comparable between WT and KO mice, this result could be due to the difference of the α(1,2)-L-fucosidases that produced by *B. infantis* and *B. bifidum*. Specifically, the α(1,2)-L-fucosidase encoded within *B. bifidum* is an extracellular enzyme, while that encoded within *B. infantis* in an intracellular enzyme. While both of α(1,2)-L-fucosidases would degrade the α(1,2)-L-fucosylated glycans, the L-fucose freed from the glycan by *B. bifidum* could be utilized by other gut commensal bacteria through competition with *B. bifidum*. This explanation is supported by other studies that related to cross-feeding among bacteria. The SPF mice utilized in this study were exposed to antibiotics prior to probiotic supplementation, and although testing on total bacterial load in faeces confirmed the elimination of most gut bacteria as a result of this antibiotic exposure, it is highly plausible that some gut bacteria persisted in the gut due to microbiome resilience. It has been reported that other commensal bacteria, other than the glycan degrader, are also able to glycan utilizers. *Enterococcus*, for example, can grow on mucin pre-digested with extracts from human stools, but not on purified mucin, suggesting its capability of utilizing mucus-derived products via cross-feeding[244]. Unlike *B. bifidum*, the L-fucose freed from the glycan by *B. infantis* only serves themselves within bacterial cells. This degradation differences therefore might contribute to the findings in this study; however, this need to be further validated in the future by other experiments. For instance, a germ-free model involving knockout (KO) mice supplemented with specific strains and α(1,2)-L-fucosidases could be developed, potentially providing clearer insights into the role of α(1,2)-L-fucosidases in the persistence of glycan-utilizing *Bifidobacterium*.

The study also has limitations. The bacterial strains employed in the study limited to *Bifidobacterium* only. It is worthy to validate whether *Fut2* gene would impact the colonization of other glycan degraders, such as *Akkermansia*, *Bacteroides*, and *Streptococcus* [132,133,135,228,245,246]. Besides, it is observed that the gut microbiome differed between *Fut2*$^{+/+}$ (WT) and *Fut2*$^{-/-}$ (KO) in Chapter 3. it is still unclear whether the altered microbiome is attributed to the impact of *Fut2* on glycan beneficiary commensal bacteria. These potential connection needs to be assessed in the future study.

Taken together, this chapter explored the impacts of host genetics (primarily focused on the *Fut2* impacts) on the colonization of gut microbes using an intervention mouse model. Results in this chapter confirmed our hypothesis. However, the mechanism is not explored. To better understand the potential mechanism why *Fut2* impacts the colonization of gut microbes, further analysis is required, which will be covered in the next chapter.

CHAPTER 5. THE IMPACT OF α(1,2)-FUCOSE SUPPLEMENTATION ON THE GUT MICROBIOME *IN VITRO*

5.1 Introduction

In the previous two chapters, variations in intestinal microbiology and *B. infantis* colonisation capability were found between *Fut2*$^{+/+}$ (WT) and *Fut2*$^{-/-}$ (KO) mice. These variations were presumed to be related to either direct α(1,2)-fucosylated glycan utilisation by microbes or indirect benefits from α-1,2-fucosylated glycan secretion. For example, *Blautia* (identified as increased in WT mice in Chapter 3), and *B. infantis* (in chapter 4) have known α(1,2)-fucosylated glycan degradation genes, while *Lactobacillus* (identified as increased in WT in Chapter 3) does not have genes that directly utilise α(1,2)-fucosylated glycans. However, these findings represent end-point observations in murine model between WT and KO mice, which does not serve as direct evidence to support the role of α(1,2)-fucosylated glycans.

To address this, in this chapter, I will further explore the mechanisms behind the *Fut2* effects on gut microbial community and bacterial colonization. Specifically, I will assess how α(1,2)-fucosylated glycans impact the growth of gut microbes and the structure and composition of gut microbial community. To achieve this, Chapter 5 includes a series of *in vitro* experiments conducted using faecal resuspensions from mice. To model α(1,2)-fucosylated glycans, supplementation with the glycan 2'-Fucosyllactose (2'-FL) was used.

While many studies have reported the importance of glycan on certain bacterial colonization using *in vitro* model [202,235], the bacteria in their studies are single bacterial strains that are widely employed in research area (mainly *Bacteroides* and *Bifidobacterium*) and do not represent the entire microbial community or the microbes in the gut. This study will be the first *in vitro* study to investigate the role α(1,2)-fucosylated glycans in gut microbial community and gut bacterial growth. The results in this chapter will provide direct evidence to explain why

Fut2 impacts gut microbial community and how do α(1,2)-fucosylated glycans impact bacterial colonization.

5.2 Methods and materials

5.2.1 Experimental model and design

An *in vitro* model was established in this study to explore the role of α(1,2)-fucosylated glycans on gut microbial community and bacterial colonization (Figure 5.1). Briefly, the collected baseline faeces (n = 64, 19 cages) from the previous mouse model were weighed and cultured in minimal media with and without the supplementation of 2'-Fucosyllactose (2'-FL, a type of α(1,2)-fucosylated glycans available on market). Optical density measurements were taken from faecal cultures to determine microbial growth. at different timepoints and were compared between *Fut2*$^{+/+}$ and *Fut2*$^{-/-}$ groups. To assess the impact of α(1,2)-fucosylated glycans on culturable gut microbes, a total of 20 faecal samples from 20 mice (10 mice/genotype, 1 mouse/cage) were employed. They were cultured in broth supplemented with/without 2'-FL and the extracted DNA after 48 hours culture was then subjected to 16S sequencing and analysis. Taxa analysis was conducted on samples cultured in minimal media with and without the supplementation of 2'-FL to identify the glycan utilizing microbes. To further confirm the results from sequencing analysis, bacterial identification using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) was performed on colonies that grew on agar.

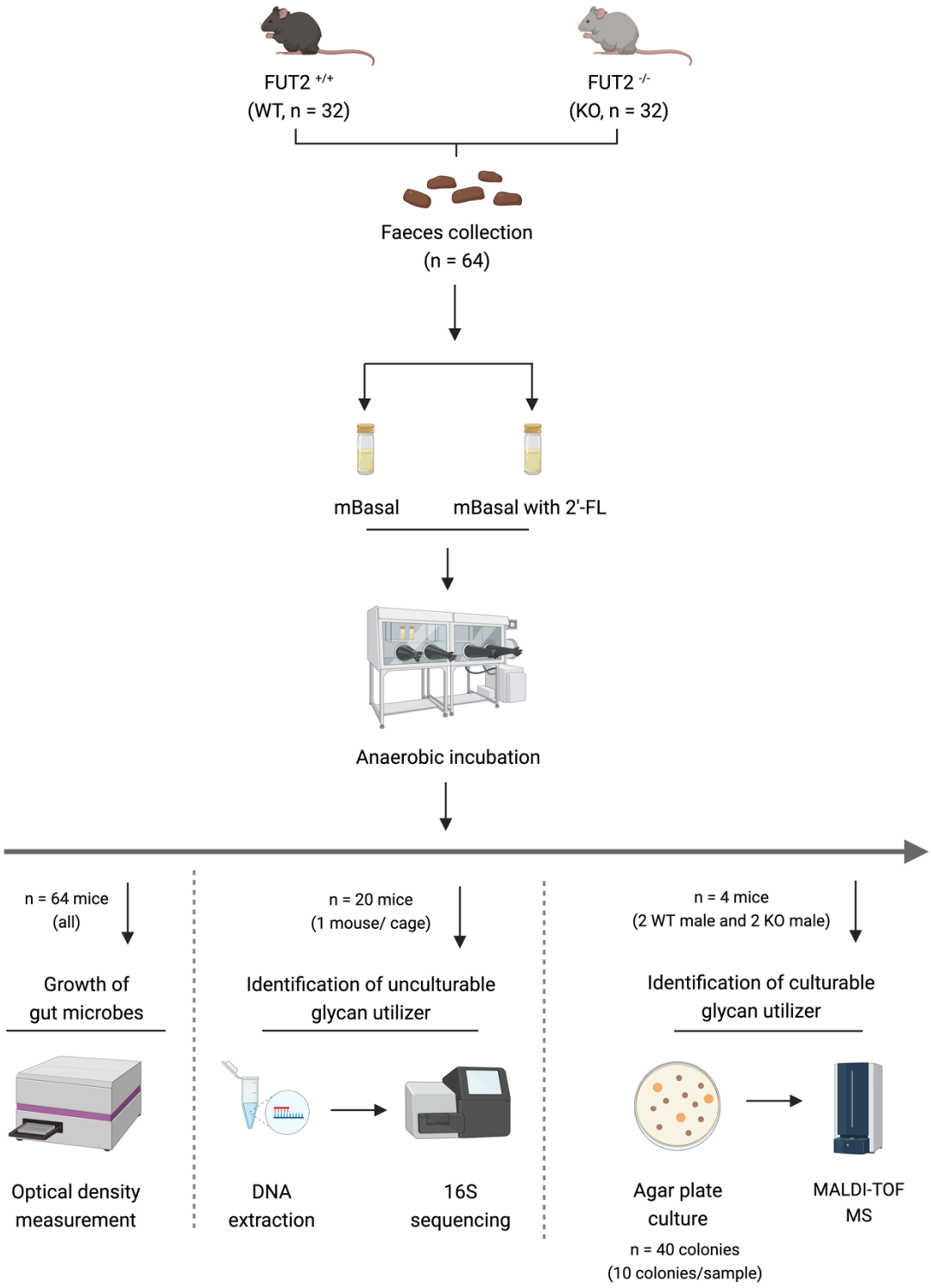


Figure 5.1 *Experiment design in Chapter 5*

5.2.2 Glycan degradation and utilization by gut microbes in faeces

Around 5 mg (± 0.5 mg) of faeces was weighted from each collection tube and placed in a 1.5 mL sterile tube. 1200 µL of PBS was added and faecal samples were resuspended by gentle vortexing. Faecal suspensions (500 µL) were transferred to a 15 mL tube along with mBasal broth (10 mL, formula see section 2.4.10) supplemented with either 5% 2'-FL or sterile milliQ water (faecal only control). Samples were incubated under anaerobic conditions (75% N2, 20% CO2, 5% H2, Coy Laboratory Products, Grass Lake, Michigan, USA) at 37°C for 120 hr (5 days). Optical density measurements (OD_{600}) were conducted using multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA) every 24 hrs. Tubes with mBasal media only was employed as a negative control. A positive control included mBasal broth supplemented with *B. infantis* JCM1222 (given its confirmed 2'-FL utilisation).

5.2.3 DNA extraction

After 5 days of incubation, cultured faecal bacteria were homogenized by a quick vortex prior to DNA extraction. A total of 300 µL of bacterial suspension were collected from each faecal 15 mL tube and subjected to faecal DNA extraction using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany), including a beat beating step at 6.5 m/s on a FastPrep (MP Biomedicals, Irvine, CA, USA), in accordance with the manufacturer's instructions. DNA were quantified using a Qubit 2.0 (Invitrogen, Carlsbad, CA, USA). Full details of DNA extraction protocol are provided in Chapter 2 (Section 3.5.1) and Appendix 2.4.

5.2.4 16S rRNA amplicon sequencing

The isolated DNA were then amplified and prepared for 16S sequencing. Basecall sequencing data were fetched and cleaned using QIIME2 pipeline. β-diversity was measured using Bray-Curtis distance matrix. The details of 16S sequencing have been described in Chapter 2 under the section of 3.6 and 3.7.

5.2.5 MALDI-TOF mass spectrometry

A total of 40 colonies were picked from 4 samples (2 cultured samples from WT male and 2 cultured samples from KO male, 10 colonies/sample) that were originally cultured in minimal media supplemented with and without 2'-FL. These colonies were subjected to MALDI-TOF MS analysis for identification.

5.2.5.1 MALDI-TOF MS analysis

One purified colony from each agar plate was picked by a sterile toothpick. A thin layer was applied on a target spot that sitting on MALDI plate using the toothpick that carried the colony. After that, 1 µL of formalin was dropped to each spot to fix the samples and the MALDI plate was incubated at room temperature until formalin became dry. Another 1 µL matrix material was applied on each spot. When the MALDI plate was completely dry, it was then placed into the MALDI Biotyper (Billerica, Massachusetts, United States) and the mass spectra obtained from each isolate were imported into software and were mapping with bacterial databases for species identification. Score values from the output that were smaller than 1.7 were considered as unreliable identification. Score values were between 1.7 to 2.0 were considered genus-level identification. Score values were larger than 2.0 were considered species-level identification.

5.2.7 Statistical analysis

All data analyses were performed using R (R Foundation for Statistical Computing; version 4.1.0). For parametric data, unpaired t test was used to compare data between two unpaired groups; One-way ANOVA was used to compare data among three or more unpaired groups. For non-parametric data, Mann-Whitney U test was used to compare data between two unpaired groups; Kruskal-Wallis test was used to compared data among three or more unpaired groups. Differences in Bray-Curtis dissimilarity between groups was performed by permutational multivariate ANOVA (PERMANOVA) and pairwise PERMANOVA, using the 'adonis' package in R, with 9,999. Linear discriminant analysis Effect Size (LEfSe) was used to identify the abundant taxa in each site [198]. Log-rank test was employed to compare survival time differences based on bacterial qPCR detection. One-tailed tests were used where differences between groups were hypothesised to be in a single direction. Statistical outcomes with p-value <0.05 were considered statistically significant. Statistically significant findings were noted according to the following cut-offs: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

5.3 Results

5.3.1 The presence of α(1,2)-fucosylated glycans altered the growth of glycan utilizing bacteria in murine gut

In this section, I hypothesised that glycan utilising microbes were present in the murine gut, contributing to the growth variation of gut flora when they were supplemented with α(1,2)-fucosylated glycans. To test this, baseline faeces collected from 64 mice (n=32/genotype) were incubated in minimal broth mBasal that was supplemented with and without 2'-FL for 120 hours (5 days).

Although OD_{600} values increased in both media supplemented with and without 2'-FL, faecal samples grown with 5% 2'-FL grew faster than without 2'-FL (median OD_{600} growth speed $_{[mBasal\ vs\ mBasal\ +\ 5\%\ 2'\text{-}FL]}$: 0.0349/day vs 0.0924/day: Figure 5.2A). This was also reflected as individual OD_{600} readings at fixed timepoints (Figure 5.2A, $p < 0.0001$ at each timepoint). To assess the overall growth of faecal microbes across the entire culturing period, area under the curve (AUC) was calculated for each group. As seen in Figure 5.2B, the area size of mBasal group is significantly smaller than that of mBasal + 2'-FL group (median area: 15.7 [IQR=14.5,17.4] vs 35.0 [IQR=30.8,38.0]; $p<0.0001$, Figure 5.2B). This result showed that faecal bacteria grew better when α-1,2-fucosylated glycan was available.

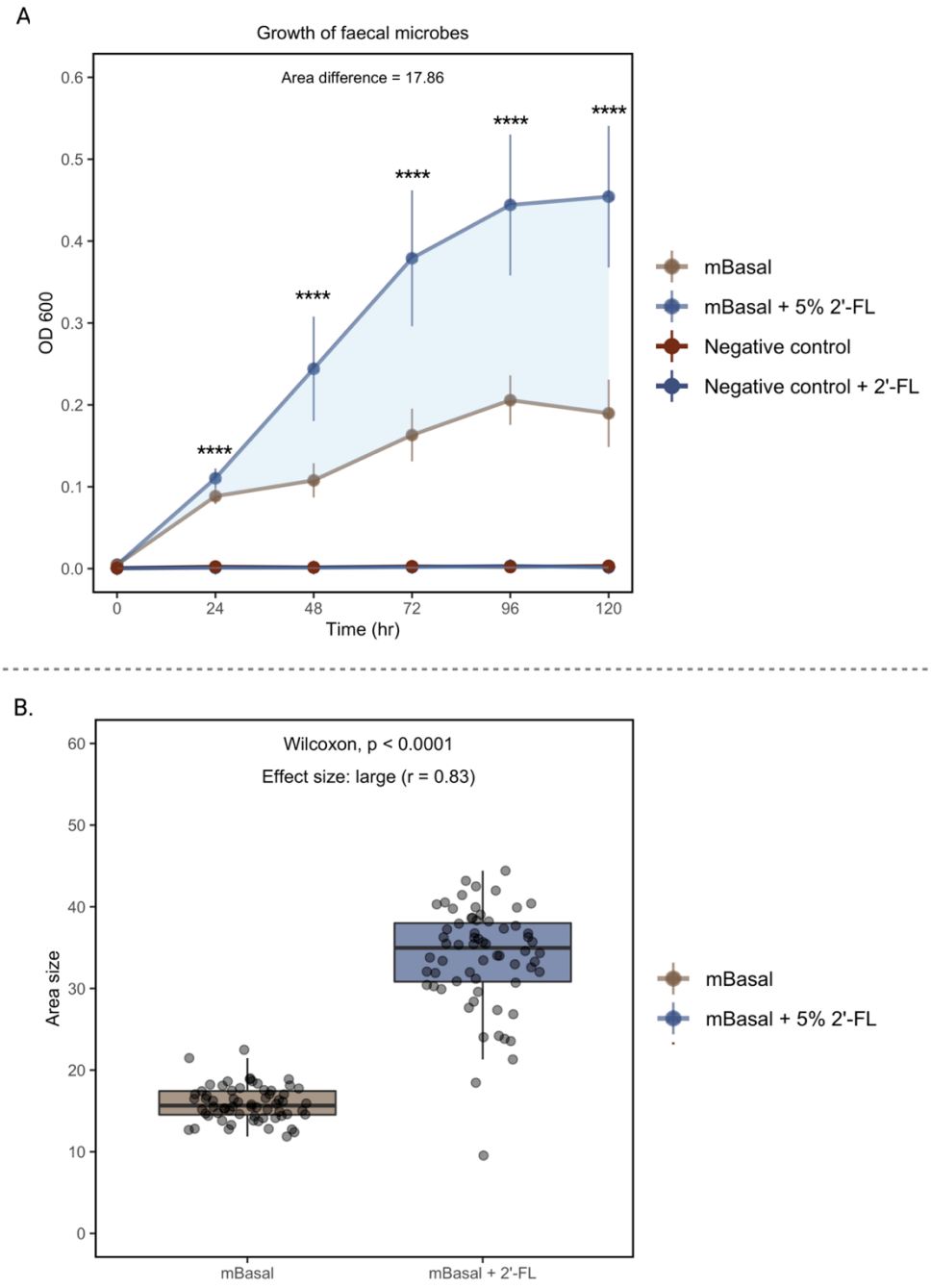


Figure 5.2 *In vitro growth of faecal bacteria*

To assess the impact of α(1,2)-fucosylated glycans on the growth of faecal bacteria, 64 faeces were cultured in mBasal broth (ingredients see section 2.4.10) with or without the supplementation of 5% 2'-FL.

The cultivation of 64 faecal samples in broth was distributed across six batches conducted on different dates, with each batch containing an equal distribution of samples cultured in mBasal broth only and same samples cultured in mBasal broth supplemented with fucose, as well as an equal representation of two genotypes (WT and KO). Each faecal sample underwent two independent replicates of cultivation under identical conditions. This approach yielded two OD600 readings per sample per treatment, and the average of these readings was utilized for visualization in the final figure. Stringent quality control

measures, including the inclusion of a non-template control (NTC control: mBasal broth without adding any faecal materials), were implemented to detect any potential contamination.

A. Growth curve of faecal bacteria grow under the condition of mBasal broth and mBasal + 5% 2'-FL group
B. Comparison of area under the curve between mBasal group and mBasal + 5% 2'-FL group

5.3.2 The presence of α(1,2)-fucosylated glycans altered the faecal microbial community

In chapter 3, significant compositional differences of gut microbiome were observed between *Fut2*$^{+/+}$ (WT) group and *Fut2*$^{-/-}$ (KO) group. To verify and recapitulate the contribution of α(1,2)-fucosylated glycans to compositional differences, faecal samples from WT mice were cultured in broth either supplemented with or without 5% 2'-FL. Non-Metric Multidimensional Scaling (NMDS) analysis of Bray-Curtis dissimilarity distance showed that faecal samples that cultured in mBasal broth only were distinguished from the same faecal samples cultured in mBasal broth that were supplemented with 5% 2'-FL (Figure 5.3A). In line with the visual observation, a PERMANOVA test based on Bray-Curtis dissimilarity distance showed that the availability of 2'-FL significantly contributed to an altered microbial composition (p=0.0001, pseudo-F=125.99, Figure 5.3A). Given that samples cultured under two different culturing conditions were originally from the same faeces and the only difference between two groups are the availability of α-1,2-fucosylated glycan, the altered composition could be attributed to the utilization of α(1,2)-fucosylated glycans by certain gut microbes.

To determine which bacteria benefit from 2'-FL, LEfSe analysis was employed to compare the relative abundance of taxa were compared between groups. Results showed that faecal samples cultured in mBasal broth supplemented with 5% 2'-FL carried a significantly higher abundance of *Bacteroides*, *Enterococcus* and *Lactobacillus* than the same faecal samples cultured in mBasal broth only (Figure 5.3B). This result suggested that these bacteria belong to these three outstanding genera are potentially 2-FL utilisers.

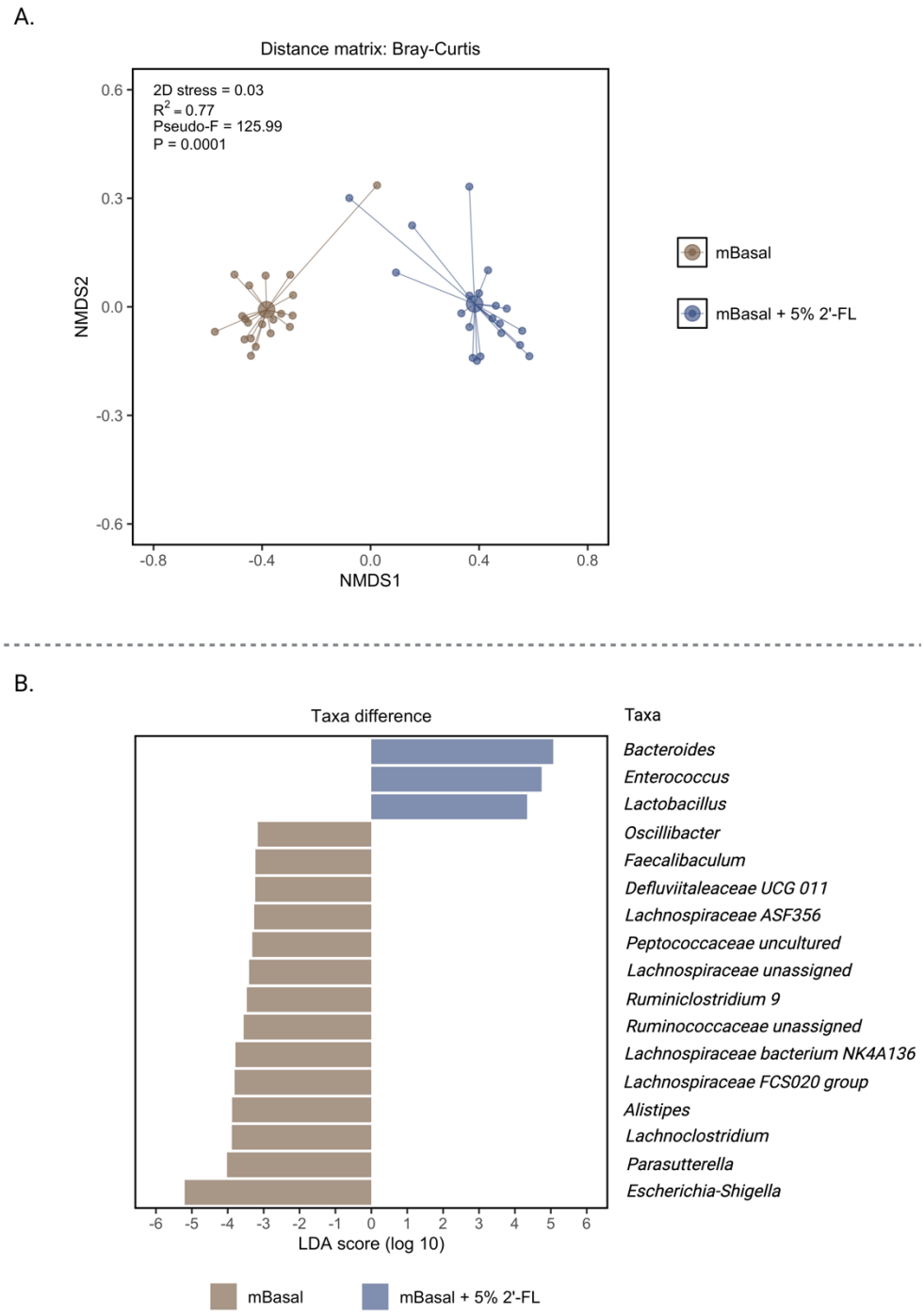


Figure 5.3 *Variation of faecal microbial community*

To assess the impact of α(1,2)-fucosylated glycans on faecal microbial community, a total of 20 faecal samples (1/cage) that cultured in mBasal broth with and without supplementation were subjected to DNA extraction and 16S sequencing. The composition difference was visualised by NMDS plot and assessed by PERMANOVA test (5.3A). Taxa difference was assessed by LEfSe analysis (5.3B).

To further assess bacteria that expanded with 2-FL supplementation, 8 faecal samples that were cultured in either mBasal alone or mBasal + 2'-FL (n=4/culturing condition) were randomly selected. These bacterial suspensions were plated on mBasal agar to allow colony identification and to avoid the interference of sufficient nutrition on assessment of glycan effects at the same time. As shown in Figure 5.4, colonies with three different morphologies (colony size: large, medium and small) were observed on agar. A total of 40 colonies (2 large colonies, 4 medium colonies and 4 small colonies per sample) were then randomly picked from agar plates and were subjected to MALDI-TOF MS analysis. Results showed that the identity of medium colonies were *Enterococcus faecalis*, the identities of small colonies were *Lactobacillus murinus* and *Lactobacillus reuteri* (Table 5.1). Given that the faeces cultured in mBasal with 2'-FL carried higher proportions of medium and small colonies than the same faeces cultured in mBasal only (Medium colony: 65.9 % vs 4.1%, Small colony: 18.3% vs 0.9%), *Enterococcus faecalis* (the medium colony), *Lactobacillus murinus* and *Lactobacillus reuteri* (the small colonies) are likely to be culturable glycan utilizers that are able grow on mBasal agar (Figure 5.5).

Taken together, three culturable glycan utilizing genera (*Bacteroides*, *Enterococcus* and *Lactobacillus*) were identified in murine gut. Of these three genera, three species that belong to *Enterococcus* and *Lactobacillus* can be isolated, which were identified as *Enterococcus faecalis*, *Lactobacillus murinus* and *Lactobacillus reuteri*. On top of that, a significant composition difference was found between faecal samples cultured in mBasal broth only and same faecal samples cultured in mBasal broth supplemented with α-1,2-fucosylated glycan. This composition difference indicates a potential glycan utilization by glycan utilizers found in murine gut.

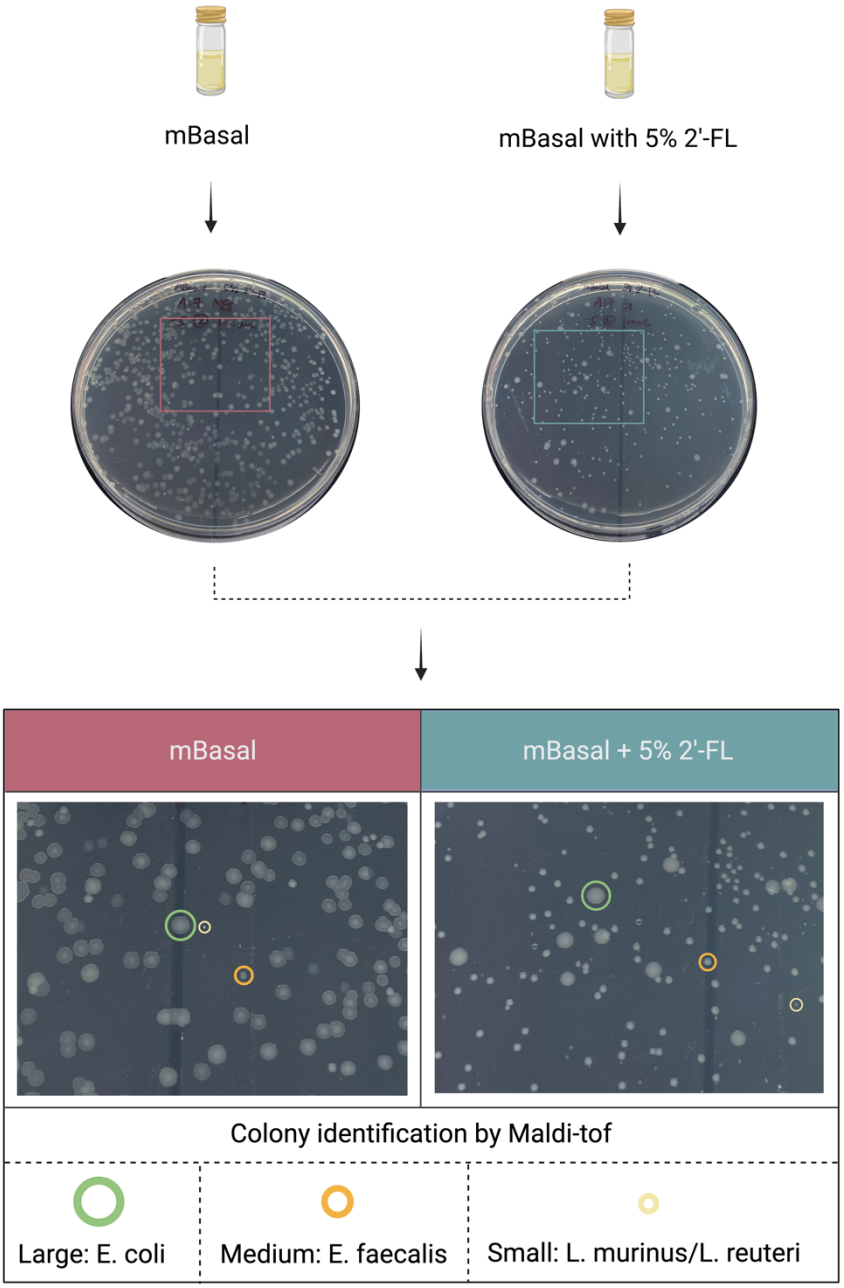


Figure 5.4 *Morphologies of colonies that grew in broth supplemented with/without 2'-FL*

Randomly chosen faecal samples (n=8) that were originally cultured in either mBasal alone or mBasal + 2'-FL (n=4/culturing condition) were plated on mBasal agar (supplemented with 5% 2'-FL). The *in vitro* experiment was conducted on 4 faecal samples with 2 replicates being used per sample. Two independent experiments were conducted, and similar results were observed.

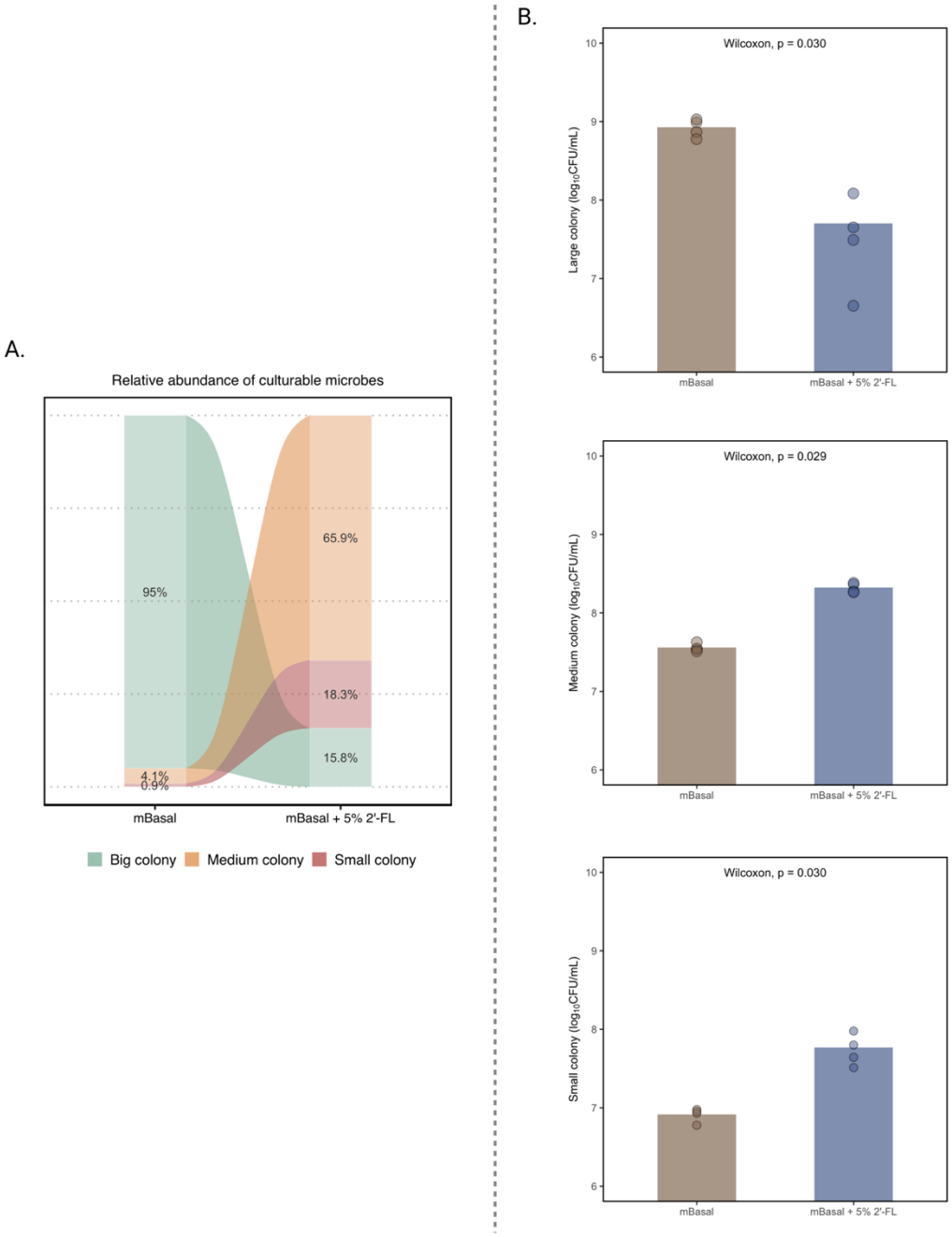


Figure 5.5 *Abundance of colonies grew in broth supplemented with/without 2'-FL*

To assess the impact of α(1,2)-fucosylated glycans on the growth of different faecal bacteria, colony numbers were counted according to the morphology size and were compared between glycan group and non-glycan group. A total of 8 samples (n=4/treatment) were included in the experiment. The colony numbers were counted twice per plate. Two replicates were counted per sample. The average count of each sample was used in the figure 5.5B.

A. Relative abundance of bacterial colonies on agar

B. Comparison of bacterial colonies abundance between glycan group and non-glycan group

Table 5.1 *Identification of colonies grew in broth supplemented with/without 2'-FL*

Colony size	Success to obtain signals	Reliability of identification	Identity	Percentage
Large	Yes	High	*Escherichia coli**	100% (8/8)
Medium	Yes	High	*Enterococcus faecalis*	94% (15/16)
	No (No peaks found)	N/A	N/A	6% (1/16)
Small	Yes	High	*Lactobacillus murinus*	81% (13/16)
	Yes	High	*Lactobacillus reuteri*	13% (2/16)
	No (No peaks found)	N/A	N/A	6% (1/16)

* Shigella and Escherichia coli are so closely related that they cannot be distinguished by MALDI-TOF MS. The accuracy of bacterial identification MALDI-TOF MS is more than 90%, which was summarised in a previous report[247].

5.3.3 The glycan utilization capability of faecal bacteria in broth associate with the variation of gut microbes between *Fut2*$^{+/+}$ (WT) group and *Fut2*$^{-/-}$ (KO) group

Many studies have reported glycan utilization capabilities of microbes. These microbes, such as *Bifidobacterium*, *Streptococcus* and *Bacteroides*, are commonly found in the gut. Considering the critical role of *Fut2* in the formation of α(1,2)-fucosylated glycans and the fact of more glycan utilizing microbes were found when faeces were cultured in broth with glycan (Figure 5.3B), it is likely that the compositional difference in gut microbiome that were observed in the chapter 3 is attributed to the abundance variation of these glycan utilizers between *Fut2*$^{+/+}$ (WT) group and *Fut2*$^{-/-}$ (KO) group.

To test this, bacterial biomass between *Fut2*$^{+/+}$ (WT) group and *Fut2*$^{-/-}$ (KO) group were compared when faeces from these two groups were cultured in broth with or without the supplementation of 5% 2'-FL. Specifically, areas under the curves were calculated to capture the growth of gut microbes across the whole incubation period. The increased bacterial biomass of faecal homogenates in each group (Figure 5.6A, highlighted in blue) represented the abundance of all glycan utilizers in that group. As seen in Figure 5.6A, the increased bacterial density associated with 2'-FL supplementation compared to minimal media alone was significantly greater when faecal homogenates were derived from WT compared to KO mice (median $AUC_{[WT]}$=20.5 [IQR=16.6, 23.5]; $AUC_{[KO]}$=16.7 [13.1, 19.7]; p=0.0046, Figure 5.6B). This result indicated a significantly glycan utilization difference by gut microbes between *Fut2*$^{+/+}$ (WT) and *Fut2*$^{-/-}$ (KO) mice.

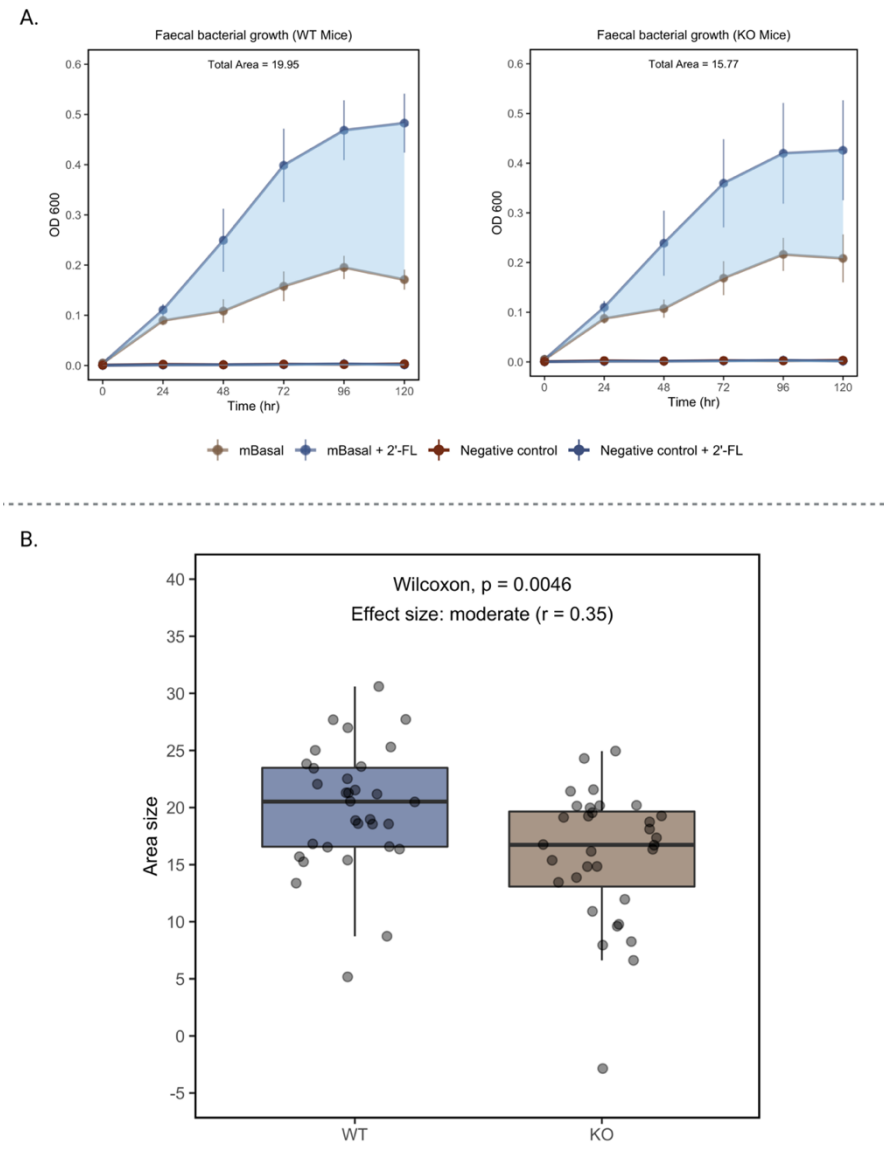


Figure 5.6 *Abundance of gut microbes in faeces grew in broth supplemented with/without 2'-FL*

The cultivation of 64 faecal samples (n=32/genotype/treatment) in broth was distributed across six batches conducted on different dates, with each batch containing an equal distribution of samples cultured in mBasal broth only and same samples cultured in mBasal broth supplemented with fucose, as well as an equal representation of two genotypes (WT and KO). Stringent quality control measures, including the inclusion of a non-template control (NTC control: mBasal broth without adding any faecal materials), were implemented to detect any potential contamination.

A. Faecal bacterial growth between WT and KO mice when faeces were cultured in mBasal broth supplemented with/without 2'-FL
B. Comparisons of increased bacterial density (AUC difference) associated with 2'-FL supplementation compared to minimal media alone (mBasal) between WT and KO mice group

5.4 Discussion

The results in the previous two chapters revealed the critical role of *Fut2* in shaping gut microbial community and impacting the adaptation of gut microbes. In this chapter, I added to these findings by exploring the contribution of α(1,2)-fucosylated glycans to a gut microbiome growth using *in vitro* culture experiments. These findings validate the mechanism behind the specific host genetic effect on the gut microbiome.

Taxa abundance results from Chapter 2 supported the presence of glycan utilizing bacteria in faeces, however, the glycan utilization capabilities within these genera varied depending on species or strain levels. For example, *B. breve* is not considered glycan utilizer when comparing with *B. bifidum* and *B. infantis*, which have been well-characterised in this project (Figure 4.2 in Chapter 4). Due to the limitation of 16S sequencing on the accuracy of species identification, it is possible that those microbes in faeces are not glycan utilizing microbes but belongs to glycan utilizing genera. Thus, prior to mechanism exploration, I firstly assessed the presence of glycan utilizing microbes using *in vitro* model developed in this study. Specifically, faecal materials were cultured in minimal broth supplemented with or without α(1,2)-fucosylated glycans (2'-FL) that are available on the market. A significantly increase of bacterial reproduction was observed when the broth was pre-supplemented with 2'-FL. The increase is reflected not only in the perspective of total biomass across the entire culturing period, but also is reflected in the perspective of growth rate. This result indicates that at least one microbe in the faeces grew better when α(1,2)-fucosylated glycans was available. Given many bacterial species that reside in the gut use glycans for adherence or as a source of energy, the microbes in the faeces grew better when α(1,2)-fucosylated glycans was available are likely to be glycan utilisers.

After confirming the presence of glycan utilisers in the faeces, microbial communities were then compared between those faeces cultured in minimal broth with supplementation of 2'-FL and the same faeces was cultured in broth without 2'-FL. In line with the findings in Chapter 3,

a significant microbiome composition difference was observed between faeces cultured in broth supplemented with 2'-FL and same faeces without (p=0.0001), indicating the potential of α(1,2)-fucosylated glycans in shaping microbial composition. The microbiome variance observed in this *in vitro* experiment is larger than the variance that directly derived from faeces. This could be due to the magnification effect caused by high concentration of 2'-FL and limited energy source in this *in vitro* experiment.

LEfSe analysis on sequencing results was employed to discover the identity of glycan utilizers. Results have shown that faeces cultured in broth with 2'-FL carried significantly higher abundance of *Bacteroides*, *Enterococcus* and *Lactobacillus* as compared with same faeces cultured in broth without glycan. Further MALDI-TOF MS analysis on colonies grow on agar confirmed the findings, unrevealing the identity of glycan utilizers at species level (*Enterococcus faecalis, Lactobacillus murinus* and *Lactobacillus reuteri*). Unfortunately, due to the difficulty of forming *Bacteroides* colony on agar with minimal nutrition, the identity of *Bacteroides* that was found from sequencing analysis at species level is still known. To support the growth of these gut bacteria, the growth medium that mimics the conditions of the gut environment would be recommended [248,249]. However, minimal media (limited carbon sources) is employed in this study for the following reasons. Firstly, sufficient carbon source would jeopardise the assessment of glycan effects on gut bacterial growth. Secondly, faecal bacteria would grow fast when they were cultured in media with sufficient nutrient, raising difficulties to capture the growth difference when faeces were cultured with and without the supplementation of glycan. Given the above arguments for minimal media, it would be expected that many gut bacterial species like *Bacteroides* cannot grow in mBasal (minimal media) even they are fastidious organisms. Another possibility that *Bacteroides* cannot be grown on agar points to the competitive exclusion when they were co-cultured with other glycan utilisers.

Apart from the glycan utilising taxa found to be more abundant in WT group, it is interesting that *Bifidobacterium*, as one of the glycan degrading genera, is not observed in the LEfSe analysis results. This could be due to the following reasons. Firstly, the rejuvenation rate of

Bifidobacterium from freezer at -80ºC is relatively low (~10% in the preliminary study) after 7 days when they were cultured in MRS-CS media (an ideal and widely used media to culture *Bifidobacterium*). Considering that the abundance of *Bifidobacterium* is at extremely low levels in the faeces that were employed in this study, the ability for *Bifidobacterium* to be selectively enriched to a detectable level may not have been possible. Secondly, the media we choose for our experiments are mBasal broth. This media has limited nutrition, *Bifidobacterium* in the faeces might lose the competition advantages over other bacteria (such as *Lactobacillus* and *Enterococcus*) when it was cultured in mBasal broth. Thirdly, only 40 colonies from 4 faecal samples were randomly picked for MALDI-TOF analysis, it is possible that *Bifidobacterium* colonies grew on the mBasal agar at low levels, however were not picked and identity determined.

After analysing the microbial community difference, the bacterial biomass was compared between WT group and KO group when faeces from these two groups were cultured in broth with or without the supplementation of 5% 2'-FL. The increased bacterial density associated with 2'-FL supplementation compared to minimal media alone was significantly greater when faecal homogenates were derived from WT compared to KO mice ($p=0.0051$). Considering that α(1,2)-fucosylated glycans was available in WT mice but not in KO mice and glycan utilising bacteria could utilise glycan for better growth, it is possible that this glycan utilisation differences by gut microbes between WT and KO mice were attributed to the initial abundance of glycan utilisers in faeces before culturing.

This study stands as the inaugural exploration into glycan utilization by gut bacteria through *in vitro* methods, following a thorough review of existing literature. To mitigate the influence of carbohydrates on our findings, fecal pellets were cultured in a minimal media environment. The composition of this media was meticulously selected, comprising trypton, yeast extract, NaCl, magnesium sulfate, and dipotassium hydrogen phosphate. While trypton and yeast extract may contain trace carbohydrates, their presence in the minimal media is negligible. These components primarily serve as nutritional substrates for bacterial growth, supplying

essential amino acids, peptides, and nutrients. Nevertheless, the study has its limitations, as the current minimal broth may not support the growth of all bacteria present in fecal samples. Many fastidious bacteria may fail to thrive in this media, potentially leading to the underrepresentation of certain glycan-utilizing species. Future investigations could focus on developing an *in vitro* cultivation system that emulates the gut environment, facilitating the survival and proliferation of a broader spectrum of gut bacteria.

Taken together, results in this chapter confirmed the presence of glycan utilisers in murine gut, and more importantly, results also suggest that the variations of gut microbiome found in Chapter 3 is related to the availability of α(1,2)-fucosylated glycans. Results in this chapter provide additional evidence of how does *Fut2*-determined α(1,2)-fucosylated glycans impact gut microbial composition and bacterial colonisation.

CHAPTER 6. DISCUSSION

6.1 Review of this PhD project

6.1.1 Rationale

As described in Chapter 1, our gut microbial community plays an integral and modifiable role in host health. The ability of therapeutic interventions to modulate host health relies on our understanding of complex host-microbiome interactions. The host-microbiome interactions can be impacted by extrinsic or intrinsic factors (**Figure 1.1**).

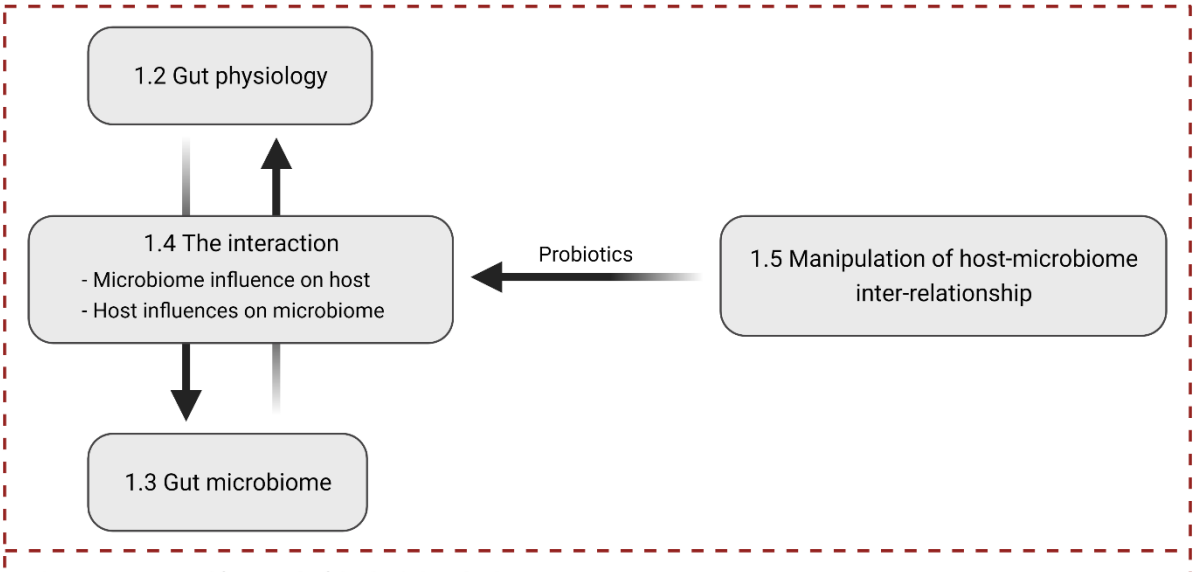


Figure 1.1 Conceptual framework of the thesis introduction

Section 1.2 and 1.3. Gut physiology and Gut microbiome. In these two sections, aspects of host physiology (including the physiology of GIT, intestinal mucosal barrier, and mucus) and gut microbiome (dynamics, influences on human health and factors influencing gut microbiome) are introduced, paving the way to better understand complex host-microbiome interactions in section 1.4

Section 1.4. The interactions between host physiology and gut microbiome. Recent findings on host-microbiome interaction will be summarised in both direction: 1) Microbiome influences on host physiology 2) Host influences on gut microbiome

Section 1.5. Manipulation of host-microbiome inter-relationship. Current therapeutic interventions targeting host-microbiome inter-relationship are characterised. Mechanisms involved and future research directions are also discussed in this section

While both factors are important, research focuses on the former due to the modifiable nature of extrinsic exposures. However, intrinsic factors like host genetics remains an active and important research area. Indeed, many genetic mutations[83-89,250], such as mutations in single candidate genes *LCT*[84], *Fut2*[93-96] and *NOD2*[90,91], impact gut microbiome composition and the abundance of bacterial taxa, directly or indirectly shifting gut microbiome to a disease-causing

state. Despite the currently findings, many knowledge gaps remain. For example, the variable findings in different healthy individual cohorts raised the concerns about the reliability of previous conclusions. Further, the mechanisms by which variation in host genes affect microbiome composition are unclear. Moreover, the impact of interactions between intrinsic and extrinsic factors on bacterial taxa are not assessed.

6.1.2 Aims

Fut2 encodes an α(1,2)-fucosyltransferase expressed in secretory mucosal cells (as described in Chapter 1, Section 1.5.2). The resulting α(1,2)-fucosylated glycans are hypothesised as playing a fundamental role in host-microbiome interactions, directly interacting with many microbes commonly found in the GI tract. Therefore, this thesis aimed to characterise how *Fut2* impacts the gut microbial community and the ability of bacteria to colonise the gut. In this thesis, I presented three studies exploring 1) the role of the *Fut2* gene on gut microbial community composition, 2) the ability for probiotics to colonize the gut, and 3) the interaction between antibiotics and probiotic colonisation.

6.1.3 Findings

The impact of *Fut2* was related to three aspects of the gut microbiome and gut bacteria: 1) the gut microbiome's structure and composition, 2) the persistence of gut bacteria in the gut and 3) the growth of gut bacteria when supplementing with α(1,2)-fucosylated glycan. Overall, the findings from this research identified that:

1. *Fut2*-related α(1,2)-fucosylated glycans contribute to an altered gut microbiome composition.
2. This altered composition consisted of taxa known to utilise α(1,2)-fucosylated glycans

3. Exposure of antibiotic and *Fut2*-related α(1,2)-fucosylated glycans impact the persistence and abundance of glycan utilising probiotic bacteria *B. infantis* following the antibiotic exposure.
4. Supplementation of α(1,2)-fucosylated glycans altered the growth of glycan utilizing bacteria in the murine gut.
5. Three culturable glycan utilising genera (*Bacteroides*, *Enterococcus* and *Lactobacillus*) were identified in the murine gut. Of these three genera, three species belonging to *Enterococcus* and *Lactobacillus* were isolated: *Enterococcus faecalis*, *Lactobacillus murinus* and *Lactobacillus reuteri*.
6. Supplementation of α(1,2)-fucosylated glycans altered the composition of the faecal microbial community

Together, the three studies in this thesis provide a deeper understanding of how *Fut2* related mucosal glycosylation shapes our gut microbiome and the persistence of the probiotic *Bifidobacterium* species. These findings can provide important information to guide probiotic usage in the clinical setting. The following sections discuss four aspects in details: 1) methods that were developed in this candidature, 2) scientific findings and clinical insights, 3) strengths and limitations and 4) future research directions.

6.2 Methods developed and optimized during candidature

To test the hypotheses proposed in this PhD project (see Chapter 1, Section 1.7), a *Fut2* KO mouse model was developed, and three individual experimental studies were designed. In these three experiments, many approaches in this PhD project (i.e., gavaging, antibiotic exposure, DNA extraction, 16S sequencing and bacterial culture) were well established by other studies. Despite this, some methods required the development of optimisation for specific purposes in this PhD, listed as follows:

6.2.1 Mouse mating and breeding strategy: Het x Het

The breeding strategy employed in this study was a critical aspect of the experimental design. While Homozygous x Homozygous mating (WT x WT and KO x KO trio mating) offers several advantages, including faster breeding and known genotypes of offspring, it introduces complexities that could confound the analysis. Specifically, this approach may facilitate genotype-specific microbiome alterations over successive generations due to vertical transmission and cage effects.

To mitigate these potential confounding factors, a Heterozygous x Heterozygous breeding strategy was adopted. This decision aimed to ensure that all dams possessed a functional *Fut2* gene. However, it is acknowledged that this approach may introduce complexities related to the cohousing of WT, heterozygous, and homozygous pups until weaning, potentially leading to exposure to stool microbiota from heterozygous dams.

Critically, the timing between weaning and analysis warrants discussion. *Fut2*-deficient pups are exposed to stool microbiota from heterozygous littermates and dams during the period from birth to weaning. However, there exists a brief window (~3 weeks) post-weaning where Fut2-deficient pups are not exposed to potential *Fut2*-utilizing microbes from the dam or *Fut2*+ littermates. This minimized exposure post-weaning can be considered a strength of the study,

as it reduces the confounding effects of vertical transmission, ensuring that any observed effects on the gut microbiome are more likely to be genuine and rapidly occurring.

Nonetheless, it is acknowledged that this design has limitations, particularly in assessing the long-term effects of prolonged *Fut2* glycan absence on the gut microbiome. Potential microbiome changes taking longer than 3 weeks to develop might not be captured adequately. Alternative breeding strategies, such as comparing wild-type (WT) with knockout (KO) weaners born to WT and KO breeders, could provide valuable insights into microbiome drift over successive generations as a consequence of vertical transmission. This longitudinal comparison may be considered for future investigations.

6.2.2 Bacterial culturing condition: MRS-CS medium

Multiple media are commonly used to culture *Bifidobacterium* species, including MRS (de Man, Rogosa, and Sharpe) and MRS-CS (MRS with added cysteine and sodium). The inclusion of cysteine and sodium in MRS-CS can help reduce oxygen toxicity and support the growth of anaerobic strains, which may be sensitive to oxygen. Given the benefits, MRS-CS is considered as a safer choice to ensure anaerobic conditions, which is widely used in studies to cultivate *Bifidobacterium* species[190,251]. All three *Bifidobacterium* species (*B. bifidum, B. infantis* and *B. breve*) employed in this PhD project are oxygen-sensitive and considered anaerobic bacteria. In my preliminary test, the growth of these strains was slower in MRS media than in the MRS-CS medium. Considering this, MRS-CS was chosen to culture *Bifidobacterium* strains.

6.2.3 Preparation of strains for oral gavage: Freshly cultured with survival test

The survival rate of *Bifidobacterium* spp. was unknown following a freeze-thaw cycle and storage at 4˚C following anaerobic culture. This was necessary to record to accurately determine how many CFU were gavaged into mice during experiments. In the preliminary test, the *Bifidobacterium* rejuvenation rate (*B. infantis* was tested as an example) is around 10%

(~one-log of bacteria died) after freezing them down at –80 °C for six days (see Figure 6.1). Given this, all *Bifidobacterium* strains given to mice were freshly cultured to ensure *Bifidobacterium* strain viability before oral gavage. Also, transportation between the microbiology lab and the mouse facility takes two hours. The *Bifidobacterium* strain survival during the 2-hour transportation on ice was also tested and considered during gavaging experiments (see Chapter 2, Section 2.4.2.2).

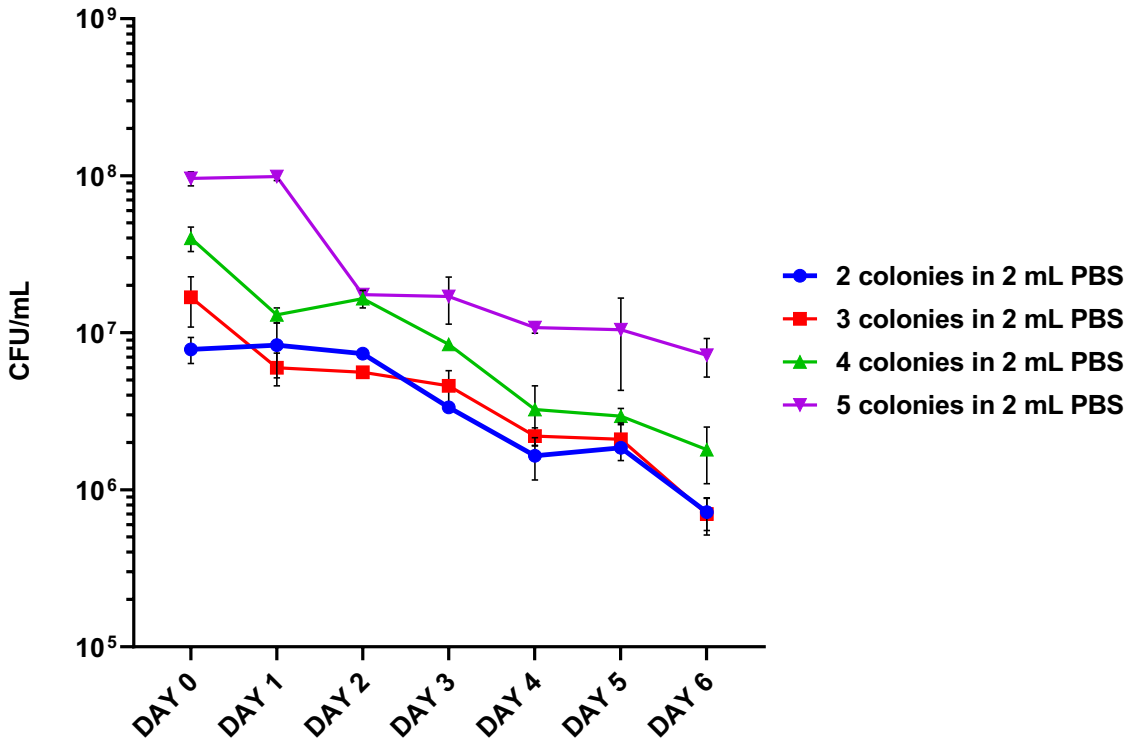


Figure 6.1. *Rejuvenation of B. infantis JCM1222*

To determine the necessity of supplementing freshly cultured probiotic strains to mice, *B. infantis* was stored in freezing media at –80 °C for 1 to 6 days. The rejuvenation rate of *B. infantis* was assessed based on the CFU count at different timepoints. Only one individual experiment with two replicates per sample was conducted.

6.2.4 Primer selection and validation

Detection and quantification of target bacterial strains is extremely important in the second study in this PhD project. All strains in the second study are within the same genus, *Bifidobacterium*. Considering bacteria within the same genus normally share a high degree of genetic similarity, it would be challenging to find three sets of primers that can perfectly differentiate and quantify them without being impacted by other strains within the same genus. To find the primers with high efficiencies and specificities, a few primer sets published in other reports were screened by conducting primer blast using NCBI database and then subjected to efficiency tests and specificity tests (see Figure 2.5, Chapter 2). Three sets of primers targeting each strain (*B. infantis*, *B. bifidum* and *B. breve*) were carefully chosen (see Table 2.2, Chapter 2).

6.2.5 Genotyping PCR: a set of primer was designed in this study

In this PhD project, a set of primers (one forward and two reverse primers) was designed targeting coding exon of the *Fut2* gene to genotype mice. Details can be found in Chapter 2, Section 2.4.1.2. Compared with other methods (e.g., sequencing-based method, mass spectrometry-based method and genomic microfluidics) for genotyping, the PCR genotyping method was found to be rapid, efficient and cost-effective.

6.2.6 *In vitro* glycan utilization assay

An *in vitro* glycan utilisation assay was developed in this PhD project to 1) assess the glycan utilisation capability of candidate *Bifidobacterium* strains employed in the second study and 2) identify the bacterial colonies derived from faecal pellets that utilised glycan for better colonisation. Specifically, bacterial strains or faecal pellets were cultured in mBasal broth with or without supplementation of α(1,2)-fucosylated glycan 2′-Fucosyllactose and bacteria growth was measured and compared by OD_{600} using a multimode plate reader. When developing this

assay, the broth selection was particularly challenging. On the one hand, if the nutrition in the broth is too rich, the complex ingredients present in richer media may confound results, interfering with the study of glycan utilisation. Also, bacteria would grow too quickly in richer media, making it hard to capture the bacterial growth differences between the group with glycan supplementation and the group without. Conversely, if the nutrition in the broth is too low, many bacteria would be unable to grow in it. After many tests using different broths, mBasal medium was selected. It has a defined and minimal nutrition that provides a consistent and standardised environment supporting bacterial growth and reduces the background metabolic activity. This made detecting and quantifying changes in bacterial growth and glycan utilisation patterns easier.

6.3 Scientific findings and clinical insights

As covered in Chapter 1 (Section 1.7), three studies were conducted to test three small hypotheses as listed below:

- **Hypothesis 1 (Chapter 3):** *Fut2* gene function impacts the gut microbiome.
- **Hypothesis 2 (Chapter 4):** *Fut2* gene function influences gut bacterial colonization.
- **Hypothesis 3 (Chapter 5):** Supplementation of α(1,2)-fucosylated glycans impacts the gut microbial community and growth of glycan utilising bacteria.

6.3.1 Scientific findings

6.3.1.1 Fut2 gene function impacts the gut microbiome composition (Chapter 3)

In Chapter 3, I investigated the impact of functional *Fut2* gene on gut microbial community. Specifically, the microbial communities were compared between *Fut2*$^{+/+}$ (WT) mice and *Fut2*$^{-/-}$ mice. As shown in the Chapter 3, microbiome composition differences between WT and KO mice were observed and this difference is more pronounced in male mice, confirming my hypothesis: *Fut2* did shape the gut microbial community in some respects. Many studies have shown the influence of secretor status on infection and disease susceptibility[171,176-178,180,181,199,252-258], highlighting the importance of secretor status in host health. Also, many studies have reported microbiome difference between patients with the above diseases and health individuals[259-265]. Given this study's results—that the functional *Fut2* gene impacts gut microbial communities—the altered gut microbiome likely mediates the association between secretor status and facets of host health.

6.3.1.2 Fut2 gene function impacts the abundance of gut bacterial taxa (Chapter 3)

Besides the microbiome composition difference, this study also identified the taxa that are more significantly abundant in WT mice. Of these taxa identified, a previous study on *Blautia* has reported its abundance was increased by α(1,2)-fucosylated glycans in the presence of

glycan degrading bacteria *B. bifidum* [202]. Together, these two results confirmed the presence of glycan degrading bacteria and commensal bacteria that benefits from glycan degradations in the murine gut.

6.3.1.3 Fut2 gene function impacts bifidobacterial colonization (Chapter 4)

In Chapter 4, I investigated the impact of the functional *Fut2* gene on the persistence and abundance of gut microbes. In this study, I chose to give three *Bifidobacterium* strains (*B. infantis*, *B. bifidum* and *B. breve*) to mice in different treatment groups. By assessing the glycan utilisation capabilities in an *in vitro* experiment, two strains (*B. infantis* and *B. bifidum*) were confirmed to be glycan-utilising bacteria, and one strain (*B. breve*) was confirmed to be a non-glycan utiliser. An antibiotic cocktail was given to mice for seven days to minimise the indigenous microbiome impact on the results. Results have shown the presence of functional *Fut2* gene impacts post-antibiotic *Bifidobacterium* colonisation, but in a species-specific pattern. Specifically, the survival and abundance of *B. infantis* are greater in WT mice than KO mice. However, this is not observed for the other two bifidobacterial species assessed. This result indicates a genotype effect on the abundance and persistence of certain microbes. While several possible explanations exist for why *B. infantis*, but not *B. bifidum* and *B. breve*, persisted longer in WT mice, the most likely explanation relates to the capacity to utilise α(1,2)-fucosylated glycans. As outlined in Chapter 1, the genes responsible for cleaving and degrading glycans are glycosyl hydrolases (GHs)[266]. *B. infantis* encodes a gene *Blon_2335* (GH95 family), which is specific for the degradation of *Fut2*-related α(1,2)-fucosylated glycans. Similar to *B. infantis*, *B. bifidum* also encode a gene *AfcA* (GH95 family) in its genome that can degrade *Fut2* related α(1,2)-fucosylated glycans; however, it is unexpected that *B. bifidum*, another glycan degrader, did not survive longer in WT mice than in KO mice. This could be due to many reasons. The most likely one is α(1,2)-L-fucosidases produced by *B. bifidum* is in extracellular form[131,267], meaning the L-fucose freed from glycan by *B. bifidum* not only can be absorbed by *B. bifidum* itself, but also can be utilised by other commensal bacteria in the gut lumen through competition. This phenomenon was reported in many studies, referred to

as cross-feeding[207,268-270]. Unlike *B. bifidum*, the α(1,2)-L-fucosidases produced by *B. infantis* are in-membrane bounded and intracellular form, meaning the L-fucose freed from the glycan by *B. infantis* only serves themselves within bacterial cells. This degradation differences therefore might contribute to the findings in this study. Besides the degradation difference between the two species, the adaptation differences might also explain the results in the study. A recent study found *B. bifidum* and *L. acidophilus*, as compared with other *Bifidobacterium* and *Lactobacillus* species, are significantly less enriched in stool during probiotics supplementation after antibiotic exposure[186], indicating that *B. bifidum* is less adapted to the GI tract than other *Bifidobacterium* species following broad-spectrum antibiotic treatment in mice. Given this, it is likely that *B. bifidum* supplemented to mice in my study were washed away by gut fluid before utilising glycan to colonise the gut.

6.3.1.4 Sex impacts gut microbiome and gut bacterial colonisation (Chapters 3 and 4)

Other than *Fut2* impacts, studies in this PhD also evidence the effect of sex on the gut microbiome and colonisation of gut microbes. In short, stratification according to sex identified a greater divergence in microbiome composition according to genotype in male mice compared to female mice, highlighting the impact of sex in the gut microbiome. Also, gut bacterial colonisation was associated with sex type in a species-specific pattern. Specifically, *B. breve* was found to be better adapted in the female murine gut, with significantly longer persistence and higher abundance observed. However, the difference was not observed in the other two treatment groups (Antibiotics + Infantis and Antibiotics + Bifidum), suggesting the impact of sex on gut microbe colonisation is species-dependent.

6.3.1.5 Supplementation of α(1,2)-fucosylated glycans impacts the gut microbial community and growth of glycan utilizing bacteria (Chapter 5)

In Chapter 5, I further investigated how *Fut2* impacts gut microbiome composition and bacterial colonisation. The microbiome and bacterial colonisation differences observed in

previous chapters (Chapters 3 and 4) represent end-point observations in the murine model between WT and KO mice. This directly evidences the glycan utilisation theory I proposed, and other variances led by the *Fut2* gene likely contribute to the results. An in vitro experiment was developed to address this using the baseline faeces collected in Chapter 3. In this chapter, I found that supplementing α(1,2)-fucosylated glycans in broth increased the faecal bacteria biomass, indicating the presence of glycan-utilising bacteria in the murine gut. Using 16S sequencing and MALDI-TOF, the identities of these glycan-utilising bacteria were confirmed as *Bacteroides*, *Enterococcus* and *Lactobacillus*. PERMANOVA analysis on 16S sequencing data identified a divergence of microbiome composition between samples cultured in mBasal broth and the same samples cultured in mBasal broth supplemented with α(1,2)-fucosylated glycans. These results align with previous results observed in Chapter 3, indicating the presence of α(1,2)-fucosylated glycans in WT mice contributes to microbiome differences between WT and KO mice. Aside from the microbiome composition, I also investigated faecal bacteria growth collected from WT and KO mice. I found that the glycan utilisation capability of faecal bacteria in broth is associated with gut microbe variation in the WT and KO mice groups. This result indicates the abundance of glycan-utilising bacteria is impacted by glycan utilisation. Taken together, all the results obtained in this study provide evidence to support glycan utilisation theory on how *Fut2* impacts the gut microbial community and bacterial colonisation.

6.3.2 Clinical and scientific insights

Studies in this PhD project highlights several important points in relation to inter-individual variance in intestinal microbiology and probiotic therapy efficacy.

6.3.2.1 Highlighting the importance of host genetics on the gut microbiome

First, the PhD project results provide evidence to support that host genetic factors impact the gut microbial community and the ability of gut microbes to colonise the human gut. These results could also explain individual heterogeneity of gut microbiome.

6.3.2.2 Helping explain the inconsistent clinical outcomes that related to probiotic therapy

Second, the effect of secretor status on *Bifidobacterium* supplementation that observed in this thesis has important implications for probiotic strategies. The usage of probiotics gained popularity among the public in recent years due to the benefits reports that probiotics can confer to host health[271,272]. However, concerns were raised on the efficacy of probiotics therapy when variable and inconsistent clinical outcomes accumulated in an increasing number of clinical studies[273,274]. Many researchers have realised the importance of probiotic strains variation and formulations on the efficacy[275]. However, the inconsistency could be attributed to other reasons and possibilities. Strain specificity and individual heterogeneity are two of the biggest assumptions. The study in this thesis raised two extra possibilities for the low efficacy of probiotic therapy, highlighting the importance of both host genetics and probiotic strain variation on the persistence of exogenous probiotics following antibiotic exposure. Since approximately one-fifth of the global population cannot express α(1,2)-fucosylated glycans on mucosal surfaces due to a lack of functional *Fut2* gene, our results indicate that secretor status, probiotic strain selection and the indigenous microbiome all impact probiotic persistence in the human gut, which might contribute to the efficacy variance of

probiotic therapy. Thus, it is crucial to consider individual host traits when designing a probiotic intervention as previously reviewed[276].

6.3.2.3 Providing a potential strategy to improve the efficacy of B. infantis-related probiotic therapy

Previous studies have shown that supplementation with human milk oligosaccharides can enhance *B. infantis* engraftment[224], with successful supplementation shown to reduce intestinal inflammation in infants[277]. These findings, along with those in this PhD project highlight that consideration of additional α(1,2)-fucosylated glycans, given as prebiotics, may be necessary for successful *B. infantis* supplementation in non-secretor individuals, contributing to an improved efficacy of probiotic therapy.

6.4 Strengths

Studies in this PhD project have many strengths, which are listed as following and explained one by one:

6.4.1 Study designs

Two murine studies and one *in vitro* study were developed to test the hypotheses I proposed.

6.4.1.1 Breeding strategy for experimental mice: het x het

The mice experiments involved SPF mice that were obtained through heterozygous mating. Such breeding was essential to allow comparison of WT and KO littermates from a maternal secretor lineage. The findings from this study are therefore independent of vertical transmission effects, which are known to influence the microbiome of the offspring[95,278], and indicate that a change in gut microbiology occurred post-weaning.

6.4.1.2 Consideration of other covariates: cage effect and sex effect

Due to the coprophagy behaviour of small animals and faeces being the sample type analysed, cage effects are important covariates, which could significantly impact the validity of conclusions if they were not controlled and well justified. Two approaches were used to minimise this impact. First, at least eight cages were used to house mice with a single genotype (four for each genotype x sex type). Unlike other studies where only two cages were used to house mice with a single genotype to control for the cage effect, the quadrupled cage size in each experimental model provided more confidence. Second, the cage effect was considered an individual covariate when assessing host genetic effects on gut microbiome and bacterial colonisation. Specifically, cages were nested in the PERMONVA model when assessing the microbiome composition differences (see Chapter 7, Appendix 3.1). Also, to minimise the chance of false positive results, all positive findings were re-analysed based on cage and not sample size (see Chapter 4, Section 4.3.6). Other than the cage effect, studies in this PhD project also considered another covariate: the sex effect. Unexpectedly, this study

identified a greater genotype divergence in male mice when assessing microbiome differences, which is not reported in previous reports.

6.4.1.3 Consideration of bacterial viability for oral gavage

The second study aims to assess the impact of the *Fut2* gene on the persistence and abundance of three different *Bifidobacterium* species in the murine gut. However, these three species were not detected in their indigenous microbial community, requiring exogenous supplementation by oral gavage. All probiotic bacteria used in the three studies were freshly cultured before experiments and subjected to survival and counting tests to ensure their gavage efficacy/bioavailability and data accuracy, as described in Section 6.2.3.

6.4.1.4 Appropriate sample size

Sample size is a critical consideration in microbiome studies because it can profoundly impact research findings' reliability, validity and generalisability. A small sample size for a microbiome study may inadequately capture the diversity present in a population. However, reducing the sample size in mouse studies is needed due to ethical, financial and logistical considerations. Thus, it is always challenging to balance obtaining statistically meaningful results and minimising the number of animals used. In our study, power calculations were performed according to three published reports[161,225,226]. A minimum sample size of eight mice/genotype x sex type/treatment group is expected to be sufficient to show an effect if the hypothesis is true. Considering the costs, the four treatment groups and four genotype x sex types, 64 mice and 128 mice, respectively, were included in the first and second studies.

6.4.2 Experimental materials

All materials, especially probiotic strains and antibiotic types were carefully chosen in the three studies.

6.4.2.1 Bacterial strains

Probiotic supplementation is needed to assess the influence of α(1,2)-fucosylated glycan on gut bacterial colonisation in the murine gut, as some *Bifidobacterium* species are well-known glycan-degrading and utilising bacteria. They are either absent or of low abundance in the murine gut. To find the appropriate strains for this study, common *Bifidobacterium* species were characterised and compared to glycan degradation genes carried in their genomes and the property of α(1,2)-L-fucosidase (extracellular, cell surface-bound or intracellular) they produced. The capability of selected bifidobacterial strains to utilise α(1,2)-fucosylated glycans was then confirmed by in vitro culture in minimal media with/without supplementation of 5% w/v 2′-Fucosyllactose (Layer Origin, Ithaca, New York, USA).

6.4.2.2 Antibiotics

Like probiotics, antibiotics are also required to test the impact of *Fut2* mutations on bacterial colonisation in the presence of a disrupted gut microbiome. Of all antibiotics, ampicillin and neomycin were carefully selected to administer to the mice. Both have broad-spectrum activity, which was tested in the preliminary data and previous murine studies in the lab (Ethic no. SAM151 and SAM218). They are clinically relevant antibiotics, often prescribed in clinical settings to treat many common bacterial infections.

6.5 Limitations and future research directions

Despite the strengths listed above, three studies also have limitations, as outlined below.

6.5.1 Method and materials

6.5.1.1 Viability of Bifidobacterium strains detected from faecal samples

While qPCR serves as a common and relatively accurate method for detecting and quantifying bacterial load in samples, it presents limitations regarding the determination of bacterial viability, as it solely targets DNA. Consequently, the detection of probiotic bacteria in faecal samples via qPCR may not necessarily indicate their viability, potentially undermining the clinical significance of findings despite the identification of host genetic effects on bacterial colonization.

To address this, the consideration of alternative methods for quantifying live faecal bacteria, such as incorporating fluorescent markers into the genome of the strain of interest and utilizing flow cytometry for quantification, emerges as a potential avenue for exploration. These methods offer distinct advantages, including real-time assessment of viability and differentiation between live and dead cells, which qPCR cannot achieve. Additionally, fluorescent labelling could prove useful for investigating difficult-to-culture bacteria or cases where selective culture is challenging.

However, it is important to note that the development and optimization of protocols for fluorescent labelling, as well as ensuring the specificity and sensitivity of the markers, require significant time and resources. Given the broader scope of the research project, the incorporation of fluorescent labelling and flow cytometry was not pursued in this study. Nonetheless, these approaches provide promising avenues for future research endeavors, particularly for more high-throughput analyses or investigations targeting difficult-to-culture microbial species.

Additionally, the utilization of selective media for culturing *Bifidobacterium* strains from collected faecal samples presents another potential solution. However, the development of an ideal selective medium poses considerable challenges, primarily due to the intricate nature of faecal microbial communities. Faecal samples harbor a diverse array of bacterial species, fostering complex interactions that can influence bacterial growth and survival. Traditional selective media may not adequately account for these interactions, potentially leading to the unintended growth of non-target bacteria. Furthermore, the reliance of certain gut bacteria on metabolic by-products produced by coexisting microbes adds another layer of complexity. Selective media designed to inhibit non-target bacteria may inadvertently disrupt essential metabolic interactions, consequently impacting the growth of target *Bifidobacterium* strains. Efforts were undertaken for several weeks to identify or develop a selective medium tailored for the cultivation of *Bifidobacterium* strains from faecal samples. However, despite extensive exploration, a perfect selective medium that effectively mitigates interference from other gut microbes in faecal samples proved elusive.

6.5.1.2 The limitation of 16S sequencing on microbiome exploration

Despite the relatively cheap cost and capability of decoding the gut microbial community, the accuracy of 16S sequencing used on bacterial identification only limit to genus level and rare species might not be well-detected due to sequencing depth limitations. Besides, it does not provide information about potential metabolic pathways and functional genes as compared with shotgun metagenomic sequencing. Shotgun metagenomic sequencing can be employed to better understand the taxa difference between groups at species and strain levels, which could provide evidence on whether the glycan utilising microbes are species specific or strain specific.[279]

6.5.2 Study modellings

All three studies were built purely on murine models, meaning many other variables are well controlled. For example, all mice were fed the same diets and raised in the same conditions.

This is a strength as researchers can focus on limited factors. However, it is also a limitation as the murine model does not mimic the situations that happen to humans.

6.5.3 Chapter 3: *Fut2* gene function impact on the gut microbiome

In Chapter 3, the baseline microbial community was compared between *Fut2*$^{+/+}$ (WT) mice and *Fut2* $^{-/-}$ mice and a significant composition difference was observed. This aligns with findings in some human studies[94,96,212], but not others[280,281]. The variable results from these studies raised more questions on this topic. For example, how big us the impact of *Fut2* mutation on the gut microbiome? Can the genetic impact be diluted by other variables (e.g., diet, race)? Moreover, previous studies also found associations between secretor status and disease and infection susceptibility[171,176-178,180,181,199,252-258]; however, it remains unknown whether this association is mediated by an altered gut microbiome, which needs to be further investigated.

6.5.4 Chapter 4: *Fut2* gene function impact on bacterial colonization

In Chapter 4, I compared the persistence and abundance of three different *Bifidobacterium* strains between WT and KO mice, and I found *B. infantis* colonise better in WT than KO mice. This result aligns with many other microbiological studies focused on the glycan utilisation capability of *B. infantis.* These studies have been well summarised in different review papers[282,283].

Despite this finding, many research gaps remained. First, the bacterial strains used were limited to three *Bifidobacterium* strains. Whether the availability of the *Fut2* functional gene impacts the persistence and abundance of other *Bifidobacterium* strains or other commensal bacterial strains is unknown. Further, it is known that the presence of an indigenous microbiome impacts the persistence and abundance of probiotic bacteria. Whether *Fut2* still impacts colonising these bacteria in the presence of an indigenous microbiome is still unclear. Moreover, whether supplementing α(1,2)-fucosylated glycans to KO mice would compensate

for the persistence and abundance difference of glycan-utilising bacteria due to the absence of a functional *Fut2* gene needs further investigation. Last, it must be noted that while humans and mice vary greatly, mice share a significant genetic similarity with humans, making them suitable for studying human diseases and biological processes. Given the study in this thesis was built solely on a murine model, our ability to observe similar findings in a human study remains unclear. Even if we observe similar results in human studies, we must still evaluate how large the genetic factor impacts are.

6.5.5 Chapter 5: Supplementation of α(1,2)-fucosylated glycans impact gut microbial community and growth of glycan utilizing bacteria.

In Chapter 5, α(1,2)-fucosylated glycans were supplemented to minimal broth with gut microbes in faeces. The growth of gut microbes was compared in WT and KO mice samples. The results showed that gut microbes from WT mice grow faster than microbes from KO mice. These results provide evidence to support glycan utilisation theory. Further, three glycan-utilising genera in the murine gut were identified.

Despite these findings, it must be noted this study is exploratory. As previously mentioned, the detailed mechanisms and pathways of how the glycan utilisation process impacts the gut microbiome composition and bacterial colonisation still need further investigation. Second, whether glycan-utilising bacteria observed from the sequencing and culturing results are glycan degraders or glycan beneficiaries remains unknown. Given that glycan-degrading bacteria are needed for glycan beneficiaries to utilise the glycan indirectly, a better understanding of this would help explain the variable results of the *Fut2* impact on the gut microbiome discussed in the previous section of this chapter. Specifically, why is the microbiome comparable between secretors and non-secretors reported in some human studies while other studies draw the opposite conclusion? Third, 16S sequencing and MALDI-TOF were employed across the study; however, both approaches have limitations, raising the question of whether the glycan-utilising properties of gut microbes are strain specific or species specific. Answering these questions would help us better understand the mechanisms behind the impact of host genetics on the gut microbiome and bacterial colonisation.

6.6 Summary

The results presented in the thesis contribute to understanding host–microbiome interactions. Scientifically, the thesis provides evidence to support the impacts of host genetic factors like the *Fut2* gene on the gut microbiome and bacterial adaptations in the gut. Clinically, this thesis also highlights several important points on inter-individual variance in probiotic efficacy, which are listed below:

6.6.1 Check on secretor status before probiotic supplementation

First, it is important to test the secretor status before probiotic supplementation. Findings in this thesis suggest that around one in five individuals who cannot express α(1,2)-fucosylated glycans on mucosal surfaces will experience different probiotic population dynamics following supplementation compared to those who can if the probiotic taken contains one of the many bacterial species able to utilise α(1,2)-fucosylated glycans. This was reflected in the significantly greater transience of *B. infantis* in the faecal and intestinal microbiome of non-secretor (KO) mice compared with secretor mice following antibiotic exposure.

6.6.2 Carefully select probiotic strains in probiotic therapy

Second, checking the glycan utilisation capability of the probiotic strains taken is always useful. Bifidobacterial species commonly used as probiotics are relatively close phylogenetically but differ in their ability to use glycans. This thesis has shown that neither *B. bifidum* nor *B. breve*, which do not internalise and degrade α(1,2)-fucosylated glycans, differ in their abundance or persistence in secretor and non-secretor animals. In contrast, *B. infantis*, which encodes fucosidases capable of internalising and degrading α(1,2)-fucosylated glycans[129], persisted significantly longer and showed a significantly higher abundance in secretor mice than in non-secretor mice. Evidence for such variance in bifidobacterial dynamics based on recipient secretor status has been reported in a previous study of breastfed infants, where maternal

secretor status was associated with the infant gut bifidobacterial community composition and abundance[284].

6.6.3 Supplementing α(1,2)-fucosylated glycans might improve clinical outcomes for non-secretors

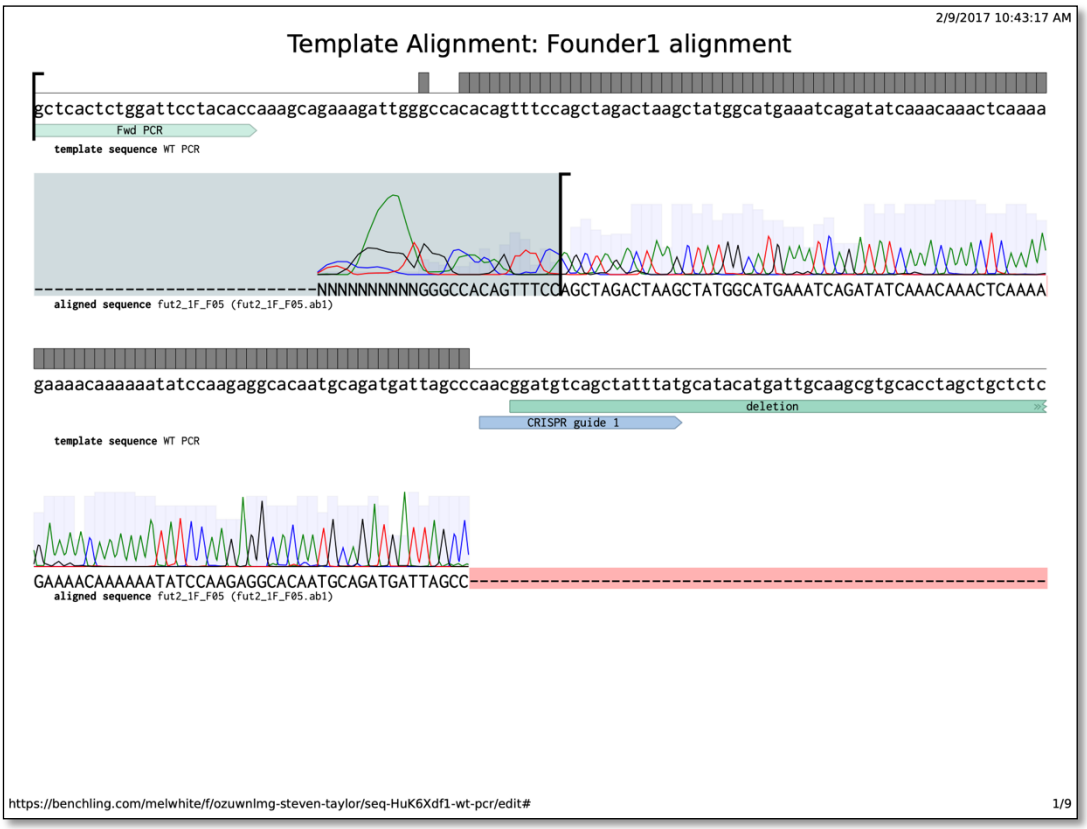
As mentioned in Section 6.6.1, around one-fifth of individuals unable to express α(1,2)-fucosylated glycans on mucosal surfaces will experience different probiotic population dynamics following supplementation compared to those who can if the probiotic taken contains one of the many bacterial species able to utilise α(1,2)-fucosylated glycans. In other words, non-secretors might have less favourable clinical outcomes when a glycan-utilising probiotic was supplemented. Thus, it is crucial to consider individual host traits when designing a probiotic intervention. A previous report has shown that supplementation with human milk oligosaccharides can enhance *B. infantis* engraftment[224], with successful supplementation shown to reduce intestinal inflammation in infants[277]. These findings together with findings in the Chapter 5 highlight that consideration of additional α(1,2)-fucosylated glycans, given as prebiotics, may be necessary for successful for successful *B. infantis* supplementation in non-secretor individuals.

CHAPTER 7. APPENDIX

In the Chapter 7, supplemental table and figures are provided. Details were presented based on the following structure:

- *Appendix 2.1* SAGC report on deletion of *Fut2* gene using CRISPR-Cas 9 gene editing technology
- *Appendix 2.2 Fut2* genotyping PCR
- *Appendix 2.3* Standard operating procedure for oral gavage
- *Appendix 2.4* Manufacturer's instruction of PowerLyzer PowerSoil DNA Isolation Kit
- *Appendix 2.5* Codes used for bioinformatic processing on 16S sequencing results
- *Appendix 2.6* Rarefaction curves of 16S sequencing
- *Appendix 3.1* Evaluation of cage effects on gut microbiome
- *Appendix 3.2* Contribution of each taxon on microbiome composition difference
- *Appendix 3.3* Microbiome composition differences
- *Appendix 3.4* Sex effect on α-diversity
- *Appendix 3.5* Genotype effect on α-diversity in single sex type
- *Appendix 3.6* Cage differences of Faith's phylogenetic diversity and Chao's richness
- *Appendix 3.7* Cage differences of Faith's phylogenetic diversity within each genotype

Appendix 2.1 *SAGC report on Fut2 knockout model development using CRISPR-Cas9 gene editing technology*

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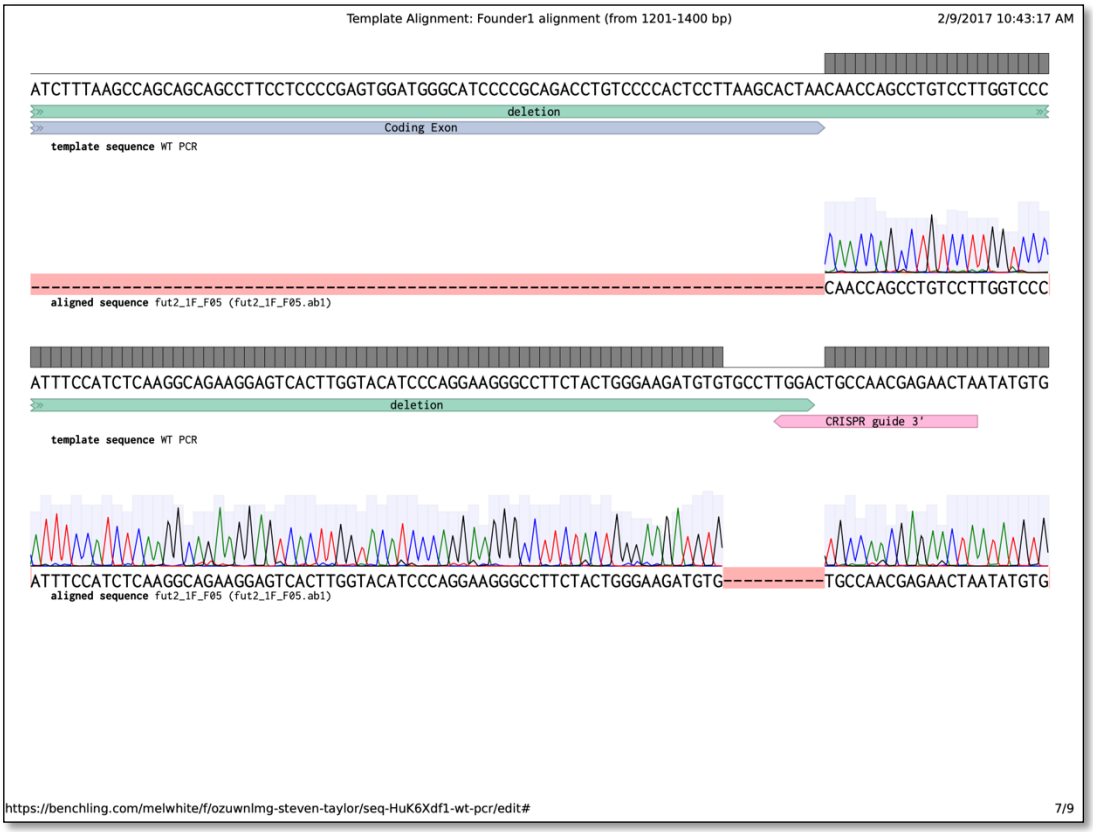
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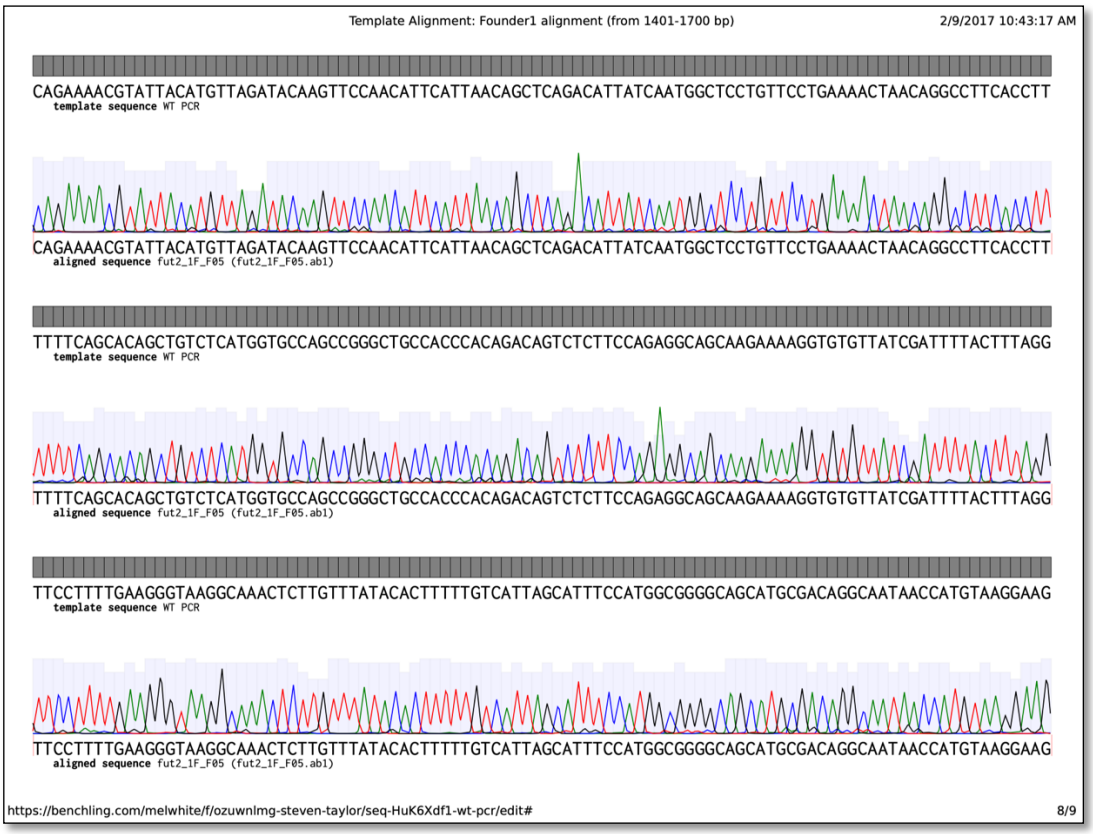
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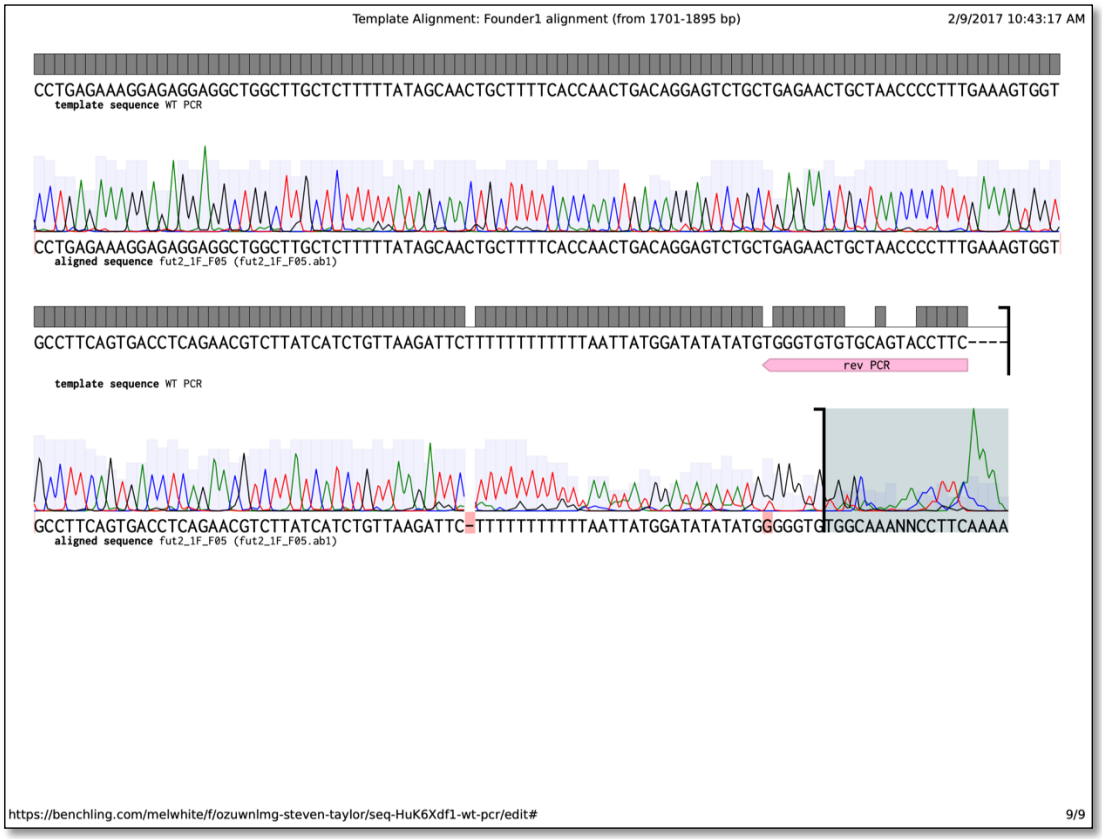
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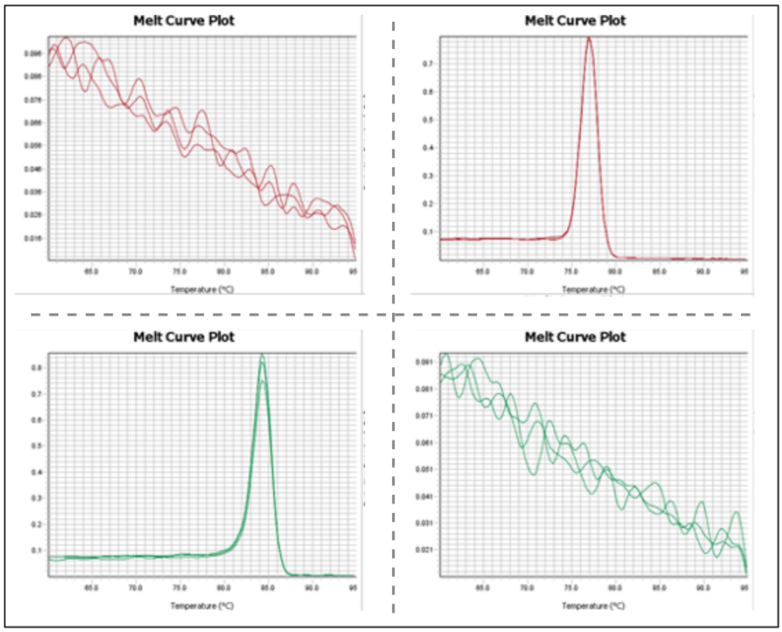
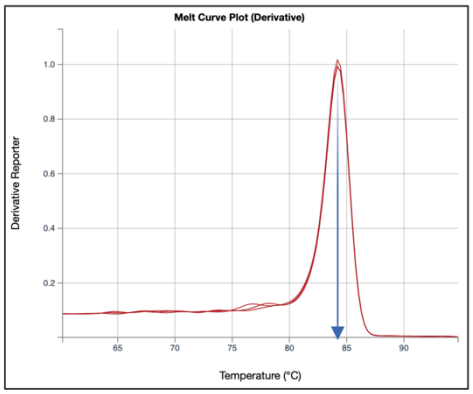
Appendix 2.2 *Fut2 genotyping PCR*

A.

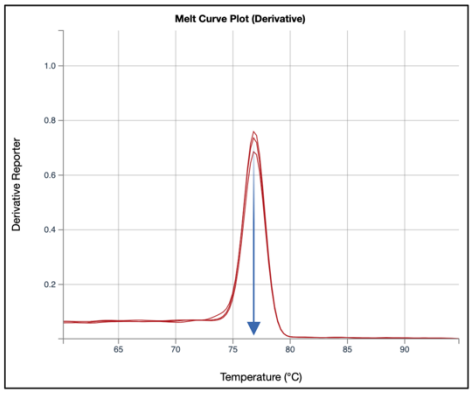


B.

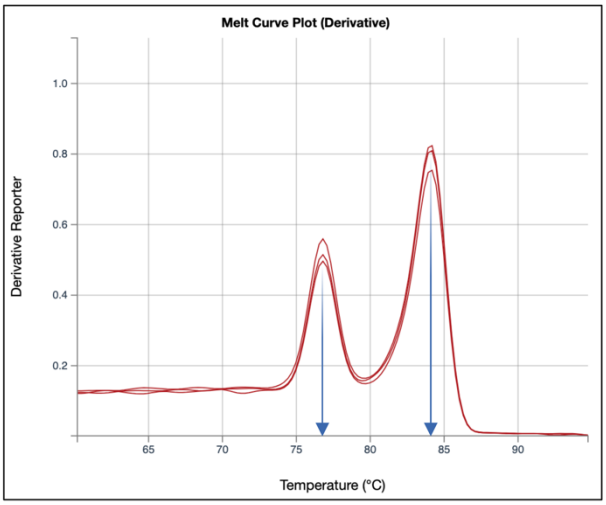
Fut2 $^{+/+}$ **(WT)**: One peak at 84.4 °C



Fut2 $^{-/-}$ **(KO)**: One peak at 76.9 °C



Fut2 $^{+/-}$ **(HET)**: Two peaks at 84.4 °C and 76.9 °C



Appendix 2.3A Excelsior Tissue Processor

Standard Operating Procedure

Excelsior Tissue Processor

SAHMRI South Australian Health & Medical Research Institute

1. Introduction

1.1. Purpose

To ensure safe operation of the Excelsior Tissue Processor, by minimising the likelihood of injury to the user and/or damage to the equipment.

To eliminate the possibility of burns due to molten paraffin wax, and to minimise exposure to chemicals including formalin, ethanol, and xylene.

To demonstrate how specimens are to be handled correctly, therefore minimising disruption to the tissue, and to ensure samples are not mixed up between cassettes.

To maintain the Excelsior Tissue Processor and its surrounding tidy, free from solidified paraffin wax build up and minimise hazards due to wax drips and chemical spills.

1.2. Scope

All users be taken through an induction procedure prior to being allowed to operate the Excelsior Tissue Processor.

Any deviation from this protocol must be reported to the Histology Technician or the Histology Manager.

1.3. Regulatory Requirements

N/A

2. Definitions

1. Screen
2. Keypad
3. Carbon filter
4. Reaction Chamber
5. Disk drive & Vapour filter
6. Wax & Reagent storage

.

3. Responsibilities

All personnel entering the laboratory are expected to abide by the information given at induction. If there are any doubts, the user is strongly urged to seek the assistance of either the Histology Technician or the Histology Manager prior to operation.

4. Safety and Personal Protective Equipment

Correct PPE must always be worn. This includes; a lab coat, nitrile gloves and enclosed rubber sole shoes.

Staff must adhere to safety information received at induction into the laboratory.

Staff must adhere to the Bioresources Lone Working Policy.

5. Equipment, Supplies and Solutions

Shandon Excelsior ES Tissue Processor.

Forceps, paraffin wax, formalin, ethanol, alcohol, water, cassette racks.

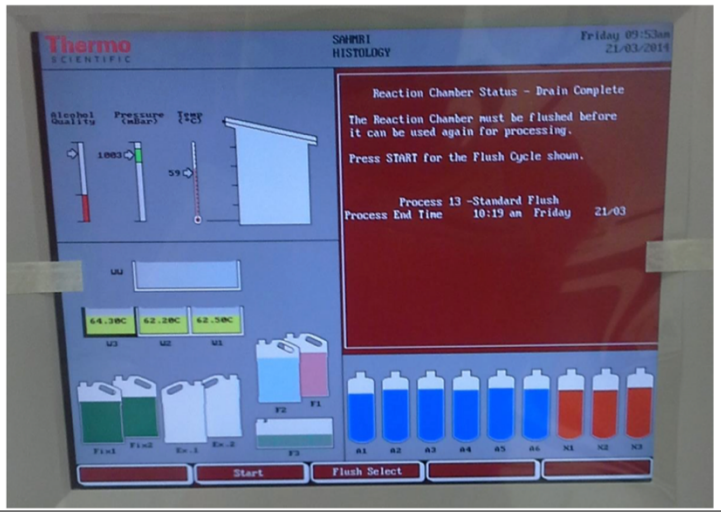
PPE (Lab coat, nitrile gloves & rubber sole shoes).

6. Procedure

6.1. Normal Everyday Operation

Ensure cassettes are adequately labelled and have undergone fixation in 10% neutral buffered formalin, or similar, for at least 24 hours.

6.2. Ensure the reaction chamber has been flushed following the previous run. If not, the following screen will be displayed. Press the button on the keypad corresponding with the "Start" option on the screen. **Flushes should be carried out immediately following the removal of cassettes after a processing run. Failure to do so may cause a blockage.**

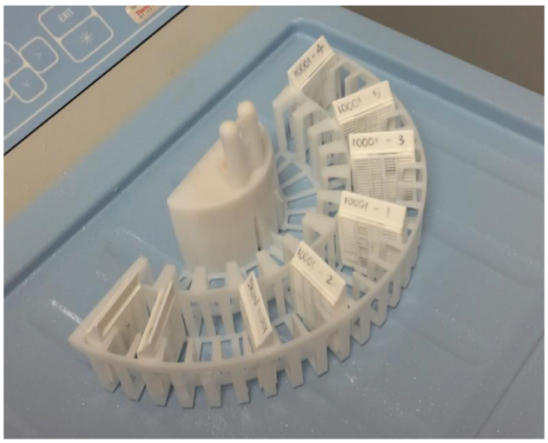


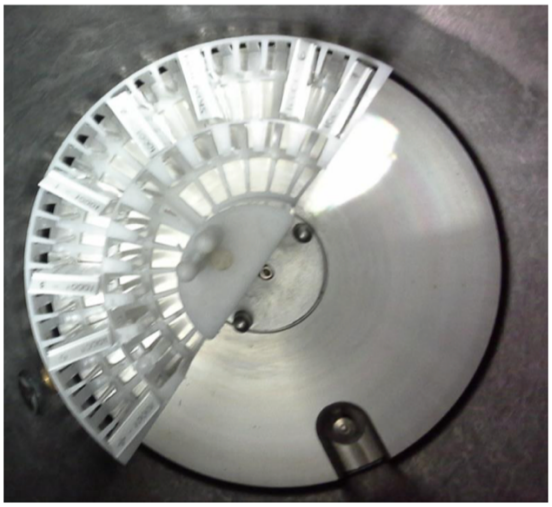
6.3. If a flush is required, ensure the 4 sensors within the reaction chamber are carefully wiped clean with the sponge found in the cupboards to the left of the processor, labelled "Processor Accessories". Please ensure seals are cleared of wax. No wax or reagent residue should be caked on the sensors. This will provide false readings of fill levels within the reaction chamber.

6.4. Open the reagent storage doors to check the wax levels. Wax levels should be kept within 2mm of the top dividing walls between wax tanks. If wax requires a refill, use the provided wax fill chute and top up with Histoplast paraffin wax. Do not overfill wax.

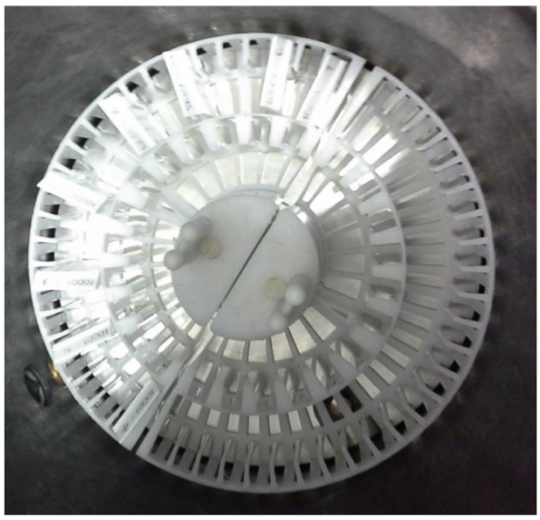
6.5. Check that all reagent pipes are correctly inserted in their corresponding bottles. (Red: Xylene, Blue: Ethanol, Green: Water).

6.6. Wearing nitrile gloves, load up cassettes into the white organised baskets. It is recommended that organised baskets are used to minimise mix ups and to allow for maximum immersion in reagents. Open reaction chamber lid by sliding the knob upwards, and place basket in the reaction chamber.





6.7. Balance out the white semi-circular organised basket with an additional semi-circular organised basket as per the image below. There is no need to evenly distribute cassettes as with a centrifuge. Slot the cover on the top with the agitation drive pins threaded though the holes in the cover. Close the reaction chamber lid.



6.8. Once the cassettes are loaded and the reaction chamber lid is closed the user will observe the following screen. Follow the onscreen instructions, starting with selecting the basket fill level by pressing the corresponding button on the keypad (1). Secondly input the cassette count by inputting the number of cassettes loaded with the number pad (2). Thirdly, select required process time for specimen type (3). Please refer to the appendices at the end of this document for a list of pre-set process runs. Finally, once the processing run has been selected set the desired end time or start process immediately. **By default, specimens will remain in formalin until processing begins.**

6.9. During a processing run, the monitoring screen will be displayed. Although not recommended, it is possible to add additional cassettes to the processing run by selecting "LID RELEASE", the reagent level may need to be adjusted accordingly. Additionally, it is possible to abort the processing run by selecting "STOP" and then, "ABORT".

6.10. Once the processing run is complete, cassettes will be submerged in paraffin wax. Follow the onscreen prompts to remove the cassettes. Firstly, drain the reaction chamber of the molten paraffin wax by pressing the corresponding button on the keypad with the onscreen "DRAIN" option. Carefully, remove the baskets and place them on the provided plastic tray. Minimise the dripping of wax on the processor. Immediately clean any wax spills or drips. **Beware of hot molten wax.** Immediately place cassettes in the embedding station tissue storage drawer (refer to Embedding Station SOP#XX). Wipe away excess wax from the reaction chamber ensuring the sensors are free from residue.

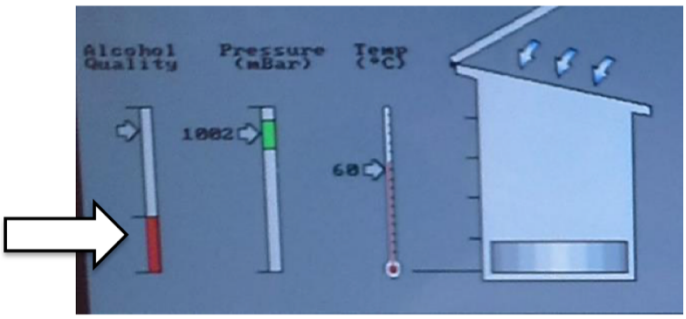
6.11. Place empty baskets back into the reaction chamber, and close the lid.

6.12. The "flush" screen will automatically be displayed. Select "Start" "Extended Flush". Once the flush is complete, follow the onscreen prompts to wipe the chamber. The processor will be ready for another run.

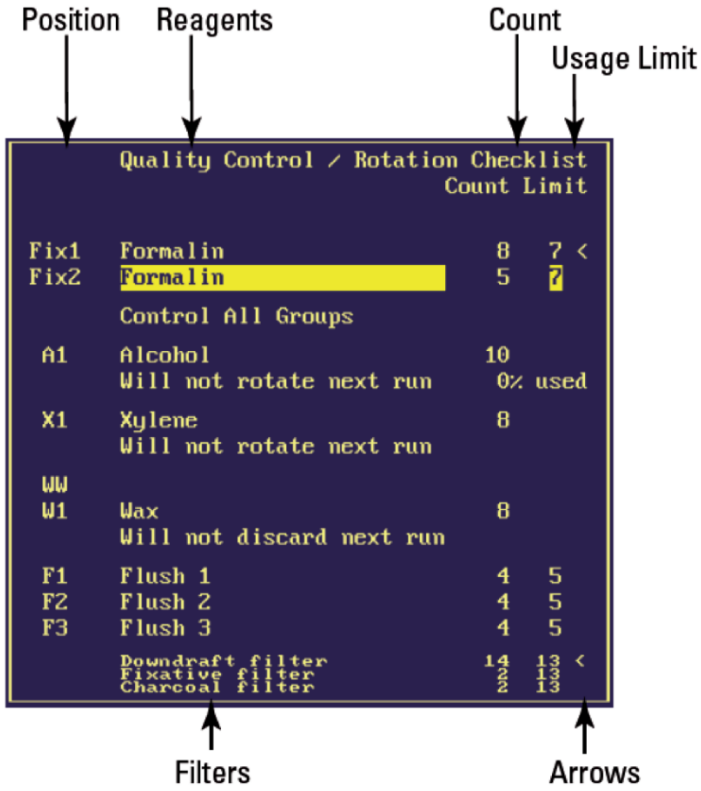
6.13. Once the flush cycle is complete, the following dialogue will be displayed. Again, ensure there is no residue adhering to the four sensors within the reaction chamber.

6.14. **Replacing Reagents – Conducted Occasionally**

The alcohol quality at bottle A1 is set as the trigger for reagent rotation. When the alcohol quality arrow reaches the red area, this will trigger reagent rotation and a wax dump.



6.15. Opening the reagent storage doors will prompt the quality control screen. By using the arrows on the keypad, one by one highlight the reagents which need to be discarded and select “REMOVE”. Remove dumped reagents bottles once prompted. Replace bottles in the storage area with new reagents and input the changes by highlighting the reagents and selecting “NEW”.



7. Associated Documents

7.1. External

- Excelsior Tissue Processor Manual, Thermofisher

7.2. Internal

- Bioresources Lone Working Policy

Appendix 2.3B HistoStar Embedding Station

Standard Operating Procedure

HistoStar Embedding Station

SAHMRI South Australian Health & Medical Research Institute

1. Introduction

1.1. Purpose

To ensure safe operation of the HistoStar Embedding Station, by minimising the likelihood of injury to the user and/or damage to the equipment.

To eliminate the possibility of burns due to molten paraffin wax and to minimise mess.

To demonstrate how specimens are to be handled correctly, therefore minimising disruption to the tissue, and to ensure sample are not mixed up between cassettes.

To maintain the embedding station tidy and free from solidified paraffin wax build up.

1.2. Scope

All users be taken through an induction procedure prior to being allowed to operate the HistoStar Embedding Station.

Any deviation from this protocol must be reported to the Histology Technician or the Histology Manager.

1.3. Regulatory Requirements

N/A

2. Definitions

N/A

3. Responsibilities

All personnel entering the laboratory are expected to abide by the information given at induction. If there are any doubts, the user is strongly urged to seek the assistance of either the Histology Technician or the Histology Manager prior to operation.

4. Safety and Personal Protective Equipment

Correct PPE must always be worn. This includes; a lab coat, nitrile gloves and enclosed rubber sole shoes.

Staff must adhere to safety information received at induction into the laboratory.

Staff must adhere to the Bioresources Lone Working Policy.

5. Equipment, Supplies and Solutions

Thermo Scientific HistoStar Embedding Station.

Forceps, spatulas, moulds, paraffin wax and paper towel.

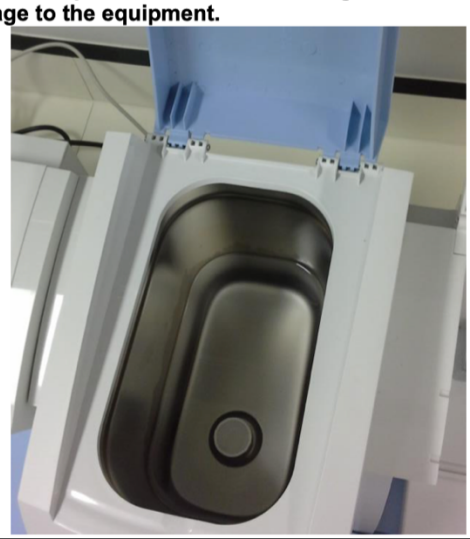
PPE (Lab coat, nitrile gloves & rubber sole shoes).

6. Procedure

6.1. Ensure the HistoStar Embedding Station is switched on both at the power outlet and the black power switches at the rear of the station. Press the "ON" button on the touch screen.

6.2. Place cassettes containing processed tissue to be embedded in the tissue storage drawer located on the right hand side.

6.3. Prior to embedding, ensure that the paraffin wax has sufficiently melted. This can be checked by opening the blue plastic lid located at the top/middle of the station and checking the wax tank. **Operation of the embedding station without molten wax may cause damage to the equipment.**



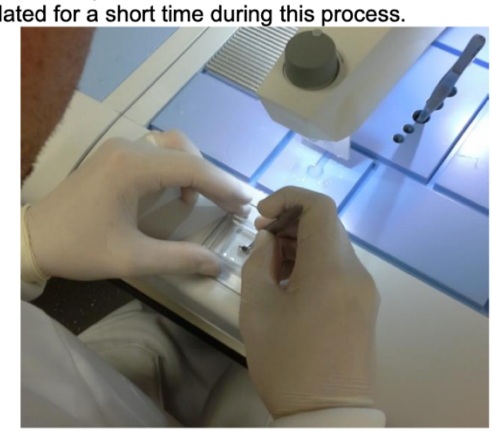
6.4. If required, the paraffin wax tank can be topped up with additional paraffin wax pellets. 1kg of Histoplast paraffin wax is more than sufficient. **Excessive amounts of paraffin wax may cause spills and increase time taken to sufficiently melt.**

6.5. Once paraffin wax has become molten and the internal temperature of the wax tank has stabilised, the embedding station is operational. Temperatures can be checked by pressing the "Status" button on the home screen. The paraffin wax tank is set to a maximum temperature of 70°C. To return to the home screen, press the back arrow button.

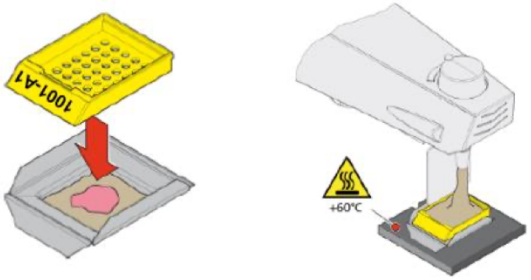
6.6. Limit embedding to **one cassette at a time** to avoid mixing up cases. Place desired cassette on the heated area in front of the tissue storage drawer. Carefully open cassette and select a clean, suitably sized mould from the heated storage compartment.

6.7. Place or hold mould under the wax dispenser. Push the lever located under the dispenser away from body to cover the base of the mould with molten wax. **Beware of molten wax ejecting from the dispenser.**

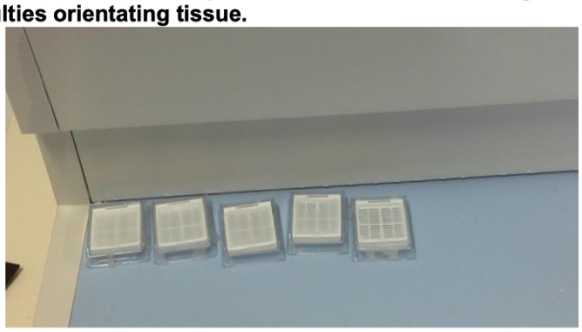
6.8. Working reasonably quickly, orientate tissue with heated forceps and place the base of the mould on the cold spot when satisfied with orientation. Tissue can be carefully manipulated for a short time during this process.



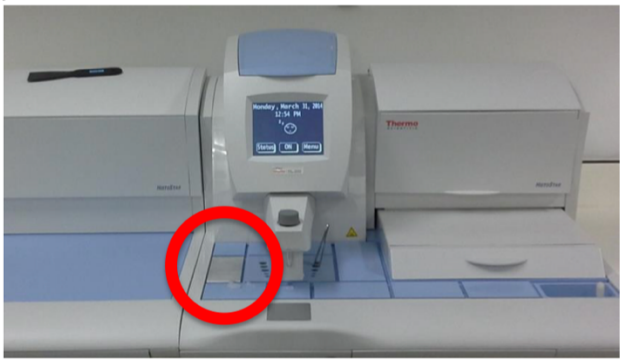
6.9. Place the labelled empty cassette on top of the mould and fill wax through the mould ensuring that wax is sufficiently covering the inside surface area of the cassette. **Too little wax will cause the specimen to break away from the cassette during sectioning. Too much wax will make it difficult to fit the cassette in the chuck at the microtome.**



6.10. Immediately place the base mould with cassette on the cold plate, being careful not to drip wax across the station. Immediately swap or replace forceps in the heated forceps blocks. **Non-heated forceps will result in tissue sticking to forceps and cause difficulties orientating tissue.**

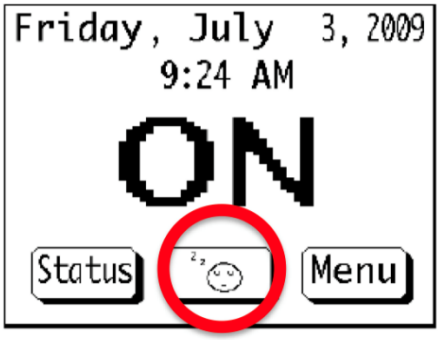


6.11. Once fully cooled and set, the cassette can be removed from the mould. Excess wax adhered to the edges of the cassette can be trimmed using the heated para trimmer. This ensures ease in fitting the cassette into the chuck for microtome sectioning.



6.12. Once finished embedding, the work station requires to be cleaned. Scrape solidified wax using the black plastic spatula labelled "WAX" onto the heated area or into a waste bin. Wipe the tissue storage drawer and the heated area with a tissue to remove excess wax. Replace unused moulds into the heated storage compartment upside down to ensure sufficient drainage of molten wax. Empty the waste wax drawer.

6.13. Press the "SLEEP" button on the touchscreen and switch off the cold plate at the small black switch located at the rear.



6.14. Scrape ice off cold plate and place paper towel on the surface to absorbed moisture.

6.15. Ensure any wax dropped on the floor is scraped off and swept into a bin. **Solidified wax can cause a slip hazard.**

7. Associated Documents

7.1. External

- N/A

7.2. Internal

- N/A

7.3. Appendices

- N/A

Appendix 2.3C Oral gavage

Standard Operating Procedure

Administration of Substance by Oral Gavage (Mice)

SAHMRI South Australian Health & Medical Research Institute

1. Introduction

1.1. Purpose

1.1.1. To use an oral gavage cannula to place material directly into the stomach of a mouse.

1.2. Scope

1.2.1. Only those individuals who have been signed off as competent in their training records are permitted to follow the procedure outlined in this SOP.

1.2.2. Any deviation from this protocol must be reported to the Bioresources Veterinary Manager.

1.2.3. Staff using this SOP must have read and understood all SOPs listed in section 7.

1.3. Regulatory Requirements

1.3.1. Prior to undertaking work/animal work within in the Bioresources facility, suitable approval from the AEC must be granted and any work carried out must be on an approved and active project.

1.3.2. The mandatory training courses must be attended, please refer to the Bioresources webpages on the SAHMRI website.

1.3.3. All personnel must be trained, competent and signed off in humane culling of mice.

2. Definitions

2.1. IVC Individually Ventilated Cages

2.2. CCS Cage Changing Station

3. Responsibilities

3.1. All personnel entering the building are expected to abide by the information given at induction, and ensure that they are not potentially carrying pathogens from another animal facility (see SOP-0176 Entering and Exiting Bioresources).

3.2. All personnel are expected to use the skills and advice given on the specific training courses in handling rodents when carrying out work in Bioresources.

3.3. All personnel conducting gavage procedures in mice must be trained and deemed competent in cervical dislocation.

4. Safety and Personal Protective Equipment

4.1. Correct PPE must always be worn. This includes; gloves, scrubs, mop cap, and unit shoes.

4.2. Staff must adhere to safety information received at induction into the facility.

4.3. Staff working outside of normal hours, must report to security and follow SAHMRI wide out of hours policy.

5. Equipment, Supplies and Solutions

5.1. Equipment

5.1.1. Individually ventilated cages.

5.1.2. Cage changing station.

5.1.3. Oral gavage cannula (flexible plastic is preferred).

5.1.4. Solution to administer in an Eppendorf or 10 mL tube.

5.1.5. 50 mL tube with Millipore water.

5.1.6. Syringes (1 mL and 5 mL) for flushing the cannula and administering the treatment.

5.1.7. Disposable paper tissues (e.g. Kleenex or similar).

6. Procedure

6.1. General Information

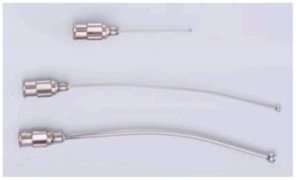
6.1.1. Oral dosing requires experience and therefore can only be carried out by a **competent person** who has been assessed by Bioresources staff.

6.1.2. Oral gavage cannula are of 2 main types:

In mice, 18-22G gavage tubes are generally used. Smaller tubes (higher gauge) for smaller mice.

Disposable flexible plastic: Plastic may be preferred by inexperienced individuals as there is less risk of tissue damage, however, there is a risk of mice chewing through the plastic and swallowing it. Plastic feeding tubes are usually meant to be single use and must be disposed of when showing signs of wear/leaks, or between each cage and each drug treatment. Please follow manufacturer's recommendations.

Stainless steel: With appropriate training, use of stainless steel gavage tubes are recommended, as mice cannot chew through it and it is reusable. There are also straight and curved stainless steel types; curved is preferred though straight can work well in experienced hands. Steel tubes must be washed out with 80% ethanol then rinsed with saline between each cage, and then autoclaved after use.



6.1.3. The amount to be dosed will depend on the AEC approved project specifications, the size and possible strain of mouse. Overnight food removal may be required if permission has been granted to do so in the AEC approved project. Recommended volume as per 2008 NHMRC Guidleines is 10ml/kg body weight, i.e. 0.2ml for a 20g mouse

6.1.4. Accidental entrance of the trachea is possible and may be indicated by the cannula not being placed as far into the animal as expected or the animal showing signs of distress i.e. extremely agitated and struggling more than normal. If this occurs remove the cannula and try to re-insert once more. If on the second attempt correct positioning does not occur return the mouse to its home cage and allow at least five minutes for the animal to calm down.

6.1.5. Accidental Lung dosing: If you do dose into the animal's lungs, on returning the animal to the cage its respiration rate will become rapidly laboured and you will be able to see the animal has some difficulty in breathing. If this occurs, euthanize the mouse immediately by cervical dislocation and report incident to Bioresources Veterinary Manager.

6.1.6. As an alternative to the gavage method, some materials may be consumed voluntarily in palatable mixtures (e.g. flavoured gelatine), however gavaging is recommended if mice require specific dosing. Material can also be dosed using a small flexible catheter introduced only into the animal's mouth

6.2. **Loading the syringe and needle**

6.2.1. Fill a sterile syringe with required amount of drug/agent (can draw up total amount required for the whole cage). Remove sterile plastic or steel cannula from its sterile packaging then attach cannula to the end of the syringe.

6.2.2. If air bubbles are present gently flick syringe to disperse them to the top and then push forward on the syringe until a small amount of agent becomes visible on the tip of the cannula.

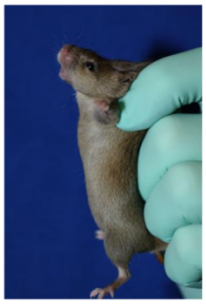
6.2.3. Wipe any excess agent from the end and sides of the cannula with paper towel/gauze.

6.3. **Restraining the animal and oral dosing**

6.3.1. Place the IVC cage inside a cleaned Class II biological safety cabinet if required as per the drug/agent's relevant risk assessment/SOP, or if the drug/agent is safe to work with, in a cage changing station or procedures room bench.

6.3.2. Check cage label, required dosage and animal identification as specified by the scientist.

6.3.3. Wearing gloves remove the mouse from its cage and scruff as in SOP-0293 Rodent Handling and Sexing ensuring that there is no movement of the animals head. (See Picture 1 below). If the scruff is sufficient, the mouth should be forced wide open and the eyes will be bulging.



A well-scruffed mouse with no head movement

Picture 1

6.3.4. Place the cannula in front of the mouse to gain an idea of how far the cannula will go before it reaches the stomach. The tube should extend from the tip of the nose to the last rib Tip of the cannula is not to be forced past this point and particular care must be taken with steel cannula.

6.3.5. Holding the animal in a vertical position (tail to the ground) with the underside of the animal facing you. Hold the syringe with the cannula attached into the animal's mouth.

6.3.6. Move the cannula over the back of the animal's tongue and slowly down the oesophagus and into the stomach keeping the syringe in an upright position. Slight pressure may be felt as the cannula moves through the sphincter muscle located at the top of the stomach (See picture 2 below). If using plastic, keep the cannula away from the teeth by holding it to the side so that the animal does not chew through the cannula. If the animal starts chewing the cannula remove it to check for leaks and replace with a new cannula if required.

Picture indicating the cannula going over the back of the tongue into the

Picture 2

6.3.7. If any resistance is felt while moving the cannula down the oesophagus of the animal, remove the cannula and try to reinsert. (see point 6.1.4).

6.3.8. Once the cannula has been positioned correctly in the stomach dispel the correct dosage, then slowly remove the cannula. Removing the cannula too soon/fast after the dosing may cause regurgitation.

6.3.9. Return the animal to its cage and monitor for any signs indicating lung dosing see 6.1.5. All animals subject to gavage should be checked again at close of business and twice the following day.

6.3.10. Reload syringe before the next animal if required (remove cannula first to keep solution sterile). If starting a new cage/treatment, wash out the steel cannula with 80% ethanol and rinse as in 6.1.2, or replace the cannula if it is plastic.

6.3.11. When finished all gavaging, wash out steel cannulas with 80% alcohol and rinse with saline, then place in a small autoclave pouch located in the dirty cage was ante-room for autoclaving, making sure to label the pouch with where it belongs and who it belongs to. Used plastic cannulas should be disposed of.

6.4. Recording

6.4.1. On E-mus, add the Oral Gavage Procedure record to the animals by selecting the checkbox of all animals who were gavaged, selecting “Modify” then completing the “Procedure” section of the form.

6.4.2. Enter a Health/Clinical Event and start a Clinical Record Sheet if required (refer to relevant ethics application).

6.4.3. Update the procedures section of the cage card including the substance and dose administered, date and initials.

6.4.4. If an animal died/was euthanised due to accidental lung dosing, record the death either in the running mortality sheet for the relevant ethics application, or enter an Incident Report on Emus. Notify the PI, Veterinary Manager, AWO/VO and colony manager.

Protocol: Experienced User

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- Shake to mix Solution C4 before use.

Procedure

1. Add up to 0.25 g of soil sample to the PowerBead Tube provided.
2. Add 750 µl of PowerBead Solution to the PowerBead Tube.
3. Add 60 µl of Solution C1 and invert several times or vortex briefly.
4. Bead beating options:
   A. PowerLyzer 24 Homogenizer: Place the PowerBead Tubes into the tube holder for the PowerLyzer 24 Homogenizer. The PowerBead Tubes must be balanced in the tube holder. Run the samples for a time and RPM suitable for your soil type.
   Note: For clay soils, 4,000 RPM for 45 s is the best starting point. For loose, granular and high organic soils, 2,500 RPM for 45 s will provide an optimal result.
   B. Vortex: Secure the PowerBead Tubes horizontally using a Vortex Adapter (cat. no. 13000-V1-24). Vortex at maximum speed for 10 min.
   Note: If you are using a 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5–10 min.
5. Make sure the PowerBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge at 10,000 x *g* for 30 s. Do not exceed 10,000 x *g*.
   Note: Centrifuge for 3 min at 10,000 x *g* for clay soils or if your soil is not completely pelleted after 30 s.
6. Transfer the supernatant to a clean 2 ml Collection Tube (provided).
   Note: Expect 400–500 µl. Supernatant may still contain some soil particles.

7. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.
   Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with the incubation we recommend you retain the step.
8. Centrifuge the tubes for 1 min at 10,000 x *g*. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube (provided).
9. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
   Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with the incubation we recommend you retain the step.
10. Centrifuge the tubes for 1 min at 10,000 x *g*. Avoiding the pellet, transfer up to 750 µl of supernatant into a clean 2 ml Collection Tube (provided).
11. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 s.
12. Load 675 µl of the supernatant onto an MB Spin Column and centrifuge at 10,000 x *g* for 1 min. Discard the flow-through and add an additional 675 µl of supernatant.
13. Centrifuge at 10,000 x *g* for 1 minute. Load the remaining supernatant onto the MB Spin Column and centrifuge at 10,000 x *g* for 1 min.
    Note: A total of three loads for each sample processed is required.
14. Add 500 µl of Solution C5 and centrifuge for 30 s at 10,000 x *g*.
15. Discard the flow-through. Centrifuge again for 1 min at 10,000 x *g*.
16. Carefully place the MB Spin Column in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the MB Spin Column.
17. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you may use sterile DNA-free PCR-grade water (cat. no. 17000-10) or TE buffer.
18. Centrifuge for 30 s at 10,000 x *g*. Discard the MB Spin Column.
19. The DNA is now ready for downstream applications.
    Note: We recommend storing DNA frozen (–90°C to –15°C) as Solution C6 does not contain EDTA. To concentrate DNA, see the Troubleshooting Guide.

Appendix 2.5 *Codes used for bioinformatic processing on 16S sequencing results*

Step 1. Load qiime2

```
module load qiime/2.2019.4
```

Step 2. Import sequencing files

```
qiime tools import --type 'SampleData[PairedEndSequencesWithQuality]' --inp
ut-path /home/wang2070/FUT2/D0_64 --input-format CasavaOneEightSingleLanePe
rSampleDirFmt --output-path S33_summary.qza
```

Step 3. Visualise reads using demux.sub to determine the cut-off value

```
qiime demux summarize --i-data S33_summary.qza --o-visualization S33_summar
y.qzv
```

Step 4. Denoise and dereplicate .qza file to remove chimeras and trim poor quality reads

```
# Change scripts in dada2. sh

qiime dada2 denoise-paired --i-demultiplexed-seqs S33_summary.qza --p-trim-
left-f 0 --p-trim-left-r 0 --p-trunc-len-f 187 --p-trunc-len-r 177 --o-repr
esentative-sequences rep-seqs-dada2.qza --o-table table-dada2.qza --o-denoi
sing-stats dada2-rep-seqs-stats.qza # Change your import file name, 250 and
 224 based on results from Step 4

# (Optional) Convert file dada2.sh to Unix format

dos2unix dada2.sh # Otherwise, errors occur

# Submit job to HPC

sbatch dada2.sh

# Check progress

squeue -l

# Important output: table-dada2.qza, save others to unimportant folder
```

Step 5. Visualise reads using visualise.sub

```
# Generate table-vis.qzv:

qiime feature-table summarize --i-table table-dada2.qza --o-visualization t
able-vis.qzv --m-sample-metadata-file mapfile_S33.tsv # Change file name

# Generate rep-seqs-vis.qzv:

qiime feature-table tabulate-seqs --i-data rep-seqs-dada2.qza --o-visualiza
tion rep-seqs-vis.qzv
```

Step 6. Create a tree for phylogenetic diversity measures using tree.sub

```
qiime alignment mafft --i-sequences rep-seqs-dada2.qza --o-alignment aligne
d-rep-seqs.qza

qiime alignment mask --i-alignment aligned-rep-seqs.qza --o-masked-alignmen
t masked-aligned-rep-seqs.qza

qiime phylogeny fasttree --i-alignment masked-aligned-rep-seqs.qza --o-tree
 unrooted-tree.qza

qiime phylogeny midpoint-root --i-tree unrooted-tree.qza --o-rooted-tree ro
oted-tree.qza
```

Step 7. Taxonomic assignment of sequences

```
# Change contents in sklearn.sh

qiime feature-classifier classify-sklearn --i-classifier silva-138-99-class
ifier-v4_q2021-11.qza --i-reads rep-seqs-dada2.qza --o-classification taxon
omy.qza

# Convert file sklearn.sh to Unix format

dos2unix sklearn.sh # Otherwise, errors occur

# Submit your job

sbatch sklearn.sh
```

Step 8. Visualise your unfiltered output taxonomy

```
qiime taxa barplot --i-table table-dada2.qza --i-taxonomy taxonomy.qza --m-metadata-file mapfile_S33.txt --o-visualization taxa-bar-plots.qzv # change mapfile type to txt.
```

Step 9. Filter out unwanted species and contaminations

```
qiime taxa filter-table --i-table table-dada2.qza --i-taxonomy taxonomy.qza --p-exclude mitochondria,chloroplast,Planctomycetes,eukaryota --o-filtered-table filtered-table.qza #Output name is filtered-table
```

Step 10. Rarefaction plotting

```
qiime diversity alpha-rarefaction --i-table filtered-table.qza --i-phylogeny rooted-tree.qza --p-max-depth 9883 --m-metadata-file mapfile_S33.tsv --o-visualization alpha-rarefaction.qzv # 10631 is your minimum reads, you want to check whether this minimum reads will cover most of OTUs
```

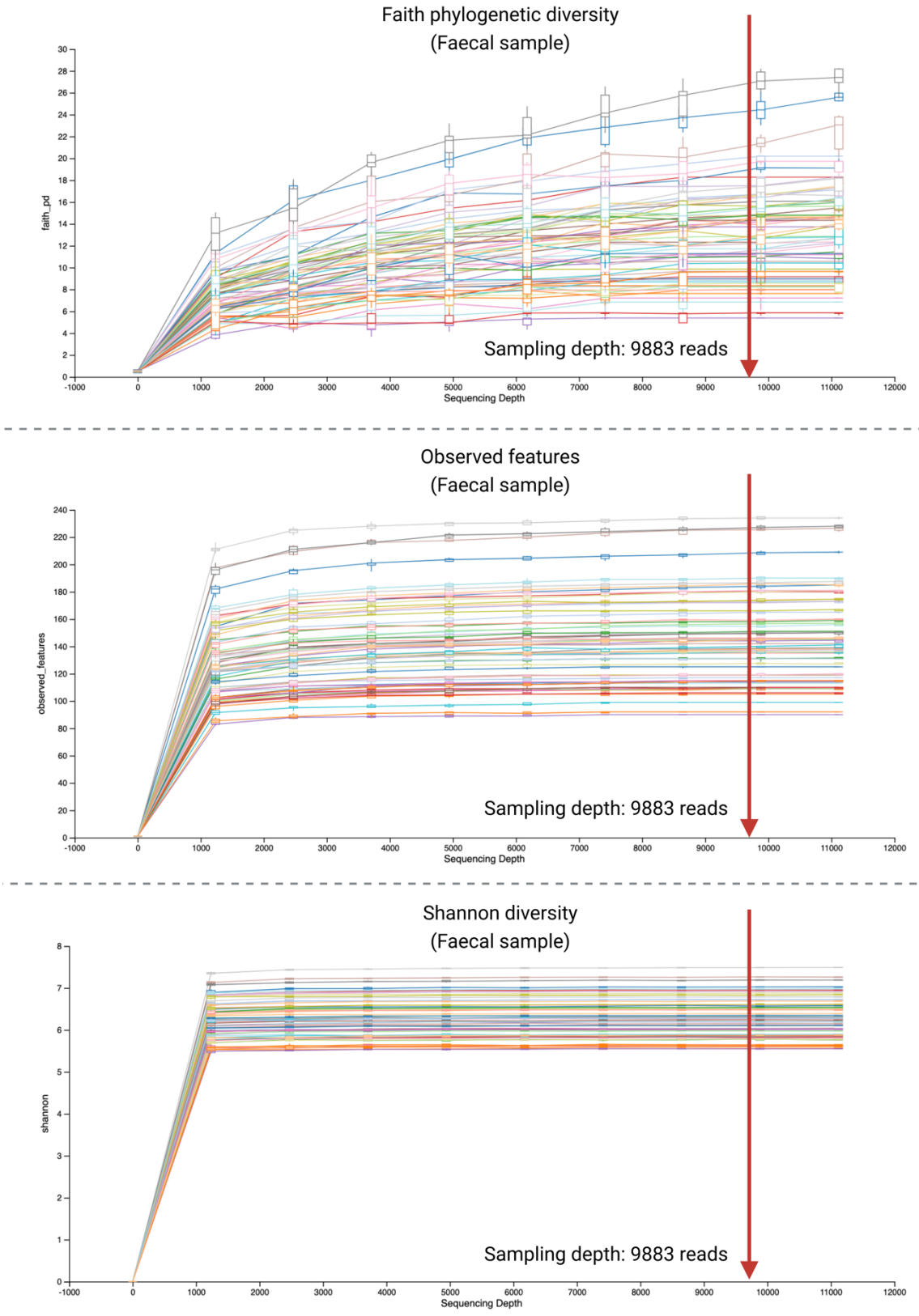
Step 11. Basic diversity metrics including rarefied table

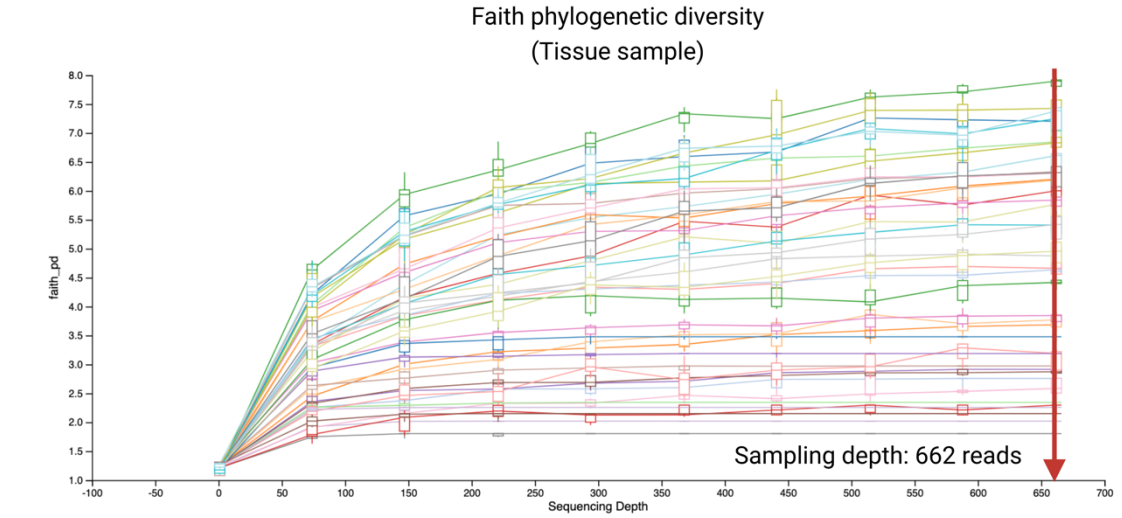
```
qiime diversity core-metrics-phylogenetic --i-phylogeny rooted-tree.qza --i-table filtered-table.qza --p-sampling-depth 9883 --m-metadata-file mapfile_S33.tsv --output-dir core-metrics-results


qiime tools export --input-path core-metrics-results/observed_otus_vector.qza --output-path /home/wang2070/FUT2/Result/observed_otus_vector #Optional


qiime tools export --input-path core-metrics-results/weighted_unifrac_distance_matrix.qza --output-path /home/wang2070/FUT2/Result/weighted_unifrac_distance_matrix
```

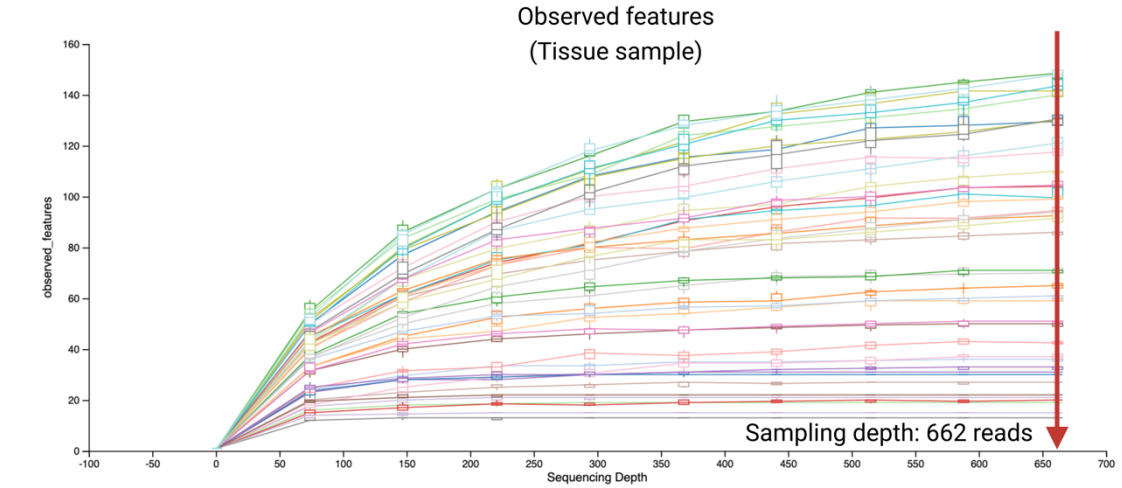
Step 12. Extract relative abundance file on filtered, rarefied table

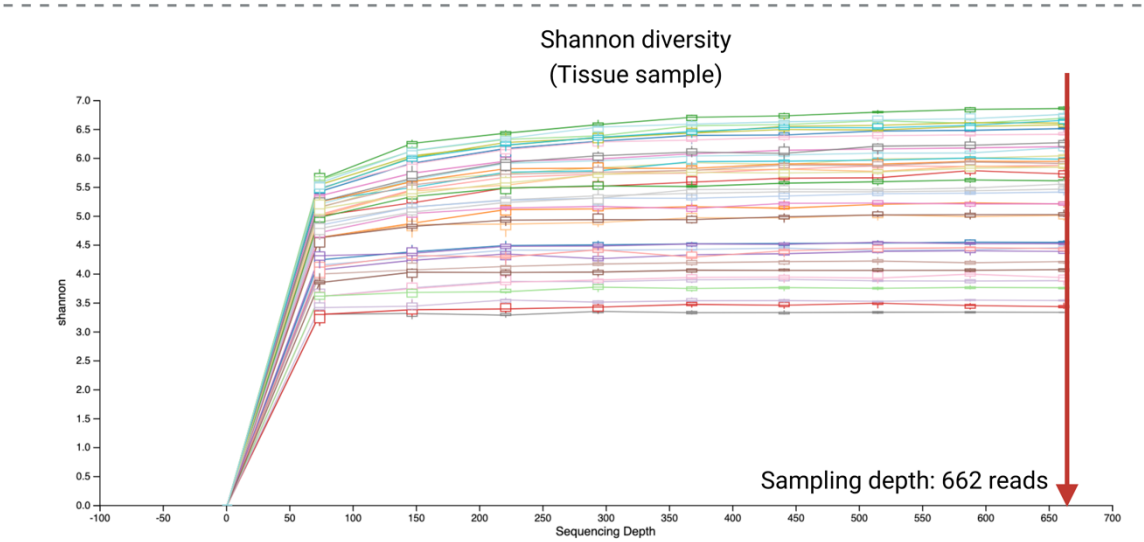
```
# Level 6 OTU table

qiime taxa collapse --i-table core-metrics-results/rarefied_table.qza --i-t
axonomy taxonomy.qza --p-level 6 --o-collapsed-table taxa-level6.qza

# Convert Level 6 OTU table to relative abundance table

qiime feature-table relative-frequency --i-table taxa-level6.qza --o-relati
ve-frequency-table taxa-rel-abu-level6.qza

# Convert relative abundance table.qza to .biom to .tsv

qiime tools export --input-path taxa-rel-abu-level6.qza --output-path taxa-
rel-abu-L6 #convert to .biom format first


biom convert --input-fp taxa-rel-abu-L6/feature-table.biom --output-fp taxa
-rel-abu-L6/table.from_biom.txt --to-tsv #converted to.txt or .tsv
```

Appendix 2.6 *Rarefaction curves of 16S sequencing*




Faith phylogenetic diversity
(Tissue sample)
faith_pd
Sequencing Depth
Sampling depth: 662 reads


Observed features
(Tissue sample)
observed_features
Sequencing Depth
Sampling depth: 662 reads


Shannon diversity
(Tissue sample)
shannon
Sequencing Depth
Sampling depth: 662 reads

Appendix 3.1 *Evaluation of cage effects on gut microbiome*

Other than sex, cage is another variable that could contribute to microbiome difference, which has been reported in many studies. To evaluate the impact of cage differences on gut microbiome, both α-diversity and β-diversity were compared among all cages used in this experimental model.

While the Chao's richness scores were comparable among all cages (p[Kruskal-Wallis: Chao's richness] = 0.090, Appendix 3.6B), an obvious cage difference was observed when comparing Faith's phylogenetic diversity scores among all cages (p[Kruskal-Wallis: Faith's phylogenetic diversity] = 0.033, Appendix 3.6A), indicating a potential cage effect on Faith's phylogenetic diversity. Considering that each cage only house mice with same genotype and genotype has been shown to significantly impact Faith's phylogenetic diversity, genotype might also contribute to the variance of Faith's phylogenetic diversity among all cages. To avoid the impact of genotype on our cage effect assessment, Faith's phylogenetic diversity scores were then compared among cages within each genotype. Results showed that cages within each genotype had a comparable Faith's phylogenetic diversity scores (p[Kruskal-Wallis: WT] = 0.076 and p[Kruskal-Wallis KO] = 0.080). Unlike the α-diversity, a significant β-diversity differences were observed among cages even when genotype and sex were adjusted in the analysis model (p[PERMANOVA] = 0.0001, Appendix 3.3).

Taken together, these results indicate that cage differences significantly impact gut microbiome in the aspect of Faith's phylogenetic diversity and microbiome composition.

Appendix 3.2 *Contribution of each taxon on microbiome composition difference*

Taxa	Average relative abundance	Standard deviation	Cumulative sum of contribution	Contribution of each taxon
Lactobacillus	0.083907	0.060228	0.252579	0.252579
Faecalibaculum	0.046166	0.037662	0.39155	0.138971
Muribaculaceae	0.031137	0.023221	0.485278	0.093729
Lachnospiraceae_unassigned	0.017511	0.016271	0.53799	0.052711
Lachnospiraceae_NK4A136_group	0.010423	0.00892	0.569364	0.031374
Clostridia_UCG014	0.00936	0.007029	0.597539	0.028175
Dubosiella	0.008561	0.007113	0.623311	0.025772
Bifidobacterium	0.007236	0.006285	0.645094	0.021783
Lachnoclostridium	0.006107	0.005669	0.663478	0.018384
Clostridium_sensu_stricto_1	0.005605	0.005778	0.68035	0.016872
Lachnospiraceae_uncultured	0.005052	0.004156	0.695557	0.015208
Alistipes	0.005037	0.003967	0.710721	0.015164
Enterorhabdus	0.004998	0.003915	0.725768	0.015046
Bacteroides	0.004946	0.003938	0.740658	0.01489
Prevotellaceae_UCG001	0.003519	0.003581	0.75125	0.010592
Turicibacter	0.003454	0.00583	0.761647	0.010397
Incertae_Sedis	0.003203	0.003215	0.77129	0.009643
Roseburia	0.003127	0.003241	0.780703	0.009413
Monoglobus	0.003012	0.002347	0.789769	0.009066
Eubacterium_siraeum_group	0.002988	0.00218	0.798765	0.008996
Peptococcaceae_uncultured	0.00289	0.002576	0.807463	0.008698
Mucispirillum	0.00287	0.00237	0.816102	0.008639
Parasutterella	0.002723	0.002791	0.824298	0.008197
Blautia	0.002482	0.002766	0.831771	0.007472
Lachnospiraceae_A2	0.002439	0.003744	0.839113	0.007342
Marvinbryantia	0.002322	0.00188	0.846101	0.006989
Candidatus_Saccharimonas	0.002321	0.001932	0.853088	0.006987
Eubacterium_coprostanoligenes_group	0.002302	0.001707	0.860017	0.006929
Atopobiaceae_unassigned	0.002231	0.002767	0.866732	0.006715
Lachnospiraceae_UCG001	0.002083	0.002581	0.873002	0.00627
Odoribacter	0.00199	0.001558	0.878991	0.005989
Lachnospiraceae_GCA900066575	0.001941	0.001787	0.884834	0.005843
Eggerthellaceae_unassigned	0.001904	0.001456	0.890565	0.005731
Romboutsia	0.00176	0.003188	0.895862	0.005297
Muribaculum	0.001736	0.001302	0.901089	0.005227
Oscillospiraceae_uncultured	0.00171	0.001731	0.906235	0.005146
Oscillospiraceae_unassigned	0.001632	0.001435	0.911149	0.004913
Candidatus_Arthromitus	0.001562	0.001814	0.915851	0.004702
Eubacterium_xylanophilum_group	0.001539	0.002174	0.920485	0.004634
Lachnospiraceae_FCS020_group	0.001393	0.00133	0.924677	0.004193
Erysipelotrichaceae_uncultured	0.001363	0.001074	0.928779	0.004102

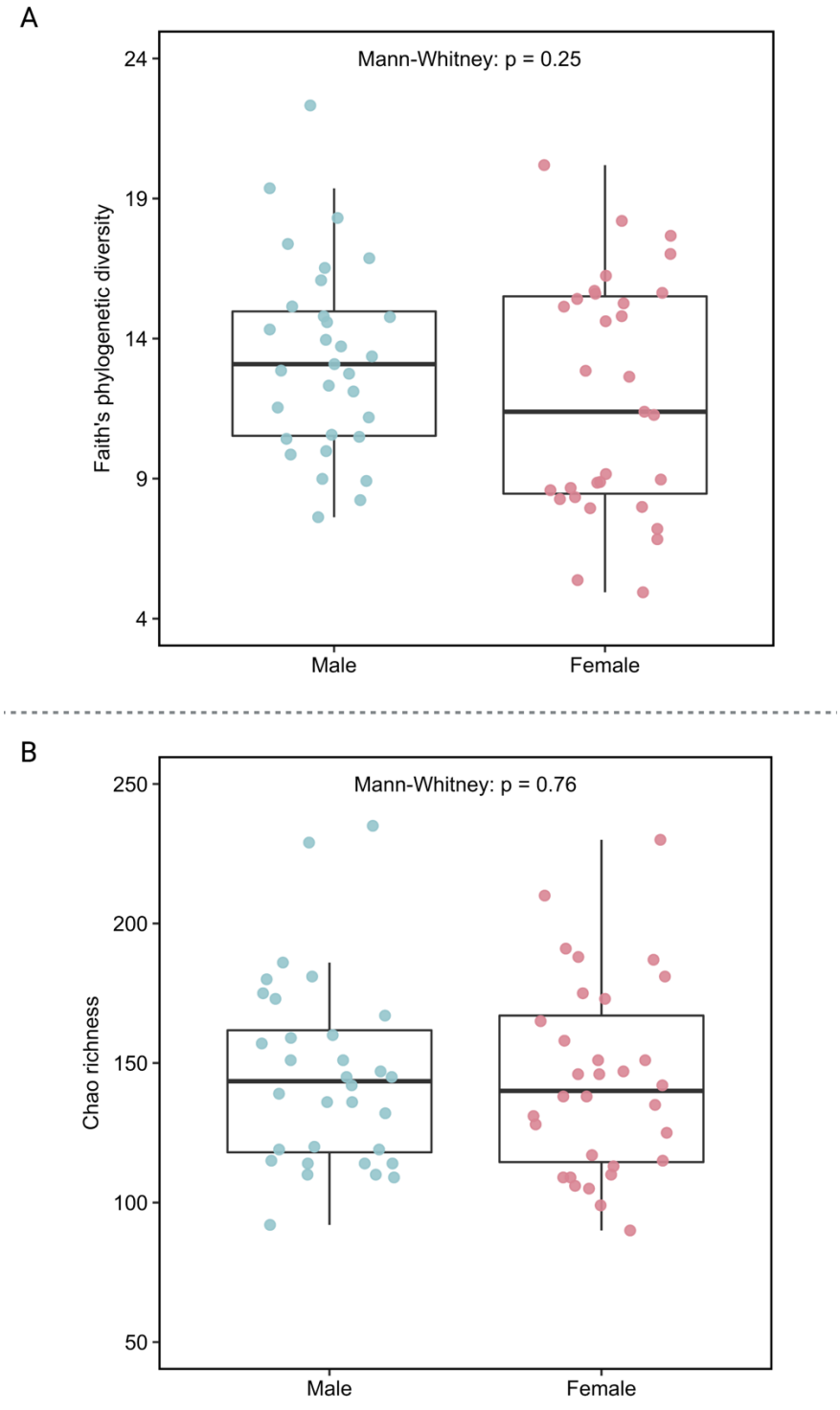
Lachnospiraceae_UCG006	0.001255	0.001664	0.932556	0.003777
Eggerthellaceae_DNF00809	0.001236	0.001028	0.936277	0.003721
Clostridia_vadinBB60_group	0.00119	0.001197	0.939859	0.003582
Dorea	0.001139	0.001141	0.943288	0.003429
Bacilli_RF39	0.001107	0.001686	0.946622	0.003333
Acetatifactor	0.001097	0.001189	0.949924	0.003302
Staphylococcus	0.001079	0.001175	0.953171	0.003247
Gordonibacter	0.000953	0.00076	0.95604	0.002869
Parabacteroides	0.000916	0.000874	0.958798	0.002758
Unassigned	0.000892	0.000655	0.961485	0.002687
Eubacterium_brachy_group	0.00087	0.00077	0.964102	0.002618
Oscillospirales_UCG010	0.00079	0.000904	0.966479	0.002377
Shuttleworthia	0.000772	0.001324	0.968804	0.002325
Colidextribacter	0.000724	0.001148	0.970984	0.00218
Ruminococcaceae_UBA1819	0.000717	0.000952	0.973141	0.002157
Ruminococcus	0.000709	0.000915	0.975276	0.002135
Oscillospiraceae_NK4A214_group	0.000682	0.000708	0.977331	0.002054
Oscillibacter	0.000655	0.001069	0.979303	0.001972
Jeotgalicoccus	0.000557	0.001099	0.980981	0.001678
Christensenellaceae_R7_group	0.000495	0.000435	0.98247	0.001489
Butyricicoccus	0.000482	0.000838	0.983919	0.001449
Eggerthellaceae_uncultured	0.000376	0.000413	0.985051	0.001132
Ruminococcaceae_unassigned	0.000342	0.00065	0.986081	0.00103
Defluviitaleaceae_UCG011	0.000342	0.000501	0.987111	0.00103
Atopostipes	0.000311	0.000785	0.988048	0.000937
Adlercreutzia	0.0003	0.00044	0.988952	0.000905
Anaerovoracaceae_Family_XIII_UCG-001	0.000293	0.000496	0.989834	0.000882
Anaerofustis	0.000276	0.000275	0.990664	0.00083
Lachnospiraceae_UCG004	0.000251	0.000583	0.991419	0.000756
Ruminococcaceae_uncultured	0.000244	0.000365	0.992154	0.000734
Tyzzerella	0.000243	0.000667	0.992885	0.000731
Streptococcus	0.000234	0.000269	0.993588	0.000703
Erysipelatoclostridiaceae	0.000219	0.000437	0.994246	0.000658
Peptostreptococcaceae_unassigned	0.000194	0.000469	0.994832	0.000585
Escherichia_Shigella	0.000183	0.000689	0.995383	0.000551
Erysipelotrichaceae	0.000161	0.000396	0.995867	0.000484
Anaerotruncus	0.00015	0.000359	0.996318	0.000452
Gastranaerophilales	0.000118	0.000445	0.996674	0.000356
Intestinimonas	0.000115	0.00028	0.99702	0.000345
Sporosarcina	0.000108	0.000351	0.997344	0.000324
Ruminococcaceae	0.000106	0.000385	0.997663	0.000319
Christensenellaceae_uncultured	7.99E-05	0.000232	0.997903	0.000241
Rhodospirillales_uncultured	7.23E-05	0.000123	0.998121	0.000218
Oscillospira	6.64E-05	0.00037	0.998321	0.0002
Planococcaceae_unassigned	6.56E-05	0.000214	0.998518	0.000198

Bacteria_unassigned	6.12E-05	0.00011	0.998703	0.000184
Erysipelotrichaceae_unassigned	4.74E-05	0.000114	0.998845	0.000143
Lachnospiraceae_ASF356	4.72E-05	0.000114	0.998987	0.000142
Eisenbergiella	4.43E-05	0.000247	0.999121	0.000133
Faecalibacterium	4.31E-05	0.000136	0.99925	0.00013
Bacillaceae_unassigned	3.79E-05	0.000155	0.999365	0.000114
Eubacterium_nodatum_group	3E-05	8.28E-05	0.999455	9.04E-05
Lachnospira	2.69E-05	0.000106	0.999536	8.09E-05
Clostridia_unassigned	1.98E-05	5.93E-05	0.999595	5.95E-05
Angelakisella	1.58E-05	5.28E-05	0.999643	4.76E-05
Paludicola	1.58E-05	8.81E-05	0.999691	4.76E-05
Corynebacterium	1.42E-05	7.93E-05	0.999733	4.28E-05
Oscillospiraceae_UCG005	1.26E-05	4.73E-05	0.999772	3.81E-05
Peptococcus	1.11E-05	4.33E-05	0.999805	3.33E-05
Curtobacterium	1.11E-05	4.33E-05	0.999838	3.33E-05
Prevotella	1.11E-05	4.69E-05	0.999872	3.33E-05
Clostridia_uncultured	7.9E-06	4.4E-05	0.999895	2.38E-05
Anaerovoracaceae_unassigned	7.9E-06	4.4E-05	0.999919	2.38E-05
Oscillospirales_unassigned	6.32E-06	3.52E-05	0.999938	1.9E-05
Harryflintia	6.32E-06	3.52E-05	0.999957	1.9E-05
Lactobacillaceae_unassigned	4.74E-06	2.64E-05	0.999971	1.43E-05
Oscillospirales_uncultured	3.16E-06	1.76E-05	0.999981	9.52E-06
Bacteroidales_unassigned	3.16E-06	1.76E-05	0.99999	9.52E-06
Pseudomonas	1.58E-06	8.81E-06	0.999995	4.76E-06
Aureimonas	1.58E-06	8.81E-06	1	4.76E-06

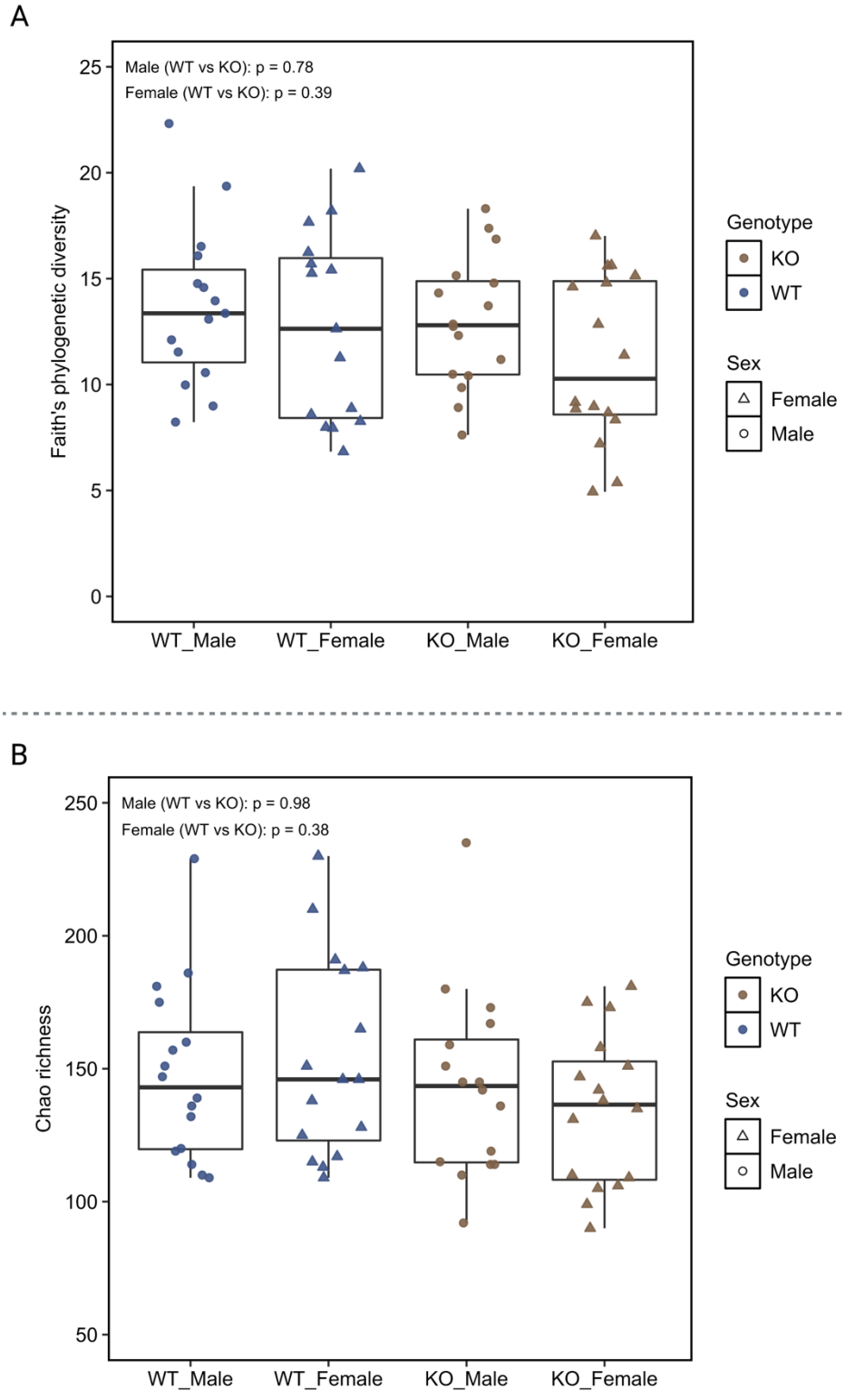
Appendix 3.3 *Microbiome composition differences*

Distance matrix	Matrix focus	Variables	R^2	Pseudo-F	P value
Bray-Curtis	Taxa abundance	Genotype	0.0278	2.7448	0.0280
		Sex	0.0217	2.1391	0.0721
		Cage	0.4948	3.0537	0.0001
		Residual	0.4557	N/A	N/A
Jaccard	Taxa presence/absence	Genotype	0.0193	1.3129	0.0346
		Sex	0.0189	1.2889	0.0391
		Cage	0.3014	1.2835	0.0001
		Residual	0.6604	N/A	N/A
Weighted UniFrac	1) Taxa abundance 2) Phylogenetic relatedness	Genotype	0.0222	2.1567	0.0763
		Sex	0.0169	1.6492	0.1498
		Cage	0.4987	3.0342	0.0001
		Residual	0.4622	N/A	N/A
Unweighted UniFrac	1) Taxa presence/absence 2) Phylogenetic relatedness	Genotype	0.0223	1.5300	0.0147
		Sex	0.0165	1.1321	0.2215
		Cage	0.3043	1.3028	0.0001
		Residual	0.6569	N/A	N/A

Appendix 3.4 *Sex effect on α-diversity*

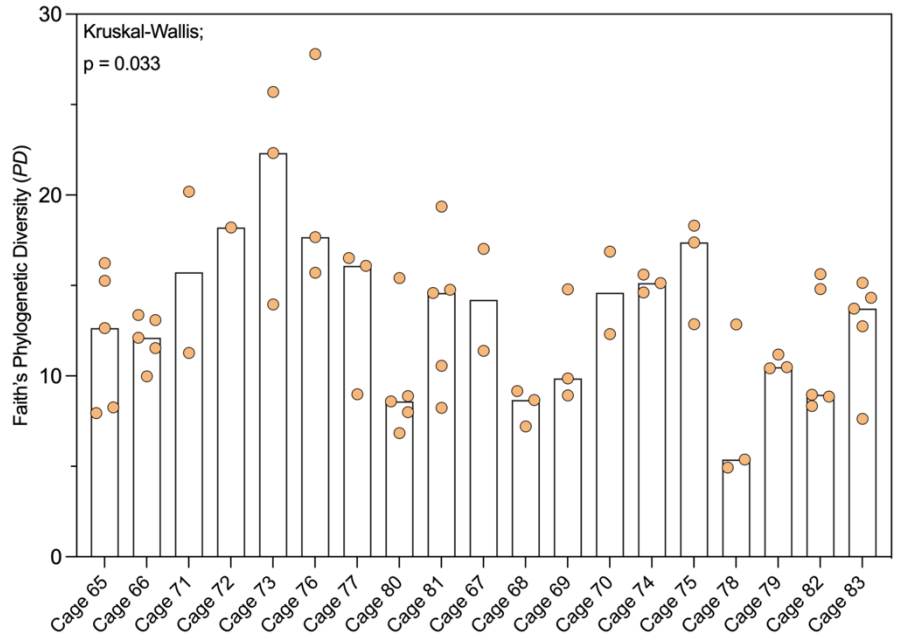


Appendix 3.5 *Genotype effect on α-diversity in single sex type*

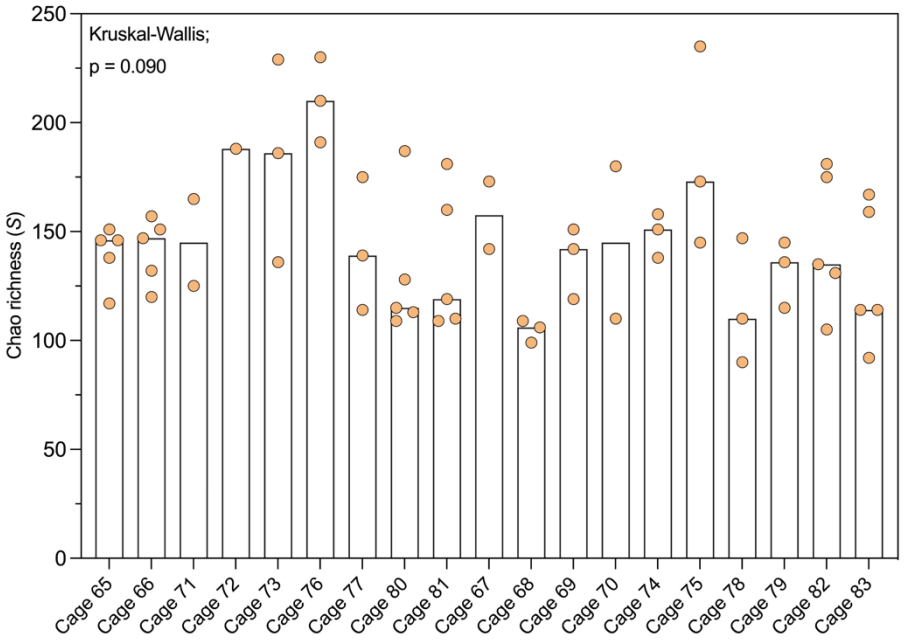


Appendix 3.6 *Cage differences of Faith's phylogenetic diversity and Chao's richness*

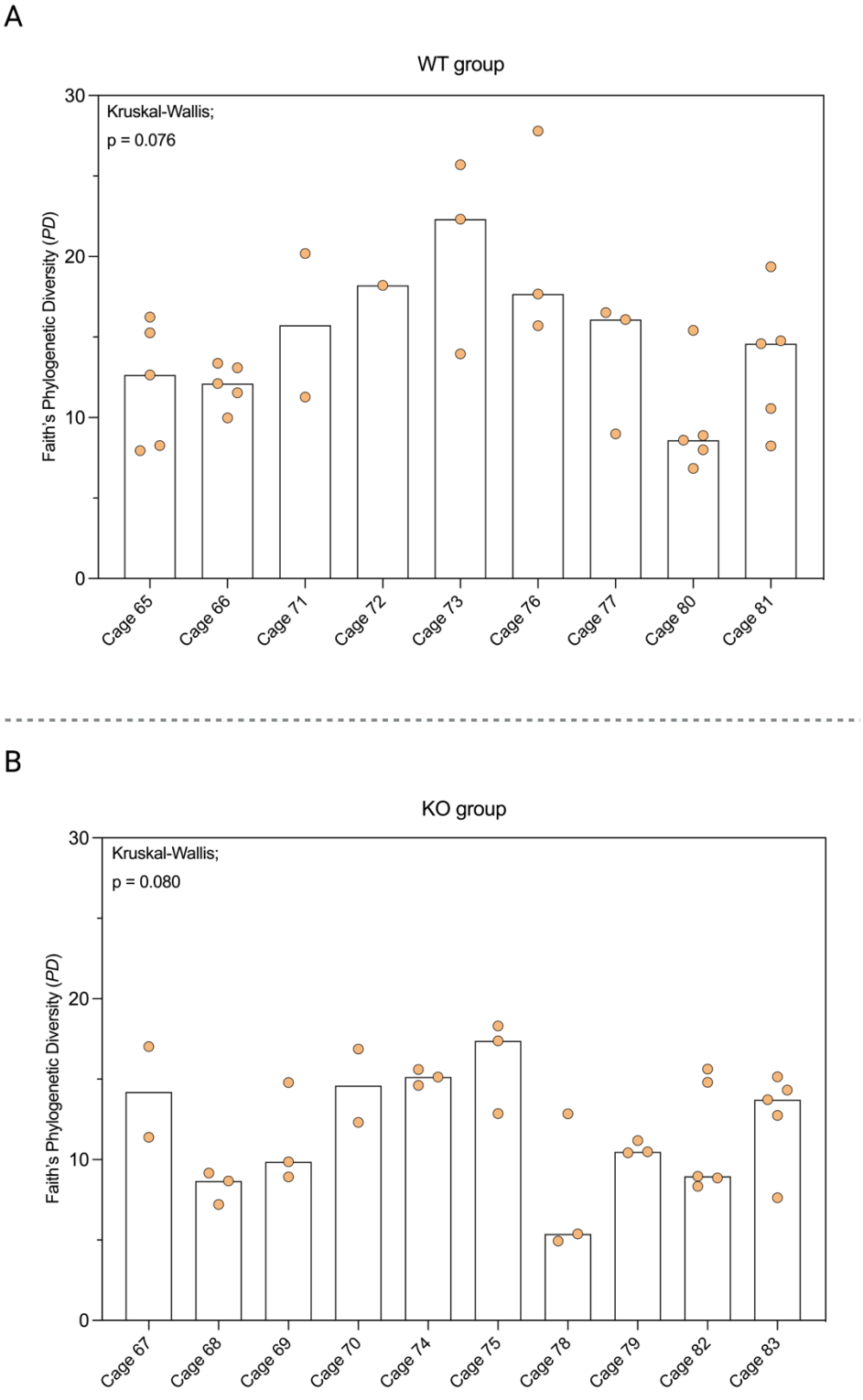
A



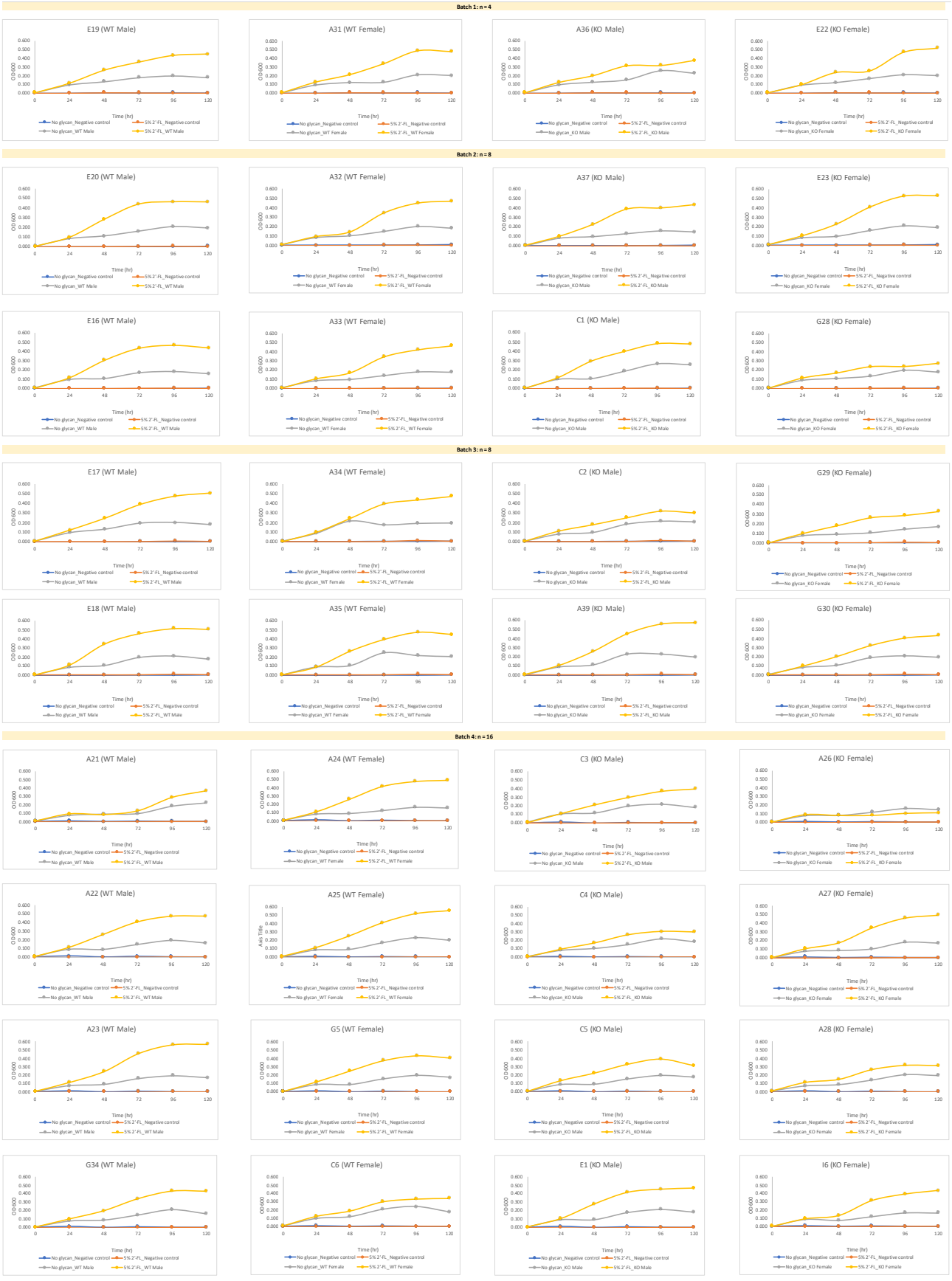
B



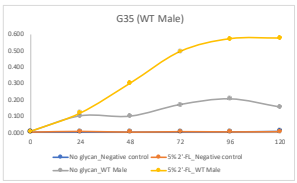
Appendix 3.7 *Cage differences of Faith's phylogenetic diversity within each genotype*

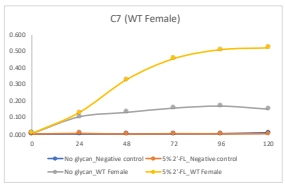


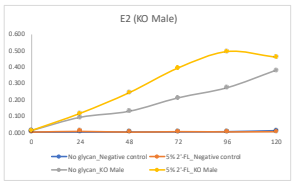
Appendix 3.8 *In vitro culture of faecal bacteria in mBasal broth with and without supplementation of α(1,2)-fucosylated glycans*

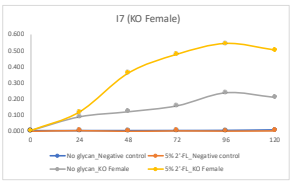


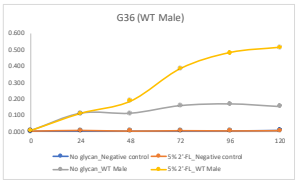
Batch 5: n = 16

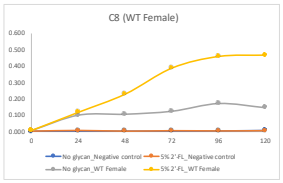


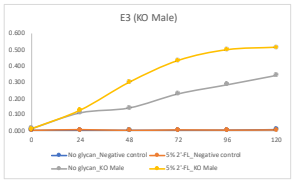


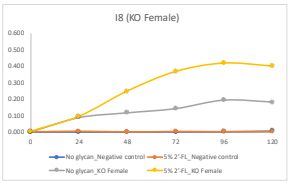


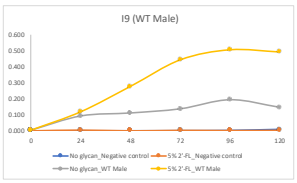


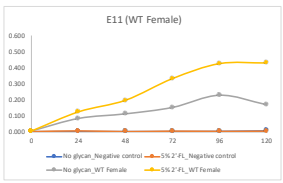


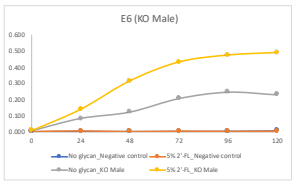


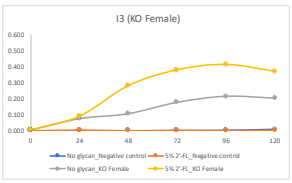


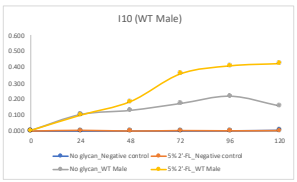


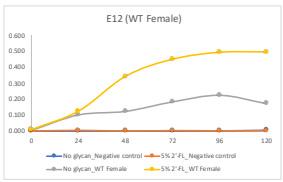


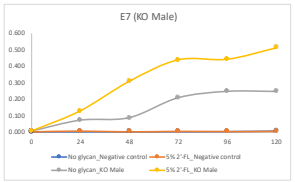


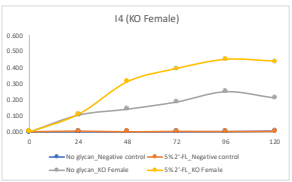












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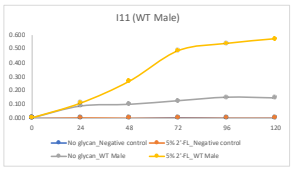

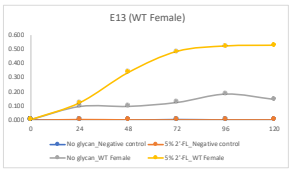

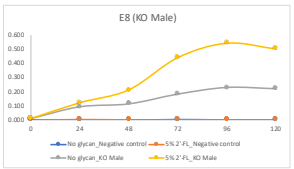

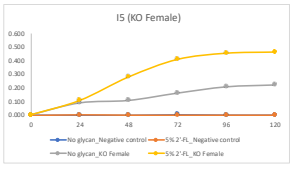

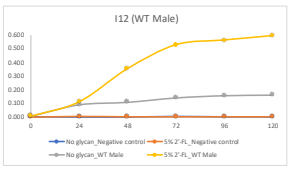

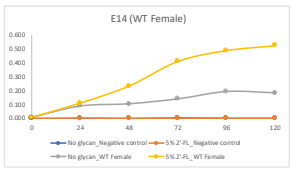

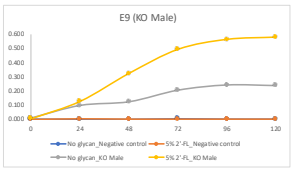

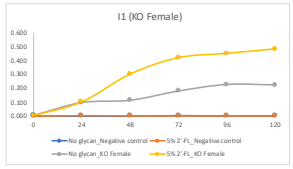

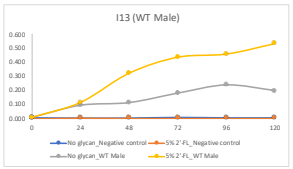

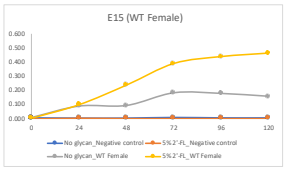

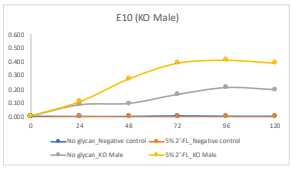

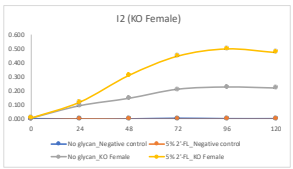

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