Lactobacillus, omega-3 fatty acids and FODMAPs influence on the gut microbiome

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By
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Declaration of Original Work

I, the undersigned, hereby declare that except where otherwise acknowledged, all work presented in this thesis is original and entirely my own. This thesis has not been submitted in whole or in part for a higher degree to any university.

Shane O'Donnell, BSc
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List of Abbreviations

Abbreviation	Meaning
°C	Degrees Celsius
ANCOM	Analysis of composition of Microbiomes
ANOVA	Analysis of variance
APC	Antigen presenting cell
AUC	Area under the curve
BAD	Bile acid diarrhea
BCFA	Branches Chain Fatty Acid
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index
BP	Base pair
CARD	Comprehensive antibiotic resistance database
CaZymes	Carbohydrate enzymes
CBT	Cognitive behavioral therapy
CCA	Canonical correspondence analysis
CD	Crohn's disease
CD	Cluster of differentiation
CFU	Colony Forming Unit
CO_2	Carbon Dioxide
CSS	Cumulative sum scaling
DDA	Data depended acquisition
DIA	Data independent acquisition
DM	Dry matter
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EM	Embden-Meyerhof
EPA	Eicosapentanoic acid
FACS	Fluorescent activated cell sorting
FBS	Fetal Bovine Serum
FDR	False discovery rate
FGID	
FHL	Formate hydrogen lyase
FID	Flame-ionisation detection
FITC	Fluorescein isothiocyanate
FODMAP	Fermentable Oligo-, Di-, Mono-saccharides And Polyols
FOS	Fructooligosaccharide
FSI	Frozen standardized inoculum
GC	Gas chromatography
GC/MS	Gas chromatography mass spectrometer
GIT	Gastrointestinal Tract
GOS	Galactooligosaccharide
GRAS	Generally recognized as safe

H_2	Molecular Hydrogen
HCl	Hydrochloric acid
HT-UHPLC	High temperature ultra high performance liquid chromatography
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IFN	Interferon
IL	Interleukin
IS	Internal standard
LAB	Lactic acid bacteria
LC/MS	Liquid chromatography mass spectrometer
LEfSe	Linear discriminant analysis effect size
MCP	Monocyte Chemoattractant protein
MIC	Minimum Inhibitory Concentration
MLN	Mesenteric Lymph Node
mM	millimolar
MRI	Magnetic resonance imagining
NADH	Nicotinamide adenine dinucleotide + hydrogen
NFOR	NADH ferredoxin oxidoreductase
NH_3	Ammonia
NMR	Nuclear magnetic resonance
OTU	Operation taxonomic unit
PBS	Phospho buffered saline
PCoA	Principle component analysis
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PFL	Pyruvate formate lyase
PFOR	Pyruvate ferredoxin oxidoreductase
PK	Phosphoketolase
PND	Post-natal day
PUFA	Poly-unsaturated fatty acid
RBB+C	Repeated bead beating + column
RCT	Randomixed Controlled trial
RDA	Redundancy analysis
rRNA	Ribosomal ribonucleic acid
SCFA	Short Chain Fatty Acid
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TCA	Tricarboxylic acid
TMAO	Trimethylamine N-oxide
TNF	Tumour necrosis factor
TSS	Total sum normalization
UC	Ulcerative colitis
VFDB	Virulent Factor Database
VPNC	Viable putative non-culturable
WT	Wild type

Thesis Publications

O'Donnell, Shane Thomas, R. Paul Ross, and Catherine Stanton. "The Progress of Multi-Omics Technologies: Determining Function in Lactic Acid Bacteria Using a Systems Level Approach." Review, *Frontiers in microbiology* 10, no. 3084 (2020-January-28 2020).

Other PhD Publications

Su, Chien Wen, Chih-Yu Chen, Lefei Jiao, Shao Rong Long, Tangyou Mao, Qiaorong Ji, **Shane O'Donnell**, Catherine Stanton, Shasha Zheng, W. Allan Walker, Bobby J. Cherayil, and Hai Ning Shi. "Helminth-Induced and Th2-Dependent Alterations of the Gut Microbiota Attenuate Obesity Caused by High-Fat Diet." *Cellular and Molecular Gastroenterology and Hepatology* 10, no. 4 (2020/01/01/ 2020): 763-78.

Thesis Abstract

Introduction

Many aspects of the human gut microbiome, the diverse combination of microorganisms that are present in the large intestine, impact human host functioning and homeostasis. As we uncover greater understanding of the links between human health and the microbial communities present within the host, we unveil new potential therapies for moderating human disorders. These potential therapies appear most pertinent for gut derived disorders such as Irritable Bowel Syndrome (IBS) and Inflammatory Bowel Disease (IBD). Dietary therapy has emerged as a popular method for managing IBS, and has become increasingly popular for managing IBS like symptoms present in IBD. The most effective dietary therapy, the low FODMAP diet, starves the microbiome of substrates that may result in the production of hydrogen *in vivo*. We hypothesise that the microbiome could be modulated with *Lactobacillus* strains to achieve this positive outcome, without the need for maintaining a diet low in Fermentable Oligo-, Di-, Monosaccharides and Polyols (FODMAP). Our research also examines new low FOMDAP foods, capable of potentially removing unwanted microbial fermentation while providing more food options for individuals on a low FOMDAP diet. Appropriate manipulation of, or starvation of, groups of bacteria present in the gut microbiome may reduce the volume of hydrogen produced, providing relief from the bloating and pain often experienced as part of these conditions.

Methods

To develop these two potential therapies, we selected *Lactobacillus* strains that were capable of degrading FODMAP carbohydrates to potentially effect change on the microbiome. Three *Lactobacillus* strains: *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083, were selected based on their ability to ferment FODMAP carbohydrates. IBS and IBD were induced in C57/Bl6 mice via the maternal separation model and DSS induced colitis, respectively. During both murine trials the three *Lactobacillus* strains were administered. We examined the compositional change within the murine microbiome and the resulting production of specific SCFAs to understand whether the the added strains were increasing or decreasing potential hydrogen production. Physiological and immunological changes were recorded and compared between groups. *Ex vivo* fermentation in the presence of a human microbiome facilitated the examination of microbial changes caused by the new low FODMAP foods in comparison to their high FODMAP counterparts. The most suitable low FODMAP foods were selected based on their impact on the human microbiome *ex vivo*.

Results

This work seeks to understand the potential for targeting the microbiome to reduce FODMAP induced IBS like symptoms. We discovered three strains that are capable of robust FODMAP degradation, survival of GI transit and effective adherence to human Caco-2 cells. The genomic potential of these strains was examined to understand carbohydrate fermentation capacity and the nature of their antibiotic resistance. This research presented a platform to explore the strains impact on *in vivo* systems using a murine model.

Assessing the impact of a highly fermentable diet and *Lactobacillus* administration in a murine model facilitated a more in depth understanding of this interaction. A diet high in FODMAP carbohydrates caused an increase in microbial diversity, an increase in carbohydrate fermenting microbial groups such as *Bifidobacterium, Lachnospiraceae* and *Roseburia,* and subsequent increase in almost all SCFAs analysed. *Lactobacillus* consumption led to a robust increase in *Lactobacillus* within the murine caecum. The proliferation of *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 resulted in an increase in CD19+ immune cells, increased abundance of microbial groups linked to anti-inflammatory effects and increased propionate levels, indicative of a reduction in net hydrogen production. Despite the maternal separation model proving to be an ineffective IBS model, increased propionate production indicates the *Lactobacillus* strains may alter the metabolism of the microbiome to reduce hydrogen production.

Previous research has revealed that probiotic strains may have a protective effect against chemically induced colitis in murine models. Due to the presence of FODMAP induced pain and bloating symptoms in IBD, we were also interested in exploring the potential protective effect these *Lactobacillus* strains may have on the physiological impact of Dextran Sulfate Sodium (DSS) induced colitis in mice. Mice that received *Lactobacillus* strains prior to DSS challenge demonstrated reduced weight loss, increased colon length and a reduced blood serum immune response. *Lactobacillus* strains persisted in the microbiome throughout DSS challenge and for the seven day recovery period after. *Lactobacillus* fed mice had significantly reduced alpha diversity after DSS treatment, indicating that the *Lactobacillus* strains became a dominant member of the microbiome. The survival of at least one *Lactobacillus* strain within the gut ecosystem, even during severe environmental perturbations, suggests that the strains occupied a niche and as such were capable of stabilising the host microbiome.

The overall scope of our research included the production of low FODMAP food alternatives such as pasta and bread. *In vitro* digestion and *ex vivo* faecal fermentation assessed the most suitable low FODMAP prototypes for use in a low FODMAP diet, based on their impact on the human microbiome

present during fermentation. Our results suggest that the low FODMAP bread, low FODMAP pasta and inulinase treated bread are suitable candidates for incorporating in a low FODMAP diet. Furthermore, malted lentils and malted buckwheat provide a low FODMAP version of their raw counterparts that do not negatively impact the microbiome.

Conclusions

The findings from this research present two potential novel therapies for FODMAP-induced IBS. *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 persist in the murine microbiome, altering the SCFA profile of the microbiome. Increased propionate, a marker for reduced hydrogen, was observed in *Lactobacillus* supplemented animals. The three *Lactobacillus* strains, selected for their ability to degrade FODMAPs, modulated the murine immune response to DSS induced colitis, moderating its severity. Low FODMAP bread, inulinase treated bread, low FODMAP pasta, malted lentils and malted buckwheat were determined to be suitable replacements for their high FODMAP counterparts as part of a low FODMAP diet, based on their impact on the human microbiome after *ex vivo* fermentation. These data require human trials to corroborate the findings, however, the results of this thesis present potential novel approaches to managing FODMAP-induced symptoms.

Chapter I

Chapter 1.1

Current and future Irritable Bowel Syndrome treatments: How targeting the Microbiome may reduce FODMAP-induced Irritable Bowel Syndrome (IBS)

1.1.1 | **Abstract**

The 100 trillion microbes that make up our gut microbiota interact with the host on an ongoing basis. This complex ecosystem is shaped by a variety of materials such as host derived glycans, non-digestible dietary components and host secreted bile acids. The broad metabolic capacity within the microbiome produces a variety of bioactive compounds from these resources. Short chain fatty acids (SCFAs) are the best-known bioactive compounds, however, other compounds such as molecular hydrogen play an underreported role in the gut. Redox balances throughout the microbiome are often maintained by producing molecular hydrogen, creating a hydrogen cycle between gut microbes. This bioactive compound negatively affects Irritable Bowel Syndrome (IBS) patients, distending the gut lumen and causing pain and bloating. IBS therapies targeting gut microbes, such as antibiotics and dietary control, are effective by reducing fermentation within the gut, in turn reducing the volume of hydrogen present. We discuss the various IBS therapies and their mechanisms of action, how hydrogen is cycled during the carbohydrate fermentation process and how specific probiotics may have potential for reducing hydrogen derived IBS symptoms.

1.1.2 | **Introduction**

Carbohydrates are not only an essential source of energy for the human body, but also for the trillions of microbes located in the gastrointestinal tract (GIT). Microbes in the colon must use the carbohydrates that are not absorbed higher up in the GIT. These compounds are often described as prebiotics, fibre, microbiota accessible carbohydrates or Fermentable Oligo-, Di-, and Mono saccharides and polyols (FODMAPs) [1]. Lactose, inulin and mannitol are examples of carbohydrates under the FODMAP umbrella. Common foods such as onions, garlic, dried fruit, wheat and rye all contain high FODMAP content. These FODMAPs act as a primary source of carbon that reaches the gut, feeding the gut microbiota that reside there [2].

The gut microbiota is comprised of more than 100 trillion microbes and over 1000 species [3]. These microbes play a key role in metabolism and impact many aspects of bodily function. Our understanding of these interactions is growing and now encompasses defence against pathogens, regulation of the immune system, maintenance of homeostasis, modulating cancer cell proliferation and regulating glucose levels [4]. These host-microbe interactions are mediated by bioactive compounds that are produced during cross-feeding within the gut [5]. FODMAP carbohydrates play a key role in the production of these bioactive compounds as they provide the primary carbon source for the microbial communities that produce them. SCFAs, the most studied bioactive compounds produced in the gut, are direct end products of FODMAP fermentation [6-8]. *Bacteroides, Ruminococcus* and *Lactobacillus* are among the genera responsible for the initial fermentation of these FODMAPs. This fermentation process releases compounds such as lactic acid that are used as energy by other microbes within the gut. This cross-feeding structure results in the production of SCFAs, increased availability of polyphenols, as well as generating other bioactive compounds such as Branched Chain Fatty Acids (BCFAs), increasing availability of antioxidants and producing ammonia [9, 10].

The bioactive compounds mentioned above have largely positive host-microbiome effects, however, some compounds that are produced during the fermentation process negatively impact the host. For example, gut microbes may increase calorie intake as they generate energy from previously non-digestible dietary components [11], trimethylamine N-oxide (TMAO) produced from choline digestion may mediate cardiovascular disease [12] and H_2 and CO_2 production may induce IBS symptoms [13-16]. The latter is largely a result of the interaction between FODMAP carbohydrates and the microbiome. During the carbohydrate fermentation process molecular H_2 and CO_2 are released by many microbes. Indeed, increased H_2 has been reported after continuous real-time analysis of exhaled H_2 when mice were fed a diet high in complex carbohydrates [17]. In a regular gut ecosystem, these gases are used during other metabolic processes, however, a dysbiotic microbiota can result in an increased volume of these gases, manifesting in IBS symptoms of bloating and pain [18, 19]. Due to this mechanism, FODMAP

carbohydrates have a direct connection to IBS symptoms of pain and bloating. This phenomenon is experienced by a large subset, perhaps as large as 70% [20], of IBS sufferers. The term 'FODMAP induced IBS' is a useful method of defining this particular subset of IBS and avoids the broad, and often difficult to define, 'IBS'. The need to define different subsets of IBS becomes more apparent as we discover more about the many causes and many therapies for IBS, as discussed in this chapter. H_2 cycling within the gut ecosystem is at the centre of these IBS symptoms.

Several approaches to treating IBS have been developed with varying degrees of success. These are presented in the broad categories of pharmacological treatments, psychological treatments, dietary treatments and more recently personalised medicine. Addressing IBS symptoms with probiotics falls within the category of personalised medicine and is currently an area of great interest. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" [21]. This review will discuss the available treatments that fit into these categories and their mechanisms of action. The complex interplay between FODMAP carbohydrates and the taxonomic groups within the gut microbiome will be discussed, identifying the largest culprits for gas production. Finally, the rationale behind probiotics reducing the negative symptoms of IBS will be discussed and compared to the low FODMAP diet.

1.1.3 | **Current IBS treatment options**

IBS treatments can be broadly divided into four different approaches, consisting of pharmacological treatment, psychological treatment, diet-based treatment and personalised medicine (summarised in Table 1). There is no gold standard approach to treating an IBS patient, although the low FODMAP diet is often the first treatment recommended. To understand the current landscape for an IBS patient looking for treatment, each of these approaches are discussed.

1.1.3.1 | Pharmacological treatment

Pharmacological treatments are often the least palatable for long standing conditions due to adverse effects. These drugs tend to target a specific type of IBS, such as IBS-Diarrhoea (IBS-D), and the sub-type will be mentioned in each case. In the case of IBS-D there are three FDA approved drugs for this sub-type, rifaximin, Eluxadoline and Alosteron [22-25]. The most popular of these is rifaximin, due to the well-reported adverse effects associated with Eluxadoline and Alosteron [22]. Despite positive trials assessing the use of the rifaximin antibiotic, a comprehensive meta-analysis determined that it is not efficacious for IBS-D treatment [26]. The IBS constipation predominant subtype (IBS-C) has several therapies focused on reducing a battery of symptoms mainly characterised by the lack of motility within the GI tract. There are two potential drug therapies. Lubiprostrone is primarily used to reduce constipation-based disorders from a variety of causes [27]. It is FDA-approved for IBS-C for women over 18 years based on the results of two randomised controlled trials (RCTs) proving its efficacy [28]. Secondly, Linaclotide, has shown a moderate improvement in IBS-C related symptoms in a meta-analysis including 3 RCTs looking at IBS-C [29, 30]. These primarily address the constipation symptom and show a moderate improvement with diarrhoea as the main side effect. The IBS mixed subtype (IBS-M), consisting of constipation or diarrhoea alternating as the primary symptom, is often treated by generic IBS drugs and as such all generic IBS drugs will be assessed together. The antibiotic rifaximin has also been described as a treatment for general IBS, reporting significant improvement in bloating in patients [31]. Loperamide has shown efficacy in treating a range of IBS symptoms among other GI disorders [32-34]. Negative outcomes associated with broad antibiotic use include inducing a dysbiotic microbiota after treatment. Interestingly, the broad-spectrum antibiotic activity against many taxonomic groups within the microbiome may be the mechanism by which these antibiotics improve symptoms such as bloating.

Anti-spasmodic peppermint oil has been reported to have a significant positive impact on IBS symptoms. Robust meta-analysis which reviewed the RCTs published to date revealed a significant improvement in symptoms after peppermint oil treatment [35, 36]. This pharmacological therapy, believed to act via a

calcium channel blockade resulting in relaxation of smooth GI muscle [37], does not result in the broad antimicrobial effect on the gut microbiota experienced after antibiotic treatment [38]. Other IBS treatments such as fluoxetine, citalopram, trimipramine, ketotifen and pregabalin have shown some benefit in isolated circumstances but are inferior to Lubiprostrone, Rifaximin, Eluxadoline and Alosteron as described above [39].

1.1.3.2 | Psychological treatment

Treating the psychological aspect associated with IBS has been a popular approach stemming from the original hypothesis that IBS is a psychological issue. This approach incorporates therapies such as Cognitive Behavioral Therapy (CBT), mindfulness and hypnotherapy. Meta-analyses of the data have reported that CBT results in a significant improvement in symptoms in comparison to basic support and medical treatment [40, 41]. Li et. al. incorporates 18 Randomized Controlled Trials (RCTs) within its framework [40], noting that CBT is not significantly better than other psychological treatments for IBS symptom reduction. Multiple RCTs are available on the efficacy of mindfulness for reducing IBS symptoms [42-46]. Despite no meta-analysis of these effects, each of these RCTs report a significant improvement in symptoms after therapy based on mindfulness. Hypnotherapy has shown short term benefits for IBS symptom management. This has been demonstrated in a meta-analysis including 7 RCT's analyzing this effect [47]. The widespread improvement of symptoms from these psychological treatments confirms that there is a substantial psychological element to this functional gastrointestinal disorder (FGID). Psychological treatments appear to target the stress-response and the salient reaction to IBS symptoms, reducing the severity of comorbidities and moderating the manner in which IBS symptoms are experienced [48][49].

1.1.3.3 | Dietary management

The therapeutic effect that a positive change in diet can have on chronic diseases has resulted in increased interest in this approach to the management of IBS [50, 51]. Dietary based therapies are prevalent for gastrointestinal disorders, largely due to the experience of negative GI symptoms after ingesting particular foods [52]. The physiological reasons for symptom generation in response to food have been reported [53]. The process of gastric accommodation expands stomach capacity via a vago-vagally-mediated reflex reaction, decreasing the stomach tone. This bloating like effect facilitates further food ingestion. Low intensity distension of the proximal stomach induces a sensation of satiety; however, a higher range of distension may induce discomfort, nausea and pain [53]. Dietary treatments are considered a more palatable option to patients in contrast to pharmacological therapies. The

difficulty involved in changing a patients diet must also be weighted when considering the best approach to treat the disorder [54].

High fibre diets represented the first popular dietary treatment for IBS. RCTs showed positive, although inconsistent results and often mentioned the difficulty in treating IBS with the high fibre diet [55-57]. The gluten free diet became popular in recent years with research backing up the claims that a gluten free diet could reduce IBS symptoms [58, 59]. One such study was carried out by researchers at Monash University, led by Muir and Gibson, who demonstrated the impact of gluten on individuals suffering with IBS [60]. Work in this area resulted in coining the term Non-celiac gluten sensitivity [61, 62].

It has also been suggested that dietary carbohydrates may be responsible for the severity of IBS symptoms. At first, very low carbohydrate diets were demonstrated to reduce IBS symptoms [63]. This quickly developed to analysing specific carbohydrates, particularly fermentable carbohydrates. Gibson and Muir described a mechanism of IBS symptom genesis that relied on an interaction between these fermentable carbohydrates and the gut microbiota [64]. In this interaction, FODMAP carbohydrates draw H_20 into the lumen of the GI tract due to osmotic pressure, causing increased luminal distension. These non-digestible carbohydrates continue to the large intestine where the gut microbes ferment them, generating CO_2 and H_2 gases. These voluminous gas compounds distend the colon, further stimulating the bloating and pain response that IBS sufferers feel. The term FODMAPs was coined to describe these non-digestible and fermentable carbohydrate sources that interacted with gut microbiota. RCT's reported a reduction in IBS symptoms after reducing these fermentable carbohydrates [65, 66]. Further research involving Gibson [67] and Muir [68], among others, demonstrated that these fermentable carbohydrates were indeed causing a substantial portion of IBS symptoms. Both researchers discovered that gluten free foods tended to be low in FODMAP carbohydrates and that the reduction in symptoms from a gluten free diet was actually due to a concurrent reduction in FODMAP carbohydrates [69].

The efficacy of the low FODMAP diet has been supported by a number of meta-analyses in recent years [13, 70, 71]. However, many come with a warning of the unknown impact that reducing FODMAPs may have on the gut microbiota. These carbohydrates act as prebiotics for many beneficial microbial groups such as Lactic Acid Bacteria and bifidobacteria. Reducing these FODMAPs may have longer term impact on the health of IBS sufferers [72]. Despite this, a low FODMAP diet has become a front line dietary approach for treating IBS [73].

1.1.3.4 | Personalised medicine

Personalised medicine focuses on the disorder in the context of the information that can be gathered on an individual. This often relies on compiling data sets such as the genome and microbiome of the patients. Due to the variable and multi-factorial nature of IBS, a personalised approach to treatment may show significant improvements. This approach combines treatment options from the previous sections after deep phenotyping has taken place. This phenotyping may describe an IBS subtype in order to direct therapy or sequence a microbiome to determine H_2 producing microbes.

IBS subtypes are often determined prior to treatment with pharmacological agents. As mentioned previously, these drugs tend to treat the major symptoms such as diarrhoea, constipation, pain, bloating etc. Each subtype can be described further with indicators such as Bile Acid Diarrhoea (BAD), thus directing the use of particular drugs as treatment [74, 75]. Gathering more information of an IBS-C subtype with pain may lead to the discovery of causative factors such as pelvic floor dyssynergia. This is the case in approximately 30% of cases, and pelvic floor rehabilitation has proven to be an effective treatment method [76]. Due to the many presentations of IBS, understanding the personal experience of the individual facilitates administration of a more complete and effective therapy.

Absorption of commonly ingested disaccharides can cause significant GI distress. These include lactose intolerance, sucrose isomaltase deficiency and even glucose-galactose malabsorption [77-79]. Lactose intolerance is simply diagnosed by a hydrogen breath test and treated by avoiding moderate amounts of lactose. A congenital genetic disorder that causes sucrose malabsorption often goes undiagnosed due to the general nature of the symptoms including chronic diarrhoea, abdominal pain and bloating. This disorder requires an assay of the duodenum or jejunum for activity against a variety of disaccharides [80]. Research analysing variants of the Sucrase-Isomaltase (SI) gene has uncovered more than seven variations, with some associated with a significantly higher proportion of IBS [81, 82]. The prevalence of a dysfunctional SI gene may cause a significant proportion of IBS cases, and genomic biomarkers for this condition provides a far less invasive diagnosis than the assay currently available. However, genetic testing currently returns variable success in diagnosis [83]. Only an in-depth analysis of the individual suffering from these generic symptoms would reveal such a disorder, highlighting the importance of a personalised approach to IBS therapy.

IBS phenotypes that are difficult to identify can be helped with imaging technologies such as MRI. These techniques provide a unique picture of motor functions within the gut, allowing a more informed diagnosis of motility-based IBS disorders [84]. MRI has been used to diagnose subtypes of IBS in a research setting [84-86], however, access to this machine is often limited to research facilities or private institutions [87]. The in depth analysis these techniques can provide at a personalised level may provide

significant advances in IBS therapies, however, access to the required infrastructure often makes these options prohibitively expensive.

We are slowly beginning to unravel the role that the gut microbiome plays in our general well-being and how this may be used to direct personalised medicine [88, 89]. This can be seen in many distinct diseases and disorders which are being connected to particular microbes, none more so than those relating to gastrointestinal disorders [90-93]. The research to date has made a link between IBS pathology and bioactive compounds such as CO_2 and H_2. We look at the mechanisms that produce these compounds from FODMAP carbohydrates and the microbes that carry out these fermentations.

Table 1. Current IBS treatments available for specific IBS subtypes. Their efficacy and the mechanisms by which they exert their influence are mentioned and referenced

Pharmacological treatment	Targeted IBS type	Pros	Cons	Mechanism of action	References
Rifaximin	IBS-D, IBS	Fast mode of action; Best studied antibiotic with some positive symptom reduction	Meta-analysis determined it not efficacious; Broad spectrum damage against the microbiome	Broad antibiotic treatment against gut microbial groups responsible for symptom reduction	[22-24]
Eluxadoline	IBS-D	Slows abdominal transit; reduces abdominal pain in some studies	Phase III trials did not demonstrate advantage over placebo at FDA endpoints	Opioid receptor effecting gut-brain signalling - Reduces gut contractions and slows transit	[22-25]
Alosteron	IBS-D	Improves global IBS symptoms and abdominal pain in women	Potentially causes adverse side effects such as ischemic colitis and severe constipation; Only shown to be efficacious in women	5-hydroxytryptamine-3 receptor agonist	[24, 25]
Lubiprostrone	IBS-C	Two RCTs have shown efficacy for women over 18	Chance of developing Diarrhoea	Activates type 2 chloride channels in intestinal epithelial cells leading to chlorine secretion	[27, 28]
Linaclotide	IBS-C	Meta-analysis shows moderate improvement for IBS-C	Chance of developing Diarrhoea	Guanylate cyclase-C receptor agonist on the surface of intestinal enterocytes.	[29, 30]
Loperamide	IBS-D	Effective anti-Diarrhoea agent	Does not improve other IBS symptoms;	Synthetic peripheral μ-opioid receptor agonist	[32-34]

Peppermint oil	IBS	Substantial evidence showing improvement in symptoms; No adverse effects	Effective for 1 in three for global symptoms and 1 in four for abdominal pain	Relaxes gastrointestinal smooth muscle by acting on gut calcium channels	[35, 36]
Psychological treatment					
Cognitive behavioural therapy	IBS	Meta-analysis shows improvement in symptoms; independent of CBT effect on general stress	Longer treatment time required than other therapy types	Potentially targets abnormal salience cognition and emotional arousal	[40, 41, 49, 92]
Mindfulness	IBS	Multiple RCTs determine IBS symptom reduction	Longer treatment time; No meta-analysis	Potentially targets abnormal salience cognition and emotional arousal	[42-46, 49]
Hypnotherapy	IBS	Benefits for symptom management	Longer treatment time; Short lasting effect	Potentially targets abnormal salience cognition and emotional arousal	[47, 49]
Dietary treatment					
High fibre diet	IBS	Proliferation of healthy microbiome; Healthy general diet	Inconclusive evidence for symptom management; Potential increase in symptom severity	Potentially proliferates microbes responsible for low gas products during fermentation	[55-57]

Gluten free diet	IBS	Evidence for symptom control; relatively easy diet to maintain	Potentially unhealthy diet if not implemented correctly; Inconsistent results for individuals	Lowers potential fermentable carbohydrate available in the gut	[58-62]
Low carbohydrate diet	FODMAP induced IBS	Effective symptom control; Easy to understand	Difficult to maintain; deleterious effect on microbiota; Incorrect implementation may result in nutrient deficiency	Lowers all fermentable carbohydrate available in the gut	[63]
Low FODMAP diet	FODMAP induced IBS	Most effective dietary therapy reducing symptoms in 70% of individuals;	Difficult diet to maintain especially in elimination phase; deleterious effect on the microbiota;	Targets the reduction of all fermentable carbohydrates that reach the gut	[64-69]
Personalised medicine					
Extensively phenotyping IBS presentation	IBS	Facilitates using the most appropriate therapy for the individual; Increases the likelihood of a successful outcome; Provides more information for therapy development	Time consuming; Difficult to carry out a number of tests in very different fields; In some cases sufficient information is not available to make conclusive decisions	Sequencing host genome for genetic disorders; Sequencing microbiome to determine microbial interactions; MRI on gut to inform motility-based disorders	[36, 74-88]

1.1.4 | **FODMAPs impact on the Microbiome - Understanding the fermentation pathways and hydrogen genesis**

Many researchers have tried to link a specific microbiome profile to IBS phenotypes often comparing IBS and IBS subtypes to healthy controls. One of the consistent results from these analyses is that of a lower microbial diversity and richness in IBS subjects compared to healthy controls [94, 95]. Despite this, a robust connection has not been made between specific taxonomic profiles and IBS. This may be due to the redundancy within the microbiome or due to the many factors at play in IBS pathology. However, as detailed above, FODMAP carbohydrates have been reported to have a significant negative impact on many IBS sufferers [96]. This negative impact is mediated by the microbiota as a result of bioactive CO_2 and H_2 produced during the fermentation process. These gaseous compounds are part of a correctly functioning gut ecosystem, however, may cause adverse effects when they are not cycled correctly.

Carbon dioxide is produced during many microbial processes and makes up 5.1% to 29% of gas within the gut by volume [97]. CO_2 is almost entirely reabsorbed by colonocytes or metabolised further as part of metabolic processes throughout the gut [98]. Molecular H_2, consists of 0.06% to 47% of intestinal gas and despite its role in many metabolic processes, 60-70% of H_2 produced by microbial fermentation remains within the gut and must be excreted externally [97, 98]. This information highlights the increased severity of H_2 compared to CO_2 in relation to IBS symptoms. The presence of these gases in large quantities causes luminal distension within the gut triggering IBS pathological symptoms such as bloating and pain. For this reason, we will look at the fermentation processes within the gut that generate these gas compounds, in particular the genesis of molecular H_2.

The microbial production of H_2occurs during three processes. During the conversion of glucose and other monosaccharides to pyruvate, NADH is produced. As part of the NADH ferredoxin oxidoreductase (NFOR) complex, NAD^+ is regenerated while H^+ ions are converted to H_2 by intercellular hydrogenases (Equation 1) [99]. Pyruvate may subsequently be converted to acetyl CoA and formate by pyruvate formate lyase (PFL). This process produces formic acid that is converted to H_2 and CO_2 by several taxonomic groups (Equation 2). Similarly, pyruvate may be converted to acetyl-CoA causing ferredoxin to be reduced as part of the Pyruvate ferredoxin oxidoreductase complex (PFOR, Equation 3). To re-oxidise this compound, hydrogenases convert H^+ ions to H_2 gas (See Figure 1). NFOR activity is dependent on hydrogen concentration. A partial pressure of >60 Pa inhibits the generation of H_2 through the NFOR complex and results in non-gaseous products such as ethanol, lactate, acetate, butyrate and butanol [100].

Equation 1. $$NADH + Fd \rightarrow NAD^+ + FdH$$

Secondly, H_2 is produced during the conversion of pyruvate to Acetyl-CoA and formate using the Pyruvate formate lyase complex [101]. The resulting formate interacts with the Formate Hydrogen Lyase complex (FHL) to produce H_2 and CO_2 [102].

Equation 2. $$\text{Formic acid (HCOOH)} \rightarrow \text{H2} + \text{CO2}$$

Finally, as pyruvate is converted to acetyl-CoA, ferredoxin oxidoreductase and hydrogenases generate H_2 molecules from H^+ ions. The PFOR complex is active at H_2 concentrations of less than 3×10^4 Pa [100].

Equation 3. $$\text{Pyruvate} + \text{CoA} + \text{Fd} \rightarrow \text{Acetyl}-\text{CoA} + \text{FdH} + \text{CO2}$$

Hydrogen generating mechanisms can be tracked through the fermentation of carbohydrates that reach the gut microbes. As complex carbohydrates become available, they are broken down to monosaccharides and organic anions by primary fermenters in the microbiome. These primary fermenters have a broad spectrum of carbohydrate enzmyes (CaZymes). The well-studied *Bacteroides thetaiotaomicron* has an arsenal of approximately 260 Glycosyl Hydrolase (GH) genes [103]. CaZymes allow microbes to hydrolyse a variety of FOMDAPs into their monosaccharide constituents. The *Bacteroides* genus generates energy mainly through the production of succinate, propionate and acetate [104, 105]. *Bacteroides* spp do not have a monopoly on this sacchrolytic activity, many genera such as bifidobacteria, *Ruminococcus*, *Prevotella* and *Lactobacillus* also ferment these FODMAP carbohydrates [106, 107]. Some CaZymes produced by these microbes are secreted into the environment and act extracellularly [108]. This type of extracellular enzyme activity further aids the cross-feeding that is present within the gut microbiome [109, 110]. Microbe-microbe signalling complements this exchange and allows groups of bacteria to develop symbiotic relationships [111, 112].

The hydrolysis of the more complex FODMAP carbohydrates, such as inulin and stachyose, to their monosaccharide constituents does not produce CO_2 or H_2. Polyols such as mannitol are converted to fructose in order to enter glycolysis. Mannitol dehydrogenation regenerates one NAD^+ molecule, however, this is used later in the process producing NADH. This renders the polyol conversion to fructose and subsequent glycolysis hydrogen neutral.

This primary fermentation is considered the rate limiting step in accessing the carbon source and produces end products such as lactate, succinate and acetate [113]. This fermentation process includes different variation of the glycolytic pathway with different hydrogen yields as a result. Lactic acid

bacteria (LAB) can use the Embden-Meyerhof (EM) pathway or the phosphoketolase (PK) pathway to generate energy from glucose molecules, the latter producing one mole of CO_2 per glucose molecule. Bifidobacteria use the bifid shunt pathway to generate 3 acetate and 2 lactate for every 2 glucose molecules [114]. Interestingly, none of the above pathways cause a net increase in hydrogen production. This is due to the regeneration of NAD^+ molecules during the conversion of pyruvate to lactate. This prevents the need for ferredoxin oxidoreductases that are driven by hydrogen producing hydrogenases [115].

Bacteroides primarily relies on the succinate pathway to metabolise hexoses resulting in succinate and propionate as end products [116, 117]. Lactate is also produced by these microbes, however, it has an inverse relationship with succinate and propionate [117]. *Clostridium* spp. are known significant producers of Hydrogen and are the subject of much biohydrogen production research [118-120]. During mixed acid fermentation, formate is also produced by *Escherichia* and other microbes as pyruvate formate lyase is used to convert pyruvate to acetyl-CoA [121]. The formate hydrogen lyase complex is present throughout the gut, best known in the *Enterobacteriaceae* family [121]. This complex hydrolyses formate, which may be taken from external sources, to produce CO_2 and H_2 [101]. Conversely; *Clostridium*, *Bacteroides*, *Ruminococcus* and *Desulfovibrio* spp. are among the many groups that use the PFOR complex as redox balance, yielding H_2 as a result [122, 123]. These comparisons serve to highlight the very different hydrogen and carbon dioxide yields depending on the pathway utilised.

The final products of many of these pathways, lactate, succinate, formate and acetyl-CoA are observed in very low quantities in faecal material. Within the gut ecosystem these could be considered intermediary elements, reused by other taxonomic groups to generate energy during production of more abundant bioactive compounds that we observe, such as butyrate and propionate. These intermediary compounds are considered bioactive compounds themselves. Lactate from LAB fermentation has been reported to modulate the inflammatory environment in intestinal mucosa [124-126]. Similarly, due to the crucial role that succinate plays in host cells during the tricarboxylic acid (TCA) cycle, microbial succinate produced by gut bacteria is hypothesised to signal immune cells and modulate the gut environment [107, 127]. Despite this, these products have a greater role as precursors to more diverse bioactive compounds.

1.1.4.1 | Hydrogen generation during SCFA production

This secondary phase of fermentation produces bioactive compounds including many SCFAs and gas compounds. The production of these SCFA compounds also includes the release of hydrogen. The net change in H_2 varies for each SCFA. Propionate production consumes 2 moles of H_2 per mole of glucose, whereas acetate produces 4 moles of H_2 from the same amount of glucose [18]. This result represents the theoretical yield and may depend on the pathway used to produce the SCFA. Given this disparity in hydrogen production, the ambient concentration of hydrogen gas may influence the SCFA produced [128].

These SCFAs play an important role in the functioning of the gut ecosystem. Butyrate has well known physiological influences on the host [126, 129, 130], whereas propionate and acetate have lesser, but still significant, known impacts [126, 131]. Butyrate is produced directly by butyrate-kinase or indirectly by using acetate with the enzyme butyryl-CoA:acetate-CoA-transferase [131]. The butyryl-Coa transferase pathway is the most prevalent, whereas only a number of known butyrate producing microbes such as *Clostridium butyricum, Coprococcus eutactus*, and *Coprococcus comes* utilise butyrate kinase [132, 133]. When directly produced from glucose, the hydrogen production is theoretically 2 mol H_2 per mole of glucose [134]. This hydrogen is as a result of converting pyruvate to acetyl CoA (Figure 1.) [134].

While Bifidobacteria and *Lachnospiraceae* can ferment dietary carbohydrates and *Akkermansia muciniphila* can digest host derived glycans, most butyrate-producing bacteria rely on the end products of primary fermenters. The most common method for butyrate generation is through butyryl-CoA : acetate CoA-transferase, converting acetate to butyrate [135]. *Eubacterium rectale*, *Eubacterium hallii*, *Roseburia intestinalis*, *Faecalibacterium prausnitzii* and *Coprococcus catus* are among the well-known butyrate producers that consume acetate [135, 136]. Butyrate produced in this manner produces 2 moles of H_2 from 1 mole of glucose [135, 137]. Members of the *Clostridium* genus have been observed utilising lactic acid and acetic acid to produce butyrate, H_2 and CO_2 [138]. Lactate utilising bacteria such as *E.hallii* and *Anaerostipes caccae* commonly produce butyrate as an end product [139]. This process requires the oxidation of lactate to pyruvate, catalysed by the EtfAB complex [140]. Duncan et al. reported reduced hydrogen production when *E. hallii* and *A. caccae* strains produced butyrate from lactate in comparison to glucose (4.7 µmol vs 12 µmol per ml of hydrogen). Formate was also not present after growth on lactate but was produced during growth on glucose [139]. This highlights the potential for limited hydrogen production in the gut, following specific metabolic pathways from complex carbohydrate to end product.

Similarly, propionate can be formed by PEP using the succinate decarboxylase pathway, or through the acrylate pathway that reduces lactate to produce propionate (Figure 1.) [131]. The succinate pathway results in varying levels of hydrogen production, depending on whether the original substrate is glucose or succinate. Bacteroidales and Clostridiales, in particular the *Lachnospiraceae* family, produce propionate as a fermentation end product [141-143]. Mucin degrading bacteria such as *A. muciniphila* are also prominent producers of both propionate and acetate [144]. Producing propionate from carbohydrates resulted in a net reduction of two H_2 molecules per mole of glucose [143]. This net reduction of H_2 is also reported when lactate is converted to propionate through the acrylate pathway [143]. Bacterial groups that produce propionate tend to generate a number of SCFAs and not just purely propionate. This phenomenon explains why *Bacteroides*, *Clostridiales* and similar propionate producers are prominent hydrogen producers *in vivo* [98, 141-143]. The role that hydrogen partial pressures play in determination of the SCFA produced in the gut is a poorly understood one. Increased partial pressure may prevent H^2 production from pathways such as the NFOR complex, resulting in an altered SCFA profile being produced [145]. Further research in this area may reveal greater understanding of how to direct gut microbial metabolism.

Acetate is produced directly from acetyl CoA, through the bifid shunt mechanism employed by bifidobacteria, from pyruvate using the *PoxB* gene (an oxygen dependent mechanism) or through the combination of H_2 and CO_2 by acetogens using the Wood-Ljungdahl pathway [146]. Direct acetate production via acetyl CoA results in 4 moles of H_2 per mole of glucose [147]. This represents the highest amount of hydrogen produced during production of the primary SCFAs, whereas pathways such as the bifid shunt, used by bifidobacteria, produce acetate with a neutral effect on H_2 levels. Interestingly, acetate can also be made from CO_2 and H_2 by acetogens within the microbiome. These microbes are part of the hydrogenotrophic consortium present in the gut.

1.1.4.2 | Hydrogenotrophic bacterial groups

We have discussed the various mechanisms by which hydrogen is derived from carbohydrate fermentation in the gut and the cross feeding of carbohydrate end products. However, a cross feeding structure is also built around the hydrogen cycle within the gut. The microbes that use hydrogen to generate energy can be divided into three primary groups: sulfate-reducing bacteria (SRB), methanogens and acetogens. These three distinct groups compete for hydrogen in this ecosystem and their success depends on the conditions present within the gut. The production of H_2S by SRB is the most energetically favourable of the three reactions, however, this requires sulfate and hydrogen in sufficient abundance. When sulfate is present, these SRB reduce sulfate as the terminal electron acceptor. This process converts $4H_2$, SO_4^{2-} and H^+ to HS^- + $4H_2O$ and releases −152.2 kJ mol^{-1} of energy

[18]. *Desulfovibrio*, *Desulfobacter*, *Desulfobulbus* and *Desulfotomaculum* all include species of known sulfate reducing bacteria [148]. In the human gut, the genus *Desulfovibrio* represents 67 – 91% of the SRB [149].

Methanogens use both hydrogen and carbon dioxide present in the gut to generate methane and energy. These archaea are capable of using H_2 as an electron donor while reducing CO_2, methanol or acetate [98, 150]. This process releases 131 kJ/mol^{-1}, less energy than SRB but significantly more than acetogens [18]. *Methanobrevibacter smithii* is the most abundant methanogen member of the human gut and may be present in concentrations of 10^9 CFU·g−1 in stool [151]. Interestingly, a bacterial pathway for methanogenesis using iron only nitrogenases has been recently reported in *Rhodopseudomonas palustris* [152]. Methanogens are also associated with slower transit time within the gut [153]. This finding is consistent with the reduction of total hydrogen levels, which have been associated with faster transit times and IBS-D [154].

Finally, acetogens complete the three groups of hydrogen using microbes. Acetogenesis generates acetate by combining CO_2 and H_2 molecules while generating 95 kJ mol^{-1} of energy [18]. These microbes use the Wood-Ljungdahl pathway and generate the least amount of energy in comparison to the other groups [155]. Acetogens rely on their ability to use the Wood-Ljungdahl pathway with a variety of electron donors which provides an ecological advantage due to redox balancing [155]. *Acetobacterium woodii* represents the model acetogen. This microbe displays the flexibility of this group as it is also able to metabolise carbohydrates, lactate and alcohols to generate energy [156]. This group is speculated to be the most abundant H_2 using group within the gut microbiome [157]. Increasing the concentration of these microbes within the gut may cause harmful effects or may be prohibitively difficult to administer. Culturing methanogens and acetogens relies on difficult growth conditions and low concentrations, parameters that are not conducive to large scale production [158].

The hydrogen cycle in the gut ecosystem has major implications for host IBS symptoms. The correlation between modulation of gut hydrogen concentration and IBS subtypes, IBS-C and IBS-D, indicates that targeting hydrogen genesis may directly affect symptoms [153, 154]. Similarly, increased breath hydrogen is considered an indication of malabsorbed carbohydrates or small intestine bacterial overgrowth [159]. This intimate connection between IBS and hydrogen production is tackled by the low FODMAP diet and antibiotics as a therapy. However, microbes may often be the solution to this fermentation problem.

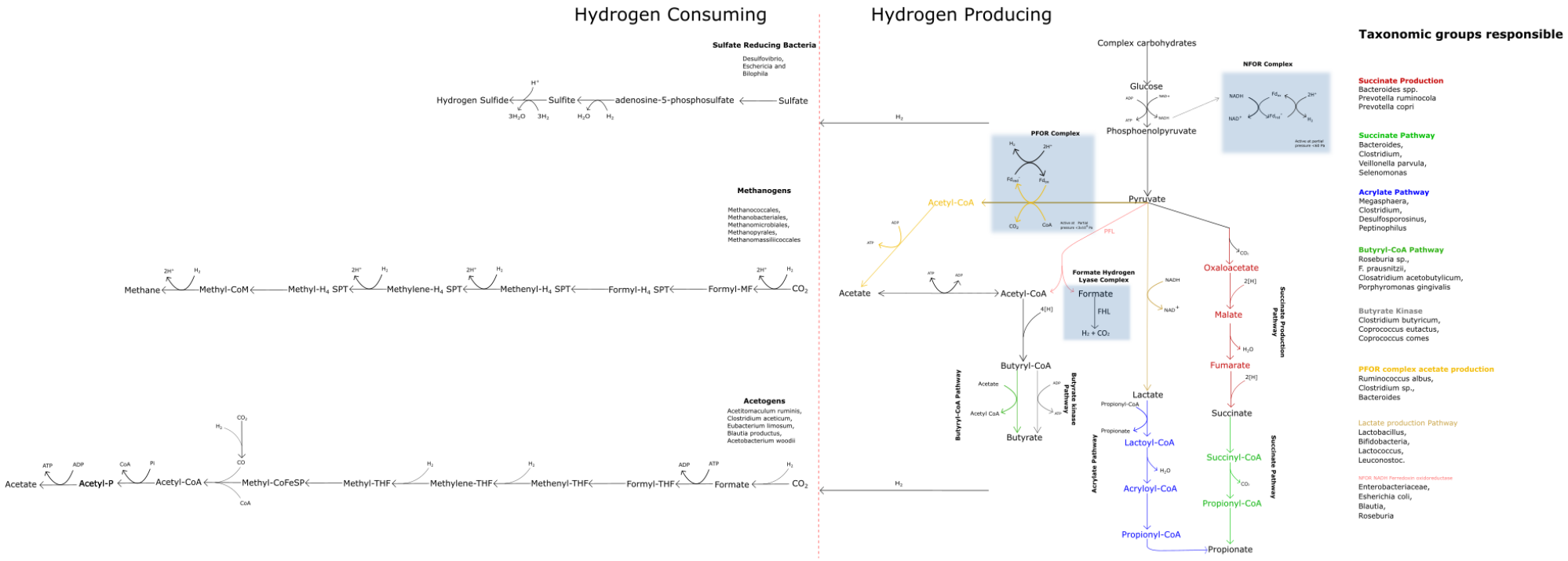

Figure 1. Hydrogen producers and consumers. For simplicity, all reactions were treated as one mole. Succinate production [160-162], Acrylate pathway [163, 164], Succinate pathway [164], butyryl CoA pathway [165-167], PFOR complex [168-170], Lactic acid pathway [171], Pyruvate Formate Lyase (PFL) [123, 172], Sulfate reducing bacteria [173], Methanogens [174], Acetogens [155].

1.1.5 | **The role of probiotics in redirecting the Microbiome**

Hydrogen production occurs within the gut under the conditions described above. The required elements are essentially an abundance of microbes capable of fermenting carbohydrates to create hydrogen and sufficient substrates for this fermentation. The low FODMAP diet successfully reduces IBS symptoms by targeting the latter of this equation. However, it is possible to disrupt the hydrogen production by introducing non-hydrogen producing species. A significant amount of work has been completed on this in the area of biohydrogen production [175]. The main characteristics of these hydrogen inhibitors should be that they 1) adeptly ferment the FODMAP carbohydrates that reach the gut, 2) have the probiotic capacity to reach the human gut and perform their function and 3) are minimal hydrogen producers during the metabolism of these carbohydrates. The first two criteria can be tested *in vitro* using standard probiotic testing such as GI transit resistance and intestinal adherence [176, 177]. The third characteristic requires an understanding of the most common metabolic pathway used by the potential probiotic. This step is possible with a genomic sequence and researching relevant papers describing the fermentation processes of the species.

Well known probiotic genera are the closest to achieving these criteria. This is due to the robust knowledge base regarding the metabolic pathways they use, their ability to reach the gut alive and their capacity to ferment carbohydrates. *Bifidobacterium* and *Lactobacillus* represent the majority of currently available probiotics and are suitable candidates for reducing hydrogen production *in vivo*. Conflicting reports exist as to the total hydrogen production of these strains. As mentioned above, *Lactobacillus* uses a hydrogen neutral pathway fermenting complex carbohydrates and glucose to lactic acid [178, 179]. However, Pandey et al. recently reported hydrogen production in a variety of *Lactobacillus* strains [100]. These reports are countered by research with identical species describing no hydrogen production [178, 180]. Analysing biohydrogen reactors with multiple cocultures indicates a role for LAB as microbes that reduce hydrogen yield. This is as a result of competitive exclusion [181], excretion of bacteriocins reducing gas producers [182, 183], hydrogen peroxide production [184] and substrate competition [185]. Increase in lactic acid has been reported to inhibit hydrogen production within biohydrogen fermentation units and destabilise hydrogen producing micro flora [181, 183, 186]. *In vivo*, a study analysing real-time hydrogen production in mice GI tracts reported a correlation between enrichment in *Lactobacillus* and a low hydrogen producing gut environment [17]. Previous research on infantile colic identifies the lack of lactobacilli in the immature infant microbiome as a potential reason for the disorder [187]. This is in accordance with reports of increased breath hydrogen in colicky infants [188]. The above research positions *Lactobacillus* as a potential probiotic strain for reduction of hydrogen in vivo.

Hydrogen utilising bacteria appear to be an appropriate probiotic for resolving this hydrogen production excess. Studies that have reported increased hydrogen production in IBS patients have previously indicated that hydrogenotrophic bacteria may present a suitable therapy [189, 190]. Increasing the concentration of these microbes in the gut is not as straightforward as consuming them as probiotics. Sulfate reducing bacteria co-occur with a variety of negative health outcomes such as inflammatory bowel disease (IBD) and rheumatic arthritis [191]. *Desulfovibrio*, the most prominent species within this group, is an opportunistic pathogen and causative factor in some cases of bacteraemia [192-194]. Increasing the supply of sulfur to the gut may result in proliferation of these microbes, however, high H_2S concentrations have also been associated with poor intestinal health [195]. For these reasons, it is not safe to administer these sulphate reducing microbes for the purpose of lowering H_2 levels within the gut.

Methanogens represent potential probiotics that may ameliorate the hydrogen derived IBS symptoms. Indeed, during the conversion of hydrogen to methane the gaseous volume is reduced by 75% [196]. However, similar to SRB, these microbes are considered opportunistic pathogens due to their presence at the sites of dental carries and their associations with intestinal disease [197-199]. Detrimental chemical and biological effects of methane have been described as their role in colorectal cancer and other intestinal diseases are investigated [200, 201]. This is most likely due to their role in reducing H_2 concentrations and facilitating fermentation by true pathogenic bacteria. The *in vitro* propagation of these microbes is also particularly difficult and growing them to a concentration that might survive GI transit may be unachievable [202]. Due to both the potential deleterious effect and growth difficulties these microbes do not qualify for potential probiotics.

Finally, acetogenic bacteria using the Wood-Ljungdahl pathway are capable of combing both CO_2 and H_2 gases to acetate. This group represent a large variety of microbes that are capable of autotrophic acetate production. Many acetogenic bacteria have been used as potential probiotic strains highlighting their safety. These potential probiotic strains have been tested *in vivo* and *in vitro* for reducing methane production [203, 204]. However, high concentrations of acetogenic bacteria do not appear to confer a greater reduction in H_2 concentration [204]. These microbes do not derive sufficient energy from the Wood-Ljungdahl pathway to outcompete methanogens. Several studies have shown that directly fed acetogens only reduce H_2 concentrations significantly when methanogensis is inhibited [205-208]. Their inability to compete with other hydrogenic microbes demonstrates the main limitation of this bacterial group when assessing their potential for H_2 reduction *in vivo*.

Due to the limitations of each group of microbes that use hydrogen directly, it appears that indirectly reducing the substrates of H_2 production may hold more potential for reducing hydrogen levels. For this

reason, we speculate that current probiotics with broad FODMAP fermentative capacity and neutral hydrogen production may fulfil this niche most effectively in the near future. These prospective probiotics may provide best effects on individuals that have shown symptom relief using a low FODMAP diet.

1.1.6 | **Conclusion**

Hydrogen gas plays an under-reported role in the gut microbial environment. The hydrogen cycle within the gut has broad reaching metabolic effects on all microbes present. A lack of balance in gut hydrogen concentration also directly impacts the host, potentially causing disorders such as IBS due to distension of the gut lumen. Many currently available IBS therapies indirectly target H_2 metabolism in order to control symptom severity, however, probiotics may provide a novel approach to targeting the overproduction of hydrogen. Hydrogen consuming microbes have several drawbacks such as opportunistic pathogenicity and difficult growth conditions. Select strains of conventional probiotics, such as LAB, may indirectly reduce hydrogen levels by fermenting the substrates that cause an increase in hydrogen. This may provide new therapies for FODMAP-induced IBS, avoiding many of the negatives associated with current solutions.

1.1.7 | **References**

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Chapter 1.2

The progress of Multi-Omics technologies: Determining function in Lactic Acid Bacteria using a systems level approach

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1.2.1 | **Abstract**

Lactic Acid Bacteria (LAB) have long been recognised as having a significant impact ranging from commercial to health domains. A vast amount of research has been carried out on these microbes, deciphering many of the pathways and components responsible for these desirable effects. However, a large proportion of this functional information has been derived from a reductionist approach working with pure culture strains. This provides limited insight into understanding the impact of LAB within intricate systems such as the gut microbiome or multi strain starter cultures. Whole genome sequencing of strains and shotgun metagenomics of entire systems are powerful techniques that are currently widely used to decipher function in microbes, but they also have their limitations. An available genome or metagenome can provide an image of what a strain or microbiome, respectively, is potentially capable of and the functions that they may carry out. A top-down, multi-Omics approach has the power to resolve the functional potential of an ecosystem into an image of what is being expressed, translated and produced. With this image, it is possible to see the real functions that members of a system are performing and allow more accurate and impactful predictions of the effects of these microorganisms. This review will discuss how technological advances have the potential to increase the yield of information from genomics, transcriptomics, proteomics and metabolomics. The potential for integrated omics to resolve the role of LAB in complex systems will also be assessed. Finally, the current software approaches for managing these omics data sets will be discussed.

1.2.2 | **Introduction**

Sequencing the first whole genome of a bacterial strain, namely *Haemophilus influenzae*, in 1995 was a milestone in molecular biology for a number of reasons [1], one of which was heralding in an era rich in information where the volume of data produced was beyond being completely interpreted. Subsequent genomic data sets derived from bacteria elucidated gene functions, metabolic networks, biological pathways, microbial evolution and genome structure significantly enhancing our understanding of bacterial function and potential [2, 3]. However, the challenge in deciphering relevant genetic information from the background genetic material was almost an impossible task. To combat this, more information was required. Information on the transcription of the genetic material and subsequent production of proteins was necessary. The targets of these proteins and the molecules they interact with had to be determined. Nuanced epigenetic triggers and the metabolites that are ultimately produced are all crucial to understanding the system as a whole. Indeed, much of the observed phenotype in a system can be explained in the context of these data sets when interpreted correctly.

Biological systems rely on a simple concept of DNA – RNA – Protein that decides the phenotype of an organism. Biologists have analysed these 'omes' for a number of years in the form of genomics, transcriptomics and proteomics. In addition to these, epigenomics and metabolomics have recently been used to answer specific questions relating to the many functions of an organism. Given the year on year advances in 'omics' technologies, the volume of information that can be gathered in individual studies is expanding rapidly. Furthermore, the current high throughput nature of these techniques has increased accessibility to this information in terms of time and cost. This has placed many researchers in a situation where they can collect several omics data sets on the same experimental samples. In order to draw more comprehensive conclusions on biological processes these data sets must be integrated and analysed as a holistic system.

Technologies involved in a multi-omics approach share several commonalities, some of which contrast with the original approach of molecular biologists. For the most part, molecular biology has utilised a reductionist approach to date. This methodology involves breaking a complex problem into its constituent parts and solving them individually. While reductionism has had significant successes, particularly when the experimental subject is controlled by a single component [4], or can be explained by interactions between single molecules [5], it also has substantial limitations. These limitations are caused by the process of isolating components of a complex system; often the nature of their role in the system is lost. This is the most significant advantage that omics technologies can have compared to a reductionist approach. Maintaining these components in the system allows observation in a realistic environment where emergent properties can also be studied. These high throughput, top down

methods also provide a phenomenal volume of data in comparison to the reductionist approach. For example, as much as six terabytes of information can be generated by processing samples in tandem on an Illumina NovaSeq 6000. Finally, omics technologies require significant computational infrastructure in the form of novel algorithms and software to process and analyse the information produced [6].

This has placed researchers in a situation where they must adapt to maximise the results from biological data. Biologists must acquire skills in managing and manipulating these vast quantities of data to resolve experimental questions outside the scope of the lab bench. An appreciation of the strengths and limitations of many of these technologies will allow researchers to answer more complex questions and generate more general conclusions. This is a constant arms race as the technologies that underpin the generation of these omic and meta'omic data sets are constantly advancing. The scope of analysis ranges from entire community samples of 10^{12} microbes to exploring the components of single cells. Higher throughput machines are facilitating deeper sequencing than ever before. This sequencing depth is opening new potential use cases such as metatranscriptomics of the gut microbiome. Integrating these large data sets to provide a systems level view will reveal previously unattainable information on the individual microbes involved.

Research on Lactic Acid Bacteria (LAB) is uniquely placed to take full advantage of these fundamental changes in approach. Notably, LAB have been the subject of extensive research exploring specific attributes and functions of isolated cultures [7, 8]. In depth analysis of individual LAB strains has provided a wealth of information on their biological processes and functionality in a variety of publically available databases. Multi-omics technologies stand ready to exploit this information on LAB to decipher and predict many functions of interest using a systems level approach. Inter-microbe interactions, particularly within starter cultures, are some of the first to witness the potential in these advances [9, 10]. Similarly, multi-omics technologies are currently being used to tackle more complex systems such as the gut microbiome and host-microbe interactions [11-13]. This emerging field is capable of providing a platform for more accurate functional prediction of LAB in a variety of complex environments.

In this review, we will discuss the current most popular combinations in different omics technologies to facilitate accurate functional prediction for LAB. The most recent advances in the relevant technologies will be mentioned, while the potential they hold for deciphering the final phenotype of these microbes will be assessed. Finally, the computational barriers associated with integrating complex and diverse data sets will be discussed.

1.2.3 | **The potential for functional prediction in LAB using omics technologies**

LAB are among the most industrially significant groups of bacteria. These versatile microbes have a variety of potential functions that are applied in many sectors. Food production, health promotion, production of antimicrobials and in-vivo fermentation all see benefit from this group of microorganisms. These diverse functions encoded within single genomes are a trove of valuable information available for exploitation. As a result, these processes have been studied extensively in vitro, in vivo and more recently in silico to determine the critical pathways underpinning the phenotypes. Analysis of these molecular pathways has resulted in more accurate use of LAB in commercial endeavours such as starter fermentation cultures and probiotic supplements [14, 15]. Deciphering the underlying biological attributes associated with these critical microbes allows greater understanding in their current roles and may reveal new applications. However, this scrutiny has often focused on a single element of interest in isolation, instead of taking the entire system into account.

A more inclusive approach is possible with diverse sets of information interrogated by omics technologies. To date many groups have utilised both omics and multi-omics data sets to advance this field and progress knowledge of LAB function [12, 16-19]. The consistent progression of new technologies and methodologies has resulted in many studies providing crucial information on the function of LAB. Progress in this manner also provides direction for future work with these microbes. Studies using multi omics for mammalian or bacterial cells will pre-empt similar work with LAB. New methodologies incorporating omics technologies are often applicable to a variety of research topics. These studies will be discussed to gain insight into the potential use cases for LAB. Translating the relevant research in multi omics research will focus on what can be discovered in LAB within ecosystems they inhabit and their common commercial applications. The influence exerted by the new technological advancements will also be assessed for their direct effect on LAB.

Basic functions of LAB are well understood, as are the cellular processes underlying them. With this foundation of knowledge, research into LAB is in a prime position to exploit the coming wave of omics technologies. The sections below document the strengths and limitations of each omics technology, the impact of each omic data set on inferring LAB function to date, the relevant advancement in technology and how these new methodologies may accelerate future progress.

Table 1: The table depicts the strengths and weaknesses of the individual omics technologies. Recent advances in their respective fields are included to illustrate the progress in this area.

Data set	Strengths	Limitations	Recent advances	Citations
Genomic	Immutable link to the organism; Databases of reference genomes often available to aid reconstruction; Provides a static image of genes of interest; High throughput sequencing as standard	Short read sequencing results in gaps in 'hard to sequence' regions; Impossible to determine the activity of the genetic elements sequenced; Difficult reconstruction of genomes with bioinformatic software	Third generation sequencing; Simultaneous epigenetic determination with Genome sequencing; Higher throughput for shotgun meta-genomics	[20]; [21]; [22]
Transcriptomic	Robust data on the requirements of a microbe in a given environment; Vast quantity of data is produced; Effective combination with single cell technologies	RNA isolation and sequencing are susceptible to handling errors; The transient nature of RNA only provides a snapshot of the needs of the organisms; Presence of RNA's does not necessarily predict the translation into proteins	Higher throughput Next Gen Sequencers (NovaSeq 6000); Meta-transcriptomics of large systems is now possible; More reliable software for integration and variant determination	[23]; [24]; [25]; [26]
Proteomic	Significant database of known proteins provide a strong platform to predict function; Robust link between an organisms proteomic profile and its phenotype; Provides a more stable image of the current requirements of the organism than other omics technologies	Throughput capabilities of proteomics lags behind other omics; Expensive MS machinery is required for proteomic research; Concessions are made in order to analyse the vast array of proteins – Splitting large proteins into smaller sections to facilitate MS analysis	Orbitrap Mass Spec facilitates ionisation of more complex proteins; Combining Liquid Chromotography with multiple MS's allows accurate depiction of specific groups of proteins; Powerful analysis tools ie. PECAN, facilitate more accurate predictions from untargeted proteomics	[27]; [28]; [29]; [30]; [31]
Metabolomic	Direct connection between phenotype and metabolomics profile; Provides an image of many well-studied metabolites simultaneously; Diverse range of applications across many fields	The transient nature of metabolites makes them susceptible to sampling artefacts; Numerous costly LC/GC and MS machines needed for processing	Back to back LC or MS machines provide higher resolution of specific groups e.g. LC-MS/MS; New machinery such as High Temperature-Ultra High Performance LC are overcoming previously difficult to detect metabolites; Single cell sorting advances are facilitating robust single-cell metabolomics in the near future	[32]; [19]; [33]; [34]; [35]

1.2.4 | **The impact of genomics on Lactic Acid Bacteria**

Genomic information has recently become an essential piece of information when studying microbes in detail. This data set provides an immutable link to the organism that for the most part remains constant. In well studied bacteria, such as many LAB, fully sequenced genomes are readily available in a variety of species [36, 37]. These provide a database of reference genomes that may be used to easily produce accurate genomes of the strain at hand. Mining these genomes alone reveals information on all traits available to the microbe. Potential products are inferred from the genetic code and viable pathways can be determined analysing the relevant genes. These pathways are categorised by their overarching function e.g. carbohydrate utilisation pathways. Comparative genomic analysis between the generic pathways available to LAB and the strain of interest may highlight the unique functional capacity of the particular strain of interest [38]. *De novo* construction of genomic information is also possible. Knowledge of characteristic motifs and patterns in specific alignment of DNA bases e.g. Shine-Dalgarno, allows software to detect important genes such as cluster specific transcription factors and the promoters associated with said genes [39]. Methods such as this can be used in conjunction with comparative genomics for difficult to detect molecules such as secondary metabolites in the form of antibiotics, toxins, immunosuppressives etc. [40, 41]. Mining genomes for well-known genes is more straightforward using BLAST or DIAMOND [42, 43]. Searching the DNA or protein sequence of the element of interest against your genome will provide probability-based results on its presence in the genome. This information is the bedrock on which the multi-omics integration is built.

Mining genomic information on its own may direct experimentation or suggest mechanisms for known functional attributes. This was exemplified by analysis of genomic data in *Lactobacillus ruminis* revealing the presence of functional flagellar apparatus in the form of 45 flagellar genes [44]. Robust flagellar apparatus suggests *L. ruminis* is a motile microbe and presents a mechanism for pro-inflammatory tendencies. Despite not expressing flagella in culture media, strains with the genomic capacity to produce flagella were observed to partially recover this ability in-vivo. Gene clusters for crucial mucus binding pili have been detected in several LAB [45]. This information led to experimental attempts to display this function, resulting in demonstrating this function in *Lactobacillus rhamnosus*. This gene cluster explains *Lactobacillus rhamnosus*' impressive capacity to adhere to the intestinal mucosa [46]. In a similar fashion, gene clusters that are capable of producing a broad range of bacteriocins have been reported. This information led to observe *Lactobacillus salivarius* outcompeting *Listeria monocytogenes* utilising these compounds [47].

Appropriate use of secondary metabolite software tools for analysing the genome has resulted in the discovery of many novel antibiotics [48, 49]. Incorporating software such as anti-SMASH [50], PRISM

[51] and GRAPE [52] has facilitated mining of the genomic data sets for crucial biosynthetic gene clusters. A very similar process unlocks the potential in genomic data sets of LAB. These microbes are capable of producing a wide variety of diverse anti-microbial peptides [53, 54]. Capitalising on these powerful analysis tools can realise much of the potential that a genomic data set provides and determine many possible functions available to the bacterium in question. Researchers can forgo the culture based issues with screening for novel anti-microbial compounds and instead direct future experiments more accurately. This process was adeptly demonstrated by Singh et al. 2015, to identify putative bacteriocins [55]. 20 LAB genomes were assessed for relevant bacteriocin producing genes. Putative operons were identified leading to further characterisation of novel bacteriocins. This simplistic process describes the exploitation of genomic material to identify these traits of interest.

The utility of genomic information is on the cusp of a generational leap forward. Third generation sequencers, such as the Sequel II and the MinIon, are set to remedy many of the intrinsic issues associated with second generation sequencers [56, 57]. These are primarily a GC bias in fragmentation and amplification [58, 59], short reads resulting in difficult to sequence repeat regions [20, 60, 61] and substantial burdens on computational rearrangement of genomes from a huge volume of short reads [21, 62]. Limitations associated with short read sequencing become apparent when analysing large insertion sequences [63] or substantial rearrangements in chromosomal or circular genomic sequences [64, 65]. Transposable elements often contain genes of interest encoding traits such as antibiotic resistance and bacteriocin production [66, 67]. In some cases, critical processes such as genome replication are also intrinsically linked to the presence of inverted repeats [68] and structure variants of large genomic transfers between species often harbour crucial mechanisms [69]. Progress in this area will have a significant impact on determining some of poorly understood traits associated with LAB [70]. Similarly, it will be possible to shed more light on up-and-coming areas for LAB such as discovering novel bacteriocins [71]. Far greater read length (>20kb) has already determined clusters coding resistance to crucial antibiotics and accurate tracking of large translocations of patho-adaptive traits from commensals to pathogenic bacteria in the microbiota [72, 73]. LAB are likely to have gained traits from similar translocation events.

However, this is the proverbial tip of the iceberg regarding the potential impact on LAB due to third generation sequencing. The most striking example of this is the extraordinary amount of information in the form of epigenetics that is lost due to the fragmentation and sequencing process in next generation sequencing. Epigenetic, post transcriptional modifications exert significant control on bacterial genomes resulting in altered phenotypes [74]. Currently, there is a significant cost in both time and money associated with determining epigenetic marks [75, 76]. Both Nanopore and PacBio report the presence of epigenetic modifications during sequencing [22]. This will result in regular sequencing reporting

epigenetic alterations to bases, in turn opening this added layer of complexity to a far greater audience [77].

1.2.4.1 | Single cell Genomics

Single cell sequencing approaches have become more frequently utilised throughout the past decade [78]. This approach holds significant promise for several reasons, primarily due to its potential to decipher cellular differences within heterogenous cell populations in any tissue or cell culture. Determining cell heterogeneity is an essential step in understanding the development, regulation and response to external influence in a population of cells. This natural heterogeneity is amassed and averaged in bulk sequencing approaches. Traditional sequencing removes much information that may indicate more nuanced reasons for phenotypes of interest. Many techniques have been developed to isolate and sequence these single cells in a cost effective and high throughput manner [79-81]. Microfluidics and Fluorescent Activated Cell Sorting (FACS) are the most popular methods to date. FACS relies on tagging and isolating fluorescent cells by capitalising on the charged nature of a fluorescently tagged cell [82]. Microfluidics focuses on the precise combinations of oil, surfactants and cells to create a droplet containing a single cell [83]. These techniques are used in a variety of fields and are perfectly designed for use in single cell sequencing. Furthermore, these techniques are adapted to include lysing of cells and to incorporate sequencing materials within the droplets encapsulating the cell components.

Single cell sequencing technologies have focused on human cells to date. This is in part due to to the ease involved in lysing them to release nucleic acids, enabling high throughput protocols. This issue is being addressed to link higher throughput cell isolation methods, such as microfluidics, including suitable lysing protocol for bacterial cells [84]. For this reason, however, LAB studies availing of high throughput analysis are not presently available. Despite this, proof of principle studies demonstrate the potential for LAB research in this area. Large sequencing attempts to explore the 'microbial dark matter' of unknown areas of the tree of life [85] in microbiome samples have been conducted. In a similar manner, single cell isolation techniques may also be employed to analyse the least abundant bacterial species within community samples. Minor community members have been observed within the fermented dairy product Koumiss using this approach [86]. The protocol, described by Yao et al. 2017, involves diluting microbiome samples and sequencing single cells. This powerful, yet simplistic, technique can be exploited to analyse pools of bacteria that are known to have a specific output or phenotype in order to isolate the cells responsible. Analysing minute quantities of DNA and RNA, sometimes as low as femtograms of material, are within the remit of these single cell techniques [87]. This knowledge has been utilised in environmental samples to isolate microbes of interest such as oil degrading microorganisms [88]. Furthermore, single cell segmented filamentous bacteria were isolated

using microfluidics from mouse gut microbiome samples [89]. This protocol provides an isolation method applicable for single cell LAB in community samples. Despite the lack of single cell sequencing studies on LAB, many of the techniques described are directly applicable to LAB. These highlight the potential advances that are attainable in this area. The rapid progress of isolation technologies, lysing protocols and sequencing depth will provide a more stable platform for targeted single cell analysis of LAB. [90].

1.2.4.2 | Metagenomics

In contrast to single cell genomics, metagenomics provides community-based genome sequences of many diverse species simultaneously. This information allows correlation-based work to compare the abundance of particular gene families to the respective environment [91]. Metagenomics provides an overview of species abundance in the microbiome and characterises common metabolic pathways available in the ecosystem [92]. The contribution LAB make to the gene pool and functional processes can be discerned using metagenomic data. Armed with this knowledge, the potential role LAB play in the community can be determined. Metagenomics is regularly used to determine the microbial diversity in order to direct further analysis of the sample at hand. Zhang et al. 2016, used metagenome sequencing to study novel fermented foods. These insights into fermented foods revealed potentially interesting Lactobacilli strains that were then isolated from the samples [93]. This targeted approach to omics data is the most effective method when working with a single omics set. However, combining other omics data unveils a more dynamic image of the metagenome. This is most commonly applied when integrating genomic and transcriptomic data. These data can be indispensable to understanding the functional role members fill in a given system.

1.2.5 | **Combining transcriptomics with genomic data sets**

The combination of genomics and transcriptomics is one of the most common in addressing experimental questions. Combining transcript data with available genomic information provides an image of the intentions of the organisms, given a specific environmental situation. The integration of genomic and transcriptomic data [94-97] is frequently used across many fields, while merging metagenome and metatranscriptome data [98, 99] is becoming far more prevalent. Genomic and transcriptomic data sets have been combined regularly to offer insight into LAB's role in food spoilage [100], potentially probiotic traits of LAB strains [101] and their ability to use alternative electron acceptors in order to respire instead of ferment [102]. Isolating strains with particular functions is readily facilitated by transcriptomics data. A systems approach was used to analyse the altered functional capacity of a mutated Lactococcus lactis strain utilising genomic and microarray data [103].The genetic component responsible for its increased thermo resistance was determined using this combination of omics data. Transcriptome analysis of *Lactobacillus* strains causing beer spoilage was performed to determine the functional pathways which enable these microbes to enter the viable putative non-culturable (VPNC) state and thus survive in beer. Analysis of three *Lactobacillus acetotolerans* strains revealed that these strains were in a heightened stress state and had reduced gene expression levels in several other regular pathways such as metabolic processes, transport and enzyme activity. Understanding this process may afford future opportunities to prevent beer spoilage by inhibiting entry into the VPNC state [23].

Fundamental processes in LAB such as amino acid and carbohydrate metabolism have been advanced using transcriptomic data. Comparative transcriptomics has been imperative in understanding amino acid metabolism in Lactococcus lactis MG1363. A codY mutant strain was used to determine the role this gene plays in regulating more than 30 genes involved in metabolising amino acids [104, 105]. This strain was further analysed using transcriptomic data to analyse its global regulatory networks during growth in milk [106]. Knowledge regarding the expression of critical genetic components in LAB such as the catabolite control protein A (CcpA) has seen considerable advancement using transcript data. Deep transcriptomic and physiological data was used to explore the differential expression between WT *Lactobacillus plantarum* and a CcpA mutant during growth phase on different carbohydrates [107]. This study reports a substantial rearrangement in the carbohydrate metabolism regulatory network and sheds new light on the complexity of this process. It is clear that incorporating this data set into LAB research has already led to an increased understanding of these microbes.

1.2.5.1 | Single cell Transcriptomics

Transcriptomics at a single cell resolution is a relatively new field that may unravel many of the changes in transcription that are altered through a cells life span. These alterations are completely masked by bulk transcriptomics [108]. With new technologies providing faster delineation of single cells [109] in conjunction with greater sequencing depth with machines such as the NovaSeq, large scale single cell transcriptomics is more accessible than ever. Progress in these complimentary fields allows researchers to explore a previously unavailable aspect of cell state heterogeneity. Many single cell transcriptomic studies focused on stem cells [110], embryos [111], tumours [112] and the nervous system [113]. The use cases for this approach in these tissue types are apparent due to the advantages of delineating the differences between differentiated and non-differentiated cells. Comparing the responders to non-responders provides a greater opportunity to isolate mechanisms that stimulate desired responses [114, 115]. This method is also efficacious when the genome and transcriptome sequencing are carried out simultaneously on the same cell [116]. Macauley et al. 2015, sequenced the DNA and RNA of single mammalian cells in parallel, demonstrating the current capacity of single cell technologies. A subpopulation of 10% within 172 single cells of human and murine origin was reported after analysis. Several genetic alterations between cells and large chromosomal translocations events were observed.

LAB are among the best understood constituents within the microbiome. However, it is difficult to determine the importance of their role in an ecosystem this large without a systems approach [117, 118]. Constituents of the microbiome are known for their ability to affect the impact of drug compounds and therapy [119], ferment and convert many components in our diet [120] and have significant impact on healthy brain function [121]. Single cell technologies may lead the way in deciphering LABs role in these functions of the microbiome.

1.2.5.2 | Metatranscriptomics

Advances in next generation sequencing technology have reached a sequencing depth that facilitates more comprehensive metatranscriptomics of large community samples such as the gut microbiome [122-124]. As mentioned above, the current NovaSeq can produce 20 billion reads in a machine run. With this volume of reads between 100-400 taxa can reach maximum saturation of reads required for the highest statistical power [125]. This step forward provides a powerful tool for gut microbiota analysis and realises a true systems biology approach to determining how this community reacts to environmental perturbations. Due to the large database of sequenced LAB genomes, LAB stand to gain the most from the analysis of ecosystems such as the gut microbiome with a systems level approach. This combination of metagenomics and metatranscriptomic data is just beginning to become relevant in larger ecosystems; however, significant results have already been attained.

To date this approach has been observed in smaller microbiome samples such as Kimchi [126] and rumen [127]. This methodology was also used in a proof of principle analysis to determine the function of a critical microbe in bacterial vaginosis. Indeed, by utilising metatranscriptomics *Lactobacillus iners* was implicated in having a functional role in the presence of this disease differentially expressing over 10% of its genome between healthy and disease states [128]. Specific commercially important processes such as cheese ripening have also seen the impact of this approach. Despite using shallower metatranscriptome data, De Filippis et al. 2016 demonstrated temperature-driven functional changes in the cheese microbiome during ripening which had a significant impact on cheese maturation rate. They indicated that "processing-driven microbiome responses" can be altered to influence product quality and production efficiency [129]. Expression data that can be tracked throughout the process and related back to the specific strains responsible is invaluable in important commercial processes such as cheese production. The potential to carry out similar research in large microbiome samples such as the gut, in a manner similar to De Filippis' experiment, is fast approaching.

Third generation sequencing platforms may have a direct impact on the RNA-seq field. Long read technologies perform better in the determination of unknown transcript abundance in single celled organisms [130], full-length splice isoforms with alternative splicing [131] and co-transcription of genes in a polycistronic fashion [132]. It is clear that there are obvious advantages to long read sequencing in reducing the difficult reassembly and loss of contextual information associated with short read sequencing. However, the lower accuracy and sheer scale of some transcriptomics and metatranscriptomics projects keeps them out of reach of current third generation sequencers.

Integrating metatranscriptomics with deep metagenomic data presents the ability to track a time mediated response to specific changes in the environment. Be it antibiotic exposure, pathogenic infection or probiotic administration, a wealth of information on how the system is reacting to the alteration will be generated. Such data could be tracked back to each species and provide information on how to produce effective therapies in similar situations. This holistic approach to studying these bacteria in their natural habitat will reveal much about the production of compounds of interest and may result in interesting revelations about the transition between the microbiomes symbiotic and dysbiotic states.

1.2.6 | **Assessing the utility of proteomics within multi-omics data**

Proteomics investigates the complete set of proteins present in a cell, tissue or organism at a molecular level. Proteomics in its own right is a powerful analysis tool that has helped resolve many functional questions regarding LAB. Proteomic analysis has determined abundant compounds that are present in the transition between growth phases in LAB [133] and has been used to study the metabolic interactions of LAB [134]. Proteomics has determined critical proteins involved in acid stress resistance in *Lactobacillus casei* comparing a known stress resistant mutant to the Wild Type (WT) strain [135]. Assessing the complete set of secreted proteins in LAB has increased our understanding of how these bacteria interact with their environment [136]. Research to determine the capacity for LAB to resist osmotic stress, a critical trait of all microbes in challenging environments, has also progressed notably utilising proteomics [137]. These examples serve to prove the flexibility and effectiveness of proteomics; however, this information is best used when combined with other omics data sets. Despite considerable advances in proteomic technologies of late [30, 138, 139], this omics data set is the limiting factor in relation to throughput when integrated with genomics and transcriptomics. Untargeted discovery proteomics is termed Data Independent Acquisition (DIA) and allows the most comprehensive combination with other omics sets [140]. This process facilitates the tracking of genes to proteins in a manner that produces functional data [141-143].

Protein abundance is intrinsically linked to the mRNA levels discussed above, however, mRNA abundance does not correlate well to protein abundance in a system [25, 28, 144]. Considering this disparity, and that proteins are the molecules that control almost all cellular processes, the benefit from integrating these technologies for a more complete image is clear [145, 146]. When combined, these data sets can answer higher dimensional questions about large scale processes such as studying the metabolism of many products simultaneously in a holistic manner [147, 148]. Complex microbial interactions such as quorum sensing may be deciphered using this approach [149]. This process was carried out for LAB to assess the complex interplay between LAB strains in yoghurt fermentations. Transcriptomics and proteomics were combined to understand how the strains present interacted to produce the desirable effects. By-products from a single strain stimulated growth in the co-culture resulting in a reliable yoghurt formation [150, 151]. Similarly, this information can be used to further understand specific traits of LAB such as their crucial ability to manage bile stress in the case of probiotic strains of *Lactobacillus [152]*. Combining proteomics with genomics and transcriptomics allows more robust biomarkers and treatments to be determined. This is observed in traits of disease phenotypes in humans [153] or functional processes in bacteria [154]. Understanding the methods that bacteria use to interact with their environment through several omic data sets develops network links between these

data sets. Primary processes such as stress responses are frequent targets for a combinatory approach and may reveal critical information that would be lost without a holistic approach [155].

1.2.6.1 | Single cell Proteomics

Single cell analysis also makes an impression on the field of proteomics. Until recently, there were merely proof of principle publications describing the ability to identify minute concentrations of proteins available in a single cell [156, 157]. Mass spectrometry, the primary method for proteomics research, is frequently used when tens of thousands of cells are available from which to extract proteins. However, a single cell has in the region of 1×10^5 protein molecules. With this in mind, it is clear why single cell mass spectrometry (MS) techniques will reveal only the most abundant of proteins present. Despite this, advances in this area have increased the scope of single cell proteomics considerably. Progress within the flow cytometry field has resulted in an increased variety of fluorescent markers [158]. While antibody based immunofluorescence confocal microscopy has been used in human cells to identify >12,000 proteins across multiple cell lines [159]. Microfluidic image cytometry can now analyse activity of specific protein groups such as kinases [160] and using photocleavable DNA barcode-antibodies to quantify various proteins from single cells is also possible [161, 162]. These techniques have enabled targeted proteomics of single cells, however, to our knowledge no single cell proteomics work has been carried out on LAB. Tracking the mechanisms and rate at which single cells adapt to environmental exposures will help define the specific triggers, systems and pathways involved in these situations. Developing knowledge of these networks while avoiding the clouded nature of bulk analysis is the most effective method to increase the accuracy at which we can predict the function and reactions of these microbes.

1.2.6.2 | Metaproteomics

Currently, meta-proteomics struggles to stack up to the comprehensive nature of metagenomics and metatranscriptomics. However, a recent study by Ting et al. [31] depicts the current power of untargeted exploratory proteomics accurately. This group demonstrated that the difference between the more comprehensive nature of Data Independent Acquisition and more accurate Data Dependent Acquisition (DDA) is lessening. After developing a novel library free peptide detection method, PECAN, this group was capable of detecting 12,767 peptides within a sample, 6,221 of which were unique compared to the targeted approach. The untargeted DIA approach detected 83% of the peptides elucidated during the targeted DDA approach. The detection accuracy was impressive as ~99.5% of the retention times were identical between both approaches. This is while simultaneously detecting more than twice the number of peptides, indicating its suitability for exploratory proteomics. Techniques such as this are facilitating more realistic, high throughput proteome analysis [163]. This represents the

future of larger scale metaproteomic work; however, it is still in its infancy. For this reason, targeted assessment of protein abundance and identification is more appropriate and useful than untargeted in its current state. As such a targeted approach must often be taken when combining the information with metagenomic and metatranscriptomics data. It is feasible to choose a specific function or set of functions to analyse as part of the system-wide metaomics approach. Important traits associated with functional features of LAB can be interrogated further with proteomics in this manner [164, 165]. Analysing functional traits has developed a significant understanding of the proteome of the LAB group to date [166]. Several studies in 2019 have combined metaproteomic data sets with the genomic counterparts to analyse fermented foods. This phenomenon has revealed much information regarding the central role plays by LAB in these functional foods [167, 168]. However, these metaproteomic approaches may only assess smaller scale community samples. Expanding this process to incorporate gut microbiome samples is beyond current proteomic technologies [169].

1.2.7 | **Completing the multi-omics picture: Metabolomics**

Metabolomics is the study of all metabolites produced in a given system. This omics technology is a natural progression from proteomics in that proteins are responsible for the presence of the majority of metabolites found in an organism. The far-reaching effect that metabolomics research may have is apparent, as all phenotypes are intrinsically linked to the metabolites involved in the system studied. This concrete connection between the phenotype and metabolome makes it an exciting data set to add to any research. The volume of information that can be gathered is substantial considering the sheer scale of all metabolites that may be present. Recent advances have resulted in more accurate systems than ever for delineating these compounds. Back to back Liquid Chromotography or Mass Spectrometry units (LC/LC-MS, LC-MS/MS) [170], High Temperature Ultra High Performance Liquid Chromotography (HT-UHPLC) [171, 172] and nanoflowUHPLC-nanoESI-MS [32] are filling in the gaps for previously difficult to detect compounds such as hydrophilic or minor metabolites. This progress results in untargeted metabolomics reaching the throughput necessary to combine them comprehensively with other omics sets.

The utility of metabolomics technology on its own is obvious and has been used to make connections between gut related diseases and metabolites produced by gut microbes [173-175]. Metabolomics has also been utilised to decipher functions of LAB such as their role in commercially important processes and revealing information on regarding their metabolism [176-178]. In a study by Hong et. al. 2011, probiotic LAB were introduced to a group of Irritable Bowel Syndrome (IBS) sufferers. NMR was used to determine the metabolic niche that these LAB fulfilled in the IBS sufferers by comparing them to a control group not receiving the probiotic [179]. The resulting data suggested a dysregulation in energy homeostasis and liver function based on the metabolites present. The potential of metabolomic data sets created are unlocked when linked to other components throughout the organism such as proteins, RNA etc.

Many functions have been determined integrating these data sets as it provides a powerful platform to provide answers to many research questions. This has been observed in several fields such as chemotherapy toxicity, toxicology, fungal phytopathology to heavy metal resistance in plants [180-183]. In LAB this combination of technologies is useful when analysing an organism-wide response such as growth efficiency and amino acid metabolism [16] or the ability to adapt to ecological niches [184]. Conversely, these omics can be used with a specific end point in mind such as probiotic potential to treat a specific issue, providing a powerful protocol for selecting suitable candidates [19]. A combination of genomics, transcriptomics and metabolomics has been used to analyse the microbial role in the fast growing area of milk whey. Milk whey has recently transitioned from un-used by-product to high value

commercial product. However, the regulation has lagged behind resulting in many unknowns regarding the microbial composition and microbial by-products present in this product. Omics data sets allowed Sattin et al. 2016, to report the reasons for poor whey quality, the potentially concerning compounds present and how to maintain higher commercial value [9]. Similar processes' were described when assessing the role multi-omics data may play in food microbial interactions [33, 185]. Indeed, genomics, transcriptomics and metabolomics aided Turroni et al. 2016, in deciphering the complex interactions that allow Bifidobacteria strains to persist in the murine gut [11]. This valuable information leads to a greater understanding of the functional requirements for probiotic strains to survive this environment.

A clear expression of this approach can be seen in many publications that target microbial-produced antibiotics. Exploiting multi-omics data sets is now a pivotal step in discovering novel antibiotics [186]. Albright et al. 2014, demonstrate a clear methodology for screening strains for novel antibiotic metabolites based on multi-omics data [187]. Figure 1 shows a generalised version of the method outlined in this paper. The flow begins with sequencing the genome of the strain of interest. This facilitates searching the genome for the presence of biosynthetic gene clusters that may indicate antibiotic production. The genome enabled proteomics used in this study produced a >15 fold increase in the number of antibiotic peptides detected compared to regular proteomic analysis. After determining the relevant proteins produced, metabolites were assessed using data-dependant acquisition. Metabolites associated with the proteomic data set that represent potential antibiotic compounds are selected for using LC MS/MS. Isolated metabolites are compared to the relevant databases to rule out the known compounds. In this manner novel natural products are isolated in an effective progression from strain to isolated compound using multi-omics data.

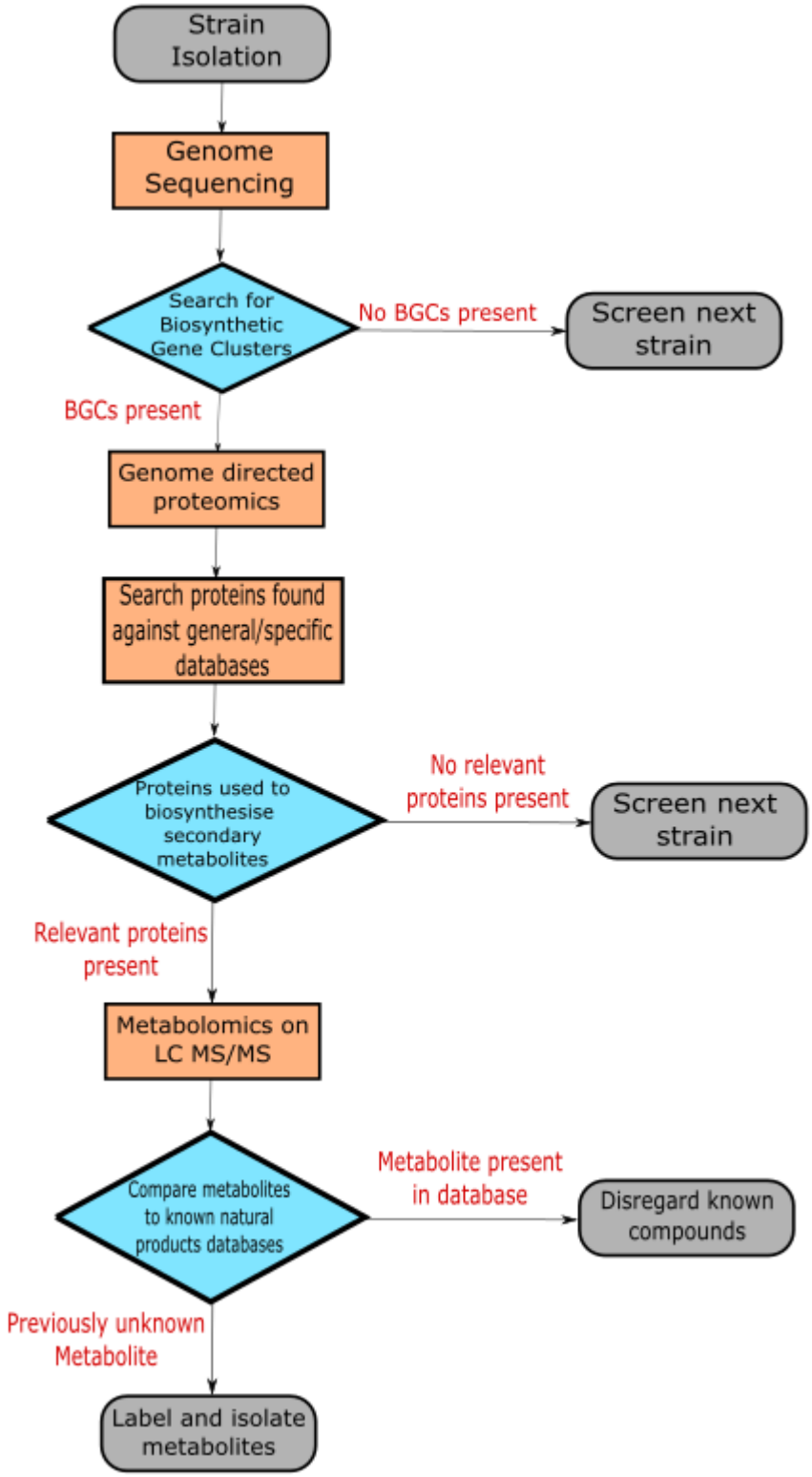

Figure 1. The flow chart depicts a generalised version of the methodology used by Albright et al. to isolate novel secondary metabolites with antibiotic potential [187].

1.2.7.1 | Single cell Metabolomics

Single cell metabolomics, although still in its infancy, can reveal the closest link to the phenotype of a single cell in a population. Sample volumes, low concentrations of analytes and sampling techniques are significant obstacles to this technology based on miniscule substances. However, in recent years, we have seen significant steps in accurate sampling of metabolites. A comprehensive review has been completed by Duncan et al. 2019, on the state of the art techniques developed over the last three years for single cell metabolomics [188]. A primary concern of these techniques is the transient nature of metabolites. A cell with particularly fast metabolic turnover can change its metabolic profile in 0.3 seconds [34]. This volatility means the cells must be maintained in the native environment and treated carefully to avoid artefacts of the sampling technique.

Live single cell mass spectrometry shows promise providing rapid, direct analysis of targets while also producing a complete annotation of the results in less than an hour [189]. Fluorescent based techniques relying on flow cytometry and direct microscopy are suitable methods for analysing specific metabolites [190, 191]. More recent developments in single cell technologies such as microfluidics are also applicable in isolating cells based on their extracellular metabolite profile [35]. With associated technologies progressing quickly it is clear that single cell metabolomics will make large steps with regard to throughput and accuracy in the near future. However, no LAB based single cell metabolomics data are available in published literature. Despite the difficulty in accessing the metabolomic data of single cells, the utility of the information is clear. This analysis allows abundance of specific metabolites to be confidently linked to traits. Moreover, developing specific links will aid functional prediction in all similar microbes with metabolomics data.

The flexibility of these data sets is apparent and with the advent of more effective and comprehensive technologies even more research will move to this culture independent, data driven approach. Further development in multi-omic databases will fuel research in improving the technologies related to each omics data set. Understanding the methods that bacteria use to interact with their environment through analysis of simultaneous omics sets will result in the development of network links between the data sets themselves. Common links that are regularly observed between omics data sets will lead to greater molecular understanding and will be incorporated into traditional interaction networks. Extracting and depicting these complex interactions is an intrinsic issue associated with big data sets that multi-omics produce. Complex software pipelines play an important role in making sense of these data. To this end, we will briefly mention the more prominent software used for these integration processes.

1.2.8 | **Data integration and computational limitations**

The integration of data sets generated in multi-omics research is by no means trivial. Each omics technology naturally consists of different types of data complicating the analysis to begin with. This is further complicated by the sheer volume of information that must be sifted through, particularly with meta-omics data. Software pipelines have been developed to manage this seemingly impossible task. Programmes are developed to create models that can predict outcomes when multi-omics data is inputted. The outcomes analysed are frequently disease states that can be described in terms of multi-omic data sets using these techniques. These pipelines generally fit into one of three approaches.

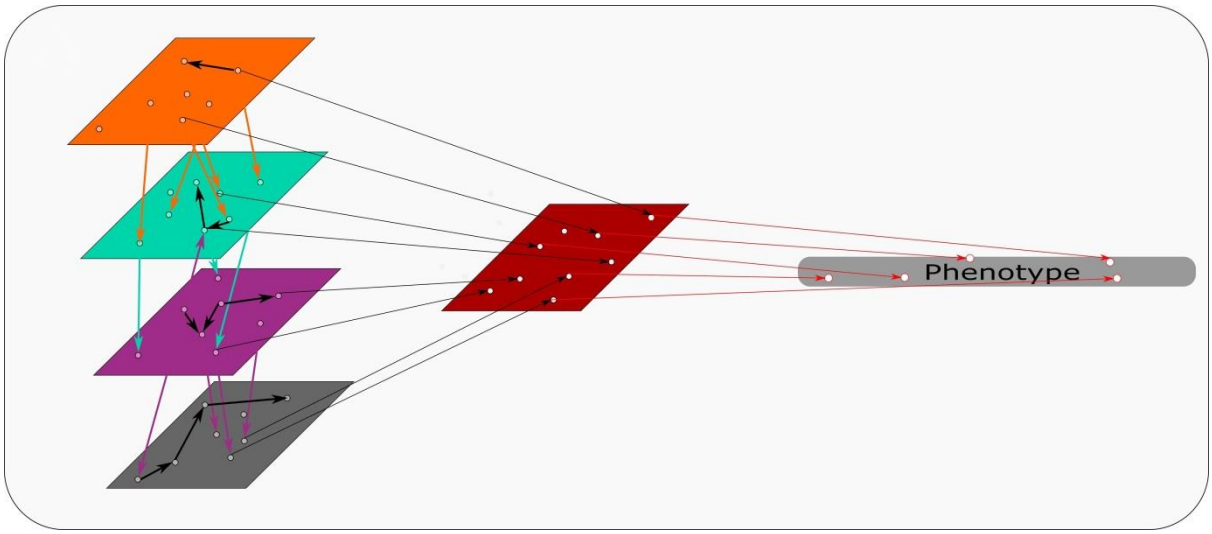

Figure 2. Concatenation based integration: Omics data sets (coloured rectangles) are combined at the beginning of concatenation based integration. Identifiers are determined between and within each omics set. These identifiers are combined and used as a model to discern specific attributes within the phenotype.

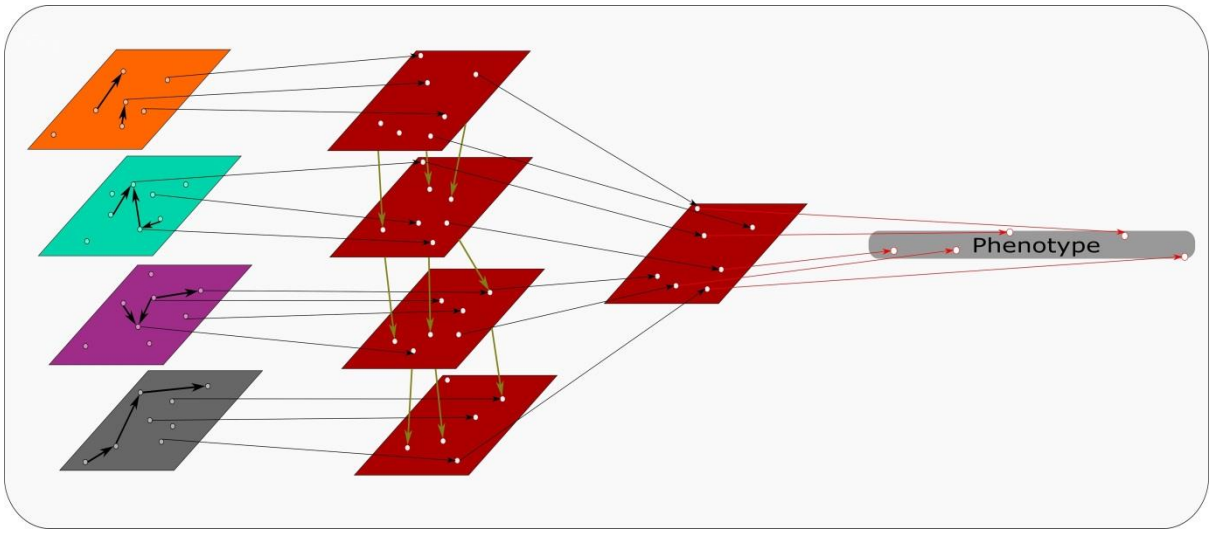

Figure 3. Transformation based integration: Each omics data set is transformed into comparable input matrices. Relevant identifiers are united to build the predictive model from all transformed data sets. This model discerns phenotypic traits that can be quantified using multi-omics data.

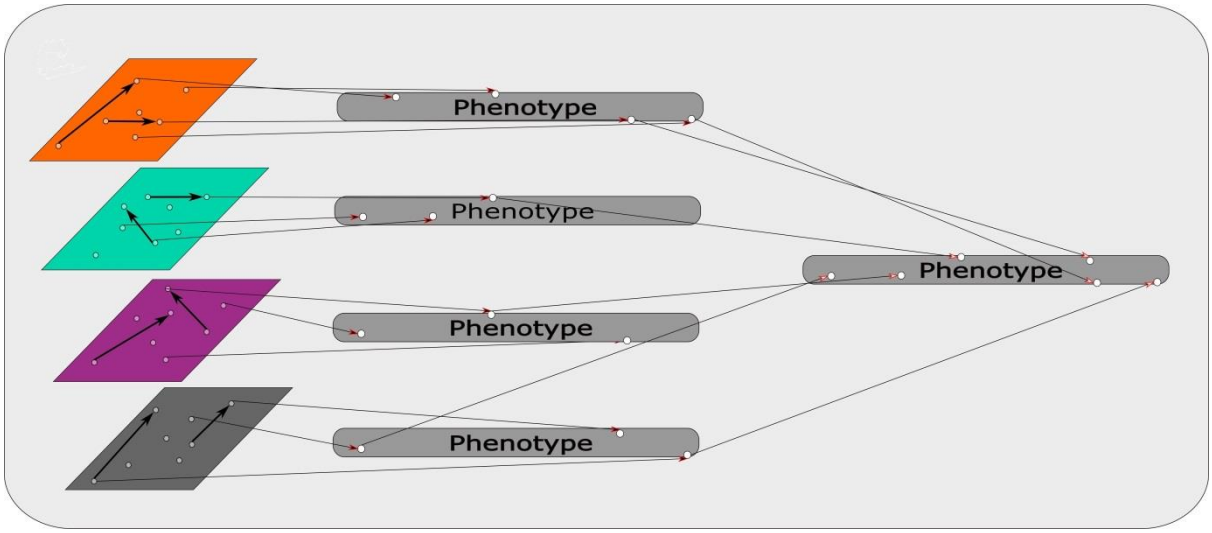

Figure 4. Model based integration: Each omics data set is used as a model to determine identifiers of traits within the phenotype. The phenotypic traits that are discerned from each omics data set/model are weighted based on their capacity to predict the phenotype. These weighted identifiers are then combined and ultimately predict the phenotype.

These models approach the issue with different methods, but all are valuable assets available to integrate the data. However, each of these approaches has limitations when negotiating these data. These limitations manifest in difficulty transforming differing data sets, combining massive input matrices and over fitting training data. Most pipelines adopt one of these approaches as a broad starting point. Researchers will have nuanced differences in how they treat types of data and how they weight connections between their data points.

A creative use of a transformation based approach has led to remarkable results in liver cancer survival prediction [192]. This model developed by Chaudhary et al. 2018 uses a deep learning method autoencoder and a single variate cox-PH model to choose features associated with survival. K-mean clustering is applied to these features to determine survival-risk groups. The omics data sets are then ranked via an ANOVA and features common with the predicting set are chosen. This step is depicted in Figure 3, where omics data sets are transformed into survival-risk predicting sets. These ranks can then be compared and combined. The final survival-risk labels are generated from the top features chosen by this method [192]. By incorporating microRNA seq, RNA seq, methylation and genomic information

this study highlights the potential powerful use for multi–omics data when appropriate software can realise its potential.

An R based software, mixOmics, presents a recent example of a modified concatenation-based approach [193]. This R based software uses its 'Diablo' process for this approach. Diablo incorporates all the input variables into a single input matrix. During this process interactions and associations between data sets are factored into the single input matrix. Figure 2 represents a schematic of this approach, combining the different omics sets while taking interactions between data sets into account. This adds another level of complexity that suits multi-omics approaches specifically, creating a more powerful input matrix from which the model is derived. This software also contains another pipeline, namely 'MINT'. MINT is a model-based integration where data sets of the same type, e.g. transcriptomics, from different studies are combined to produce a model. Each type of data set predicts aspects of the phenotype as illustrated in Figure 4. The predictions determined by each model are weighted based on their propensity to determine the phenotype. These input models are subsequently combined to generate a final prediction model [193].

These exciting new software packages encompass each of the major methods of generating models for integrating omics data. Many developers are constantly updating and upgrading their platforms to incorporate the latest data sets and produce the most effective models [194, 195]. Many concatenation based approaches have been developed to address nuanced different data sets [196, 197]. Cancer survival prediction has seen significant success utilising transformation and model based approaches. This progress has been observed using multiple genomic data sets to accurately assess ovarian cancer survival and in predicting instigators of melanoma from gene expression data and chromosomal copy number variation [196, 198]

More powerful models are constantly being developed, incorporating ever expanding data sets while integrating even more types of omics data. These advances are essential to keep up with the rapidly increasing volumes of data and to address the current limitations associated with many original data sets [199]. Several studies have emphasised the over estimation of significant results and several contradictory outcomes in multi-omics data sets in many high impact publications [200, 201] . In these cases genuine heterogeneity within samples in genome wide association studies is considered statistically important disease specific information [202, 203]. Software tools must understand and incorporate these issues, while avoiding the risk of false negatives due to too harshly correcting data. For more information on the challenges associated with integrating these data sets, the reader is directed to the following review articles [204-206]. For the reasons detailed above, there must be an

element of responsibility on biologists to negate some of these limitations by developing a workable level of understanding regarding the most suitable software model for their experimental questions.

1.2.9 | **Conclusion**

The mechanisms responsible for generating omics information have seen considerable progress in recent years. The advent of new technologies, such as third generation sequencing, is capable of transforming the level of information available to researchers. These advances have placed the integration of multiple omics data sets within reach of more scientists. This availability will result in substantial advances in all aspects of microbial work from delineating specific functions to understanding their role in complex ecosystems. This review assesses the advantages to a high dimensional systems level approach when analysing organisms and systems simultaneously. The fortuitous position that LAB research finds itself in is also discussed. LAB are a group of microbes with a wealth of data already available in a variety of databases. Due to this position, research focused on this group is poised to take full advantage of the progress in multi-omics research. As the field of molecular biology becomes a data intensive one, it is critical that biologists keep up with this trend. Researchers must develop skills in data processing, develop an understanding of the mechanisms behind software they utilise and be flexible to incorporating new technologies into their workflows. This task is challenging, but the rewards available with multi-omics are substantial.

1.2.10 | **References**

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Chapter II

Selecting *Lactobacillus* strains capable of reducing the burden of a low FODMAP diet.

2.1 | **Abstract**

Members of the *Lactobacillus* genus were assessed for their ability to ferment non digestible carbohydrates in order to provide an alternative to a low Fermentable Oligo-, Di-, Mono-saccharides and Polyol (FODMAP) diet for Irritable Bowel Syndrome (IBS) sufferers. *Lactobacillus* spp. can ferment these non-digestible FODMAPs without producing the molecular hydrogen and carbon dioxide associated with IBS symptoms. *In vitro* FODMAP fermentation assays were carried out on 32 strains previously isolated from adult and neonatal gastrointestinal (GI) tracts and Sicilian cheese. From these 32 strains, 10 were chosen based on their ability to ferment a variety of FODMAP carbohydrates, such as mannitol, inulin and raffinose. Varied FODMAP fermentation profiles, the ability to survive simulated gastrointestinal transit, appropriate antibiotic resistance and robust adherence to Caco-2 cells were observed during further testing. The genetic components responsible for these traits were analysed and linked to the phenotypic results where possible. Correct orientation of the mannitol operon was demonstrated to be necessary for moderate and strong growth on mannitol. Chromosomally encoded antibiotic efflux pumps and beta-lactamases were correlated with moderate *in vitro* antibiotic resistance. Two *Lactobacillus casei* strains and one *Lactobacillus rhamnosus* strain were selected following *in vitro* experiments. These three strains will be used to determine the potential for reducing hydrogen yielding fermentation *in vivo* (Chapter 3). By targeting this diet-microbiome interaction, these three strains show promise for treating FODMAP induced IBS via a novel probiotic led approach.

2.2 | **Introduction**

Irritable Bowel Syndrome (IBS) has garnered significant research attention due to its prevalence and disruption to quality of life. The prevalence of this chronic functional gastrointestinal disorder (FGID) within the Western world is between 10-15 % [1, 2]. IBS is characterised by abdominal pain, bloating, discomfort and a change in symptom severity following defecation. The diagnosis of this FGID is made on the basis of these symptoms after excluding organic diseases such as inflammatory bowel disease (IBD) and celiac disease [3]. IBS has been described as having no underlying biochemical basis to the disorder, however, this view has shifted in recent years. An increased understanding of the factors intimately linked to IBS, such as the gut microbiota and the gut brain axis, has developed our knowledge of this FGID [4, 5]. The high prevalence of this disorder also causes a substantial economic impact. The cost to industry for each IBS patient ranges from approximately €500 to €1000 annually [6]. This is as a result of patients being twice as likely to take days off work due to the illness [7] while as many as 12 % of sufferers report giving up their work completely due to IBS [8]. There is also substantial direct cost incurred by the patient and a reduction in quality of life that this disorder brings [6]. IBS has a significant impact on the lives of patients that suffer with it; however, there are very few treatments available. One of the most prevalent methods for ameliorating the severity of symptoms is through dietary intervention. These treatments include low carbohydrate, gluten free and high fibre diets [9-11], while the most prominent and effective dietary intervention is now understood to be the low FODMAP diet [12, 13]. FODMAPs are non-digestible carbohydrates that reach the gut intact. Lactose, inulin and mannitol are examples of carbohydrates within the FODMAP umbrella. Common foods such as onions, garlic, dried fruit, wheat and rye all contain high FODMAP content. FODMAPs act as a primary source of carbon for the gut microbiota that reside there [14]. FODMAP carbohydrates alter the gut ecosystem as they are fermented by various gut microbes.

We now have an increased understanding of how the microbial consortia present in the gut, the gut microbiota, plays an important role in IBS pathophysiology. The gut microbiota comprises of more than 100 trillion microbes and over 1000 species [15]. These microbes have been the subject of intense research in recent years and has been implicated in many disease states [16-19]. The link between IBS and the gut microbiota has also been explored [5, 20]. Host-microbe and microbe-microbe interactions contribute to the pathology of this disorder, while external factors such as stress and diet also play a significant role [21]. A breakdown in regulation of host-microbe interactions leads to an increased host defence response against bacteria and increased bacterial engagement with the host [22]. Dietary components have a profound impact on the microbiome and IBS symptomology. This is particularly true for the non-digestible compounds within our diet, such as FODMAPs. *Bacteroides*, *Ruminococcus* and *Lactobacillus* are among the genera responsible for the first fermentation step of

these FODMAPs. This fermentation process releases compounds such as lactic acid that are used as an energy source by other microbes within the gut. This complex cross feeding structure produces a range of bioactive compounds such as Short Chain Fatty Acids (SCFAs), Branched Chain Fatty Acids (BCFAs) and ammonia [23]. SCFAs are well known for their potential health benefits such as maintaining homeostasis, reducing inflammation and suppressing carcinogenesis [24]. However, this FODMAP fermentation process also has potentially negative outcomes such as causing symptoms in individuals that suffer with IBS due to the genesis of CO_2 and H_2 within the gut. These compounds are present in all individuals, however, ineffective consumption of H^2 or excessive production of H^2 lead to the symptom generation seen in IBS patients. The low FODMAP diet has been shown to reduce symptoms severity in over 70% of IBS patients in some studies [25, 26]. However, the low FODMAP diet is particularly restrictive during the initial phases.

Due to the difficulty in adhering to the low FODMAP diet, only individuals with severe IBS symptoms find it a palatable option. For this reason, we focused on providing an alternative to this diet for IBS patients, while still delivering the reduction in FODMAP load that the diet provides. Bacterial strains that can enzymatically digest these FODMAP carbohydrates may provide this alternative to the low FODMAP diet. Research on previous probiotic strains supports their role in managing IBS symptoms such as higher levels of anxiety and pain [27-29]. We hypothesise that selecting the correct bacterial strains may also result in amelioration of FODMAP induced IBS. The correct strains may confer this effect as lactobacilli can proliferate using both the Embden Meyerhof (EM) pathway and the Phosphoketolase (PK) pathway [30-32]. The latter pathway produces CO_2 to create energy from glucose, whereas the EM pathway does not [33]. Selecting *Lactobacillus* strains that preferentially use the EM pathway may cause a reduction in CO_2 and H_2 concentrations in the gut through a number of mechanisms such as competitive exclusion [34], excretion of bacteriocins reducing other gas producers [35], competitive ethanol production [36] and substrate competition [37]. *Bacteroides* and *Clostridium* spp., among others, produce large volumes of H_2 gas during the fermentation of FODMAPs [38]. H_2 gas plays a significant role in causing IBS symptoms of luminal distension and pain [38]. Lactobacilli produce no detectable H_2 during fermentation and present as an ideal candidate to redirect the microbiome from H_2 production [39]. This presents an opportunity to control H_2 production by introducing these strains to take over the role of primary fermenters.

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit to the host” [40, 41]. Members of the *Lactobacillus* group were chosen due to their generally recognised as safe (GRAS) status, formidable enzymatic capacity and robust ability to survive GI transit. Probiotic candidates should meet specific criteria in order to be selected [42], including

ability to survive bile acid exposure and low pH present in the upper GI tract, resistance to a pre-defined range of antibiotics should be below guidelines set by the European Food Safety Authority (EFSA) and should be non-transferable. We also assessed the ability of the strains to adhere to intestinal epithelial tissue and their ability to ferment FODMAP carbohydrates as these traits help the probiotic candidates exert their benefits on the host. These selection criteria are generally determined by individually testing the traits of interest *in vitro* and following whole genome sequencing of strains, we investigated the potential for *in silico* prediction of probiotic phenotypes based on their genomes. We assessed the genomes manually, looking for the presence of specific genes pertaining to the traits discussed above. We also used the genomic information from these strains with web-based software, ProbioPred and DBCAN2, to predict their potential to survive GI transit, adhere to intestinal cell walls, resist antibiotics, express virulence factors and degrade carbohydrates.

Lactobacillus rhamnosus DPC6071, *Lactobacillus casei* DPC6072 and *Lactobacillus casei* DPC6083 were chosen due to their GRAS status, their robust growth on varied carbohydrates and resilience to the processes associated with preparing probiotics for commercial use [43-45]. However, species included in the *Lactobacillus* genus have recently been spread over 25 genera, including 23 novel genera [46]. We will mention here the new names for the most frequently discussed strains in this manuscript. Both *Lactobacillus casei* and *Lactobacillus rhamnosus* have been reclassified to the genus *Lacticaseibacillus*. Whereas *Lactobacillus delbrueckii* has changed to *Lactobacillus delbrueckii* subsp. *delbrueckii.* For ease of reference we will continue to name the strains based on their old classification throughout this manuscript.

In this study, we aim to identify a combination of food grade strains that may be used to reduce the FODMAP load *in vitro* and *in vivo* while assessing the accuracy of *in silico* predictions based on genomic information.

2.3 | **Materials and Methods**

2.3.1 | Culture and Propagation of strains

Thirty two *Lactobacillus* strains were isolated previously from adult and neonatal GI tracts and Sicilian dairy cheeses and characterised as *Lactobacillus* by sequencing the 16S rRNA V3-V4 region [47, 48]. Strains were stored at -80°C in Phosphate Buffer Saline (PBS, Merck) with 25% glycerol added as a cryoprotectant. Strains were routinely grown in MRS media (Difco) with 0.05% L-cysteine-hydrochloride (Sigma Aldrich) added, using a 1% inoculum at 37 °C for 24 hours [49]. Before experimentation strains were cultured twice. *Lactobacillus rhamnosus* DPC6071, *Lactobacillus casei* DPC6072 and *Lactobacillus casei* DPC6083 isolated originally from Sicilian dairy cheese [48] were the most effective fermenters of FODMAP carbohydrates from 32 identified strains isolated from GIT of neonates, adults [47] and further dairy cheese strains [48]. These three strains showed many desirable attributes *in vitro* such as resistance to simulated bile acid and gastric juice; production of anti-microbial peptides; antibiotic resistance; freeze dry resistance and limited gas production. These *Lactobacillus* strains were routinely cultured in an anaerobic environment for 24 hours at 37 ° C.

2.3.2 | FODMAP carbohydrate utilisation

Table 1. Characteristics of the eleven FODMAP carbohydrates used as substrates for carbohydrate fermentation testing. All carbohydrates were sourced from Merck.

FODMAP	Type	Nomenclature	Bonds between monosaccharides	Monosaccharides	Degree of Polymerisation
Fructose	Monosaccharide		N/A	Fructose	1
Sucrose	Disaccharide		α-1,2 Glycosidic bond	Fructose, Glucose	2
Lactose	Disaccharide		β-1,4 Glycosidic bond	Glucose, Galactose	2
Raffinose	Galactooligosaccharide	GOS	α-1,6, α-1,2 glycosidic bonds	Glucose, Fructose, Galactose	3
Stachyose	Galactooligosaccharide	GOS	α-1,6, α-1,2 glycosidic bonds	Glucose, Fructose, Galactose	4
Inulin	Fructooligosaccharide	FOS	β-2,1, α-1,2 Glycosidic bonds	Fructose, Glucose	3 to 60
Fructooligosaccharides	Fructooligosaccharide	FOS	β-2,1, α-1,2 Glycosidic bonds	Fructose, Glucose	2 to 20
Mannitol	Sugar alcohol	Polyol	N/A	Mannitol	1
Sorbitol	Sugar alcohol	Polyol	N/A	Sorbitol	1
Maltitol	Sugar alcohol	Polyol	α-1,4 Glycosidic bond	Sorbitol, Glucose	2
Xylitol	Sugar alcohol	Polyol	N/A	Xylitol	1

Carbohydrate fermentation potential was determined using a modified protocol previously described for lactobacilli [50]. A minimal media derived from MRS was used, supplemented with an individual FODMAP carbohydrate as the sole carbon source. Cultures were incubated using anaerobic incubation conditions (10% H_2, 10% CO_2 and 80% N_2) in a chamber Mac 500 (Don Whitley Scientific, West Yorkshire, UK) at 37 °C for 24 hours. Optical Density was observed at 600nm using a Synergy 2 plate reader (BioTek Instruments, Inc., Vermont, US). Plates were removed from the anaerobic chamber and placed in the plate reader in order to determine the optical density of the culture. Plates were shaken for 10 seconds prior to reading in order to mix the cultures. These readings were taken at 6 hours, 12 hours and 24 hours. Cultures were grown in triplicate and the data represent the mean of those replicates.

2.3.3 | Simulated bile acid and gastric juice resistance

Five ml bacterial cultures were grown overnight at 37 °C under anaerobic conditions. Then, cells were harvested, washed twice with PBS and resuspended in 1ml of the same solution. 100 µL of bacterial suspensions were added to 900 µL of simulated gastric juice (125 mM NaCl, 7 mM KCl, 45 mM NaHCO3, and 3 g/L pepsin [Sigma], adjusted to pH 2.5 with HCl) or simulated bile acid (45 mM NaCl, 1 g/L pancreatin [Sigma] and 3 g/L Oxgall [Sigma], adjusted to pH 8.0 with NaOH). Suspensions were then incubated in anaerobiosis for 90 or 180 minutes with gastric juice and simulated bile acid, respectively. Plate counts in MRSc were done at time 0 and after incubation. The results are represented as percentage survival with regard to the initial volume of cells added for each test (CFUs alive after the test/CFUs inoculated to start the test).

2.3.4 | Antibiotic resistance assay

Ten *Lactobacillus* strains, i.e. nine *Lactobacillus casei* and one *Lactobacillus rhamnosus*, were tested on their ability to resist antibiotics during growth (Table 3). This was assessed using microdilution in VetMIC plates for LAB (SVA, Uppsala, Sweden), following the manufacturer's recommendations. Strains were cultured twice before 100ul was inoculated into the VetMIC plates resulting in a final concentration of $5x10^5$ CFU/ml. Plates were incubated for 24 hours, anaerobically at 37°C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of a given antibiotic that completely inhibited the growth of the strain.

2.3.5 | Caco-2 adherence assay

The adhesion capability of the individual strains and in combination (pool) was assessed with the epithelial intestinal cell line Caco-2 cells from the ATCC® culture collection (HTB-37™). Caco-2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum

(FBS), 0.5% penicillin and 0.5% streptomycin. Cells were maintained at 37° C and 5% CO_2. Culture media were changed every two days and the cell line was trypsinized with 0.25% trypsin-EDTA solution (Sigma) following standard procedures. For adhesion assays, Caco-2 monolayers were prepared in 12-well tissue culture plates. Cells were inoculated at 1 X 10^5 CFU per well and allowed to reach confluence (approximately 1 X 10^7 CFU/ml) and differentiate. *Lactobacillus rhamnosus* 6071, *Lactobacillus casei* 6072 and *Lactobacillus casei* 6083 were grown to a 1 X 10^9 CFU/ml concentration. *Lactobacillus rhamnosus* GG was included as a reference strains for comparison. 100 µl of each culture was mixed with 10ml DMEM to produce a 1 X 10^7 CFU/ml inoculation. 1ml of the 1 X 10^7 CFU/ml inoculation of *Lactobacillus* inoculum was added to wells of differentiated Caco-2 cells. The negative control consisted of sterile PBS added to similar wells of differentiated Caco-2 cells. After 2 hours incubation at 37° C the supernatant was discarded and Caco-2 cells were softly washed 5 times with PBS to remove non-attached bacteria, after which ice cold H_2O was used to break up monolayer. The remnants of the monolayer were plated on MRS agar for enumeration to determine the number of adhered bacteria. Results were expressed as a percentage of adhered bacteria with respect to the initial number of bacteria added. Experiments were carried out in triplicate.

2.3.6 | Bacteriocin screening

The three *Lactobacillus* strains (*Lactobacillus rhamnosus* DPC6071, *Lactobacillus casei* DPC6072 and *Lactobacillus casei* DPC6083) were subject to a bacteriocin production screen by overlaying with 10 ml "sloppy" MRS agar (7.5 g · liter−1 agar) tempered to 50°C and seeded with an overnight culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 (0.25% [vol/vol]) (as previously described [51]). Strains were observed to determine if clear zones of inhibition were produced.

2.3.7 | Freeze Drying

Lactobacillus pre-culture was transferred from frozen glycerol stocks to 10 ml of MRS and grown for 24 hours for each strain. The 10ml pre-culture was used to inoculate 50 ml falcons of MRS with a 1% inoculum. These 50ml falcons were incubated for 24 hours to a concentration of 1 x 10^9 CFU/ml confirmed after by plate counts. Contents of 50ml falcons were centrifuged at 4,000g for 35 minutes at 4°C using a Sorvall Lynx 4000 centrifuge. The pellet was washed with PBS and resuspended in 5ml of 15% trehalose to a final concentration of 15% trehalose and 1 x 10^{10} CFU/ml. One ml of this suspension was distributed to each freeze dry vial (Fisher Scientific, Ballycoolen, Dublin, Ireland) after which the lid (Fisher Scientific, Ballycoolen, Dublin, Ireland) of each vial was placed on top. The lid was placed to allow a gap to facilitate gaseous exchange. Vials were then placed in the Virtis wiz 2.0 freeze dryer overnight (freeze temp. −40°C, condenser set point −60°C, vacuum set point 600 m Torr). Freeze dried cultures were stored at -20°C and reconstituted in 1ml of PBS. To determine viability, freeze

dried samples were reconstituted at 2 days, 6 weeks and 3 months. These samples were spread on MRS solid media and plate counts were observed to determine viable concentration remaining.

2.3.8 | Genome sequencing

Ten strains, nine *Lactobacillus casei* and one *Lactobacillus rhamnosus,* were selected following promising FODMAP fermentation results *in vitro* results and grown in MRS as described in the methods above. Pure cultures were extracted with GenElute™ Bacterial Genomic DNA Kit using the protocol included with this kit. After extraction DNA was eluted in Tris-HCL pH 8.0 and stored at -20°C until sequencing commenced. Genome sequencing was provided by MicrobesNG (http://www.microbesng.uk) which is supported by the BBSRC (grant number BB/L024209/1) using the following protocol. DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input and PCR elongation time was increased to 1 minute from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 [52]. De novo assembly was performed on samples using SPAdes version 3.7 [53] and contigs were annotated using Prokka 1.11 [54].

2.4 | **Results**

2.4.1 | Carbohydrate utilisation profiles of *Lactobacillus* strains

A total of 32 *Lactobacillus* strains were previously isolated from the adult and neonatal GI tract and from artisanal cheese [47, 48]. All isolated *Lactobacillus* strains were previously identified by sequencing of the 16S rDNA fragment and belonged to seven species: *Lactobacillus casei* (18), *Lactobacillus casei*/para*casei* (6), *Lactobacillus rhamnosus* (4), *Lactobacillus* gasseri (1), *Lactobacillus salivarius* (1), *Lactobacillus brevis* (1) and *Lactobacillus ruminis* (1) [47, 48]. In this study, these strains were screened for their capacity to ferment FODMAP carbohydrates (Table 2) [55].

*Table 2 Carbohydrate utilisation profiles. Results were determined using OD. The OD reached within 24 hours of growth is depicted below. * indicates the ten strains that were selected for further testing.*

DPCC No.	16S rRNA	Total number of +'s	Number of Carbohydrates digested	Fructose	Sucrose	Lactose	Raffinose	Stachyose	Inulin	FOS	Mannitol	Maltitol	Sorbitol	Xylitol
6063	*casei*	1	1	+	-	-	-	-	-	-	-	-	-	-
6064	*casei*	2	1	++	-	-	-	-	-	-	-	-	-	-
6065	*casei / paracasei*	3	3	+	+	+	-	-	-	-	-	-	-	-
6066	*casei*	3	2	+	-	-	-	-	-	-	++	-	-	-
6067	*casei*	1	1	-	-	+	-	-	-	-	-	-	-	-
6068	*casei*	3	2	-	+	++	-	-	-	-	-	-	-	-
6069	*casei*	5	2	++	-	+++	-	-	-	-	-	-	-	-
6070	*rhamnosus*	8	4	+	-	+++	-	-	-	-	+++	+	-	-
6071*	*casei*	18	7	+++	+++	+++	+	-	+++	+++	++	-	-	-
6072*	*casei*	18	8	+++	+++	+++	+	-	+++	++	++	-	-	+
6073*	*casei*	12	5	+++	-	+++	+	+	-	-	+++	-	-	+
6074*	*casei*	12	10	+	+	+	+	+	+	+	+++	+	+	-
6075	*casei*	1	1	-	-	+	-	-	-	-	-	-	-	-
6076	*casei*	12	6	+++	-	+++	+	+	+	-	+++	-	-	-
6077*	*casei / paracasei*	8	4	+++	-	++	+	-	-	-	++	-	-	-
6078*	*casei*	13	7	+++	-	+++	+	+	-	-	+++	+	+	-
6079*	*casei*	16	9	+++	+	+++	+	+	+	-	+++	++	+	-
6080*	*casei*	10	6	+++	-	-	+	+	-	-	++	+	++	-
6081	*casei*		4	+++	-	-	+	-	-	-	++	-	++	-
6082*	*casei*	12	6	+++	+++	++	-	-	++	+	+	-	-	-
6083*	*casei*	22	10	+++	+++	+++	+	+	++	++	+++	++	++	-
6084	*casei / paracasei*	10	7	+	-	+++	+	+	-	-	++	+	+	-
6106	gasseri	7	5	++	-	++	-	-	+	-	+	-	+	-
6107	salivarius	1	1	-	-	+	-	-	-	-	-	-	-	-
6108	brevis	2	2	+	-	+	-	-	-	-	-	-	-	-
6109	*casei / paracasei*	0	0	-	-	-	-	-	-	-	-	-	-	-
6110	*rhamnosus*	11	6	+++	-	+++	+	+	-	-	++	-	+	-
6113	ruminis	7	5	+	++	++	-	-	+	-	+	-	-	-
6114	*casei / paracasei*	0	0	-	-	-	-	-	-	-	-	-	-	-
6115	*casei / paracasei*	0	0	-	-	-	-	-	-	-	-	-	-	-
6116	*rhamnosus /casei*	0	0	-	-	-	-	-	-	-	-	-	-	-
6117	*rhamnosus /casei*	7	3	+++	-	++	-	-	-	-	++	-	-	-

No Growth <0.25 (-) Low Growth 0.25-0.5 (+) Moderate Growth 0.5-0.8 (++) Strong Growth >0.8 (+++)

Strains were selected for further testing if they were deemed to be adeptly able to ferment FODMAPs. This was demonstrated by strong growth (OD > 0.8 after 24 hours) on 2 or more FODMAP substrates or some growth on six or more FODMAP substrates. The ability to use less commonly fermented FODMAPs, such as mannitol and inulin, was considered more valuable than fermenting lactose, fructose or sucrose. This distinction allows numerous strains with different fermentation profiles to be combined to provide a more complete fermentation of all FODMAP carbohydrates. Moreover, strains with the enzymatic capacity to grow on inulin or mannitol are likely to have similar activity on other FODMAP carbohydrates such as FOS and sorbitol. Nine *Lactobacillus casei* and one *Lactobacillus casei*/para*casei*, characterised by 16s rRNA, were selected based on these criteria and as such were chosen for further tests (selected strains marked with an *, Table 2). Interestingly, all ten chosen

strains were previously isolated from artisanal cheeses and none of the selected came from the GIT of adults and neonates. These ten strains underwent further probiotic relevant tests to determine the most effective combination of strains that would provide maximum FODMAP reduction *in vivo*.

2.4.2 | Phenotypic and genomic analysis of ten selected strains

Simulated bile acid and gastric juice resistance assay

Ten *Lactobacillus* strains capable of robust FODMAP fermentation (Table 2) were subjected to simulated gastric digestion using a slightly modified protocol previously described by Arboleya et. al. [56]. Samples were lyophilized prior to testing to simulate the state they would be provided as a probiotic. Plate counts were taken before and after 180 minutes in simulated Bile acid and 90 minutes in Gastric juice. The survival rate of each strains is reported in Table 3.

Table 3 Percentage survival of ten Lactobacillus strains exposed to simulated gastric juice and simulated bile acid for 90 minutes and 180 minutes, respectively.

	Tolerance to gastric juice	Tolerance to bile acid
Lactobacillus rhamnosus DPCC 6071	0.5000	0.2000
Lactobacillus casei DPCC 6072	2.6364	0.0012
Lactobacillus casei DPCC 6073	1.2727	0.0240
Lactobacillus casei DPCC 6074	1.6842	0.1923
Lactobacillus casei DPCC 6077	60.0000	0.0125
Lactobacillus casei DPCC 6078	0.0600	0.0065
Lactobacillus casei DPCC 6079	3.1250	0.6364
Lactobacillus casei DPCC 6080	0.6000	0.0227
Lactobacillus delbrueckii DPCC 6082	0.0167	0.6667
Lactobacillus casei DPCC 6083	1.0000	0.1000

Antibiotic resistance assay

Antibiotic resistance was determined using VetMic plates that are based on minimum inhibitory concentration (MIC). The European Food Safety Authority (EFSA) have set antibiotic resistance cut off points for *Lactobacillus* due to intrinsic resistance within the genus to certain antibiotics. Table 4 shows the MIC for each strain beside the cut off value in square brackets []. Strain 6074 was sensitive to the buffer solution used in the VetMIC plates and as such failed the quality control. Strain DPC 6071, DPC 6078 and DPC 6083 were above the cut off value for resistance to at least one antibiotic.

Table 4. Antibiotic resistance illustrated for each strain. Figures in brackets represent the EFSA cut off concentration for Lactobacilli to be considered safe.

	Gm	Km	Sm	Tc	Em	Cl	Cm	Am
Lactobacillus rhamnosus DPCC 6071	4 [32]	64 [64]	16 [64]	2 [4]	0.5 [1]	0.5 [1]	**8 [4]**	4 [4]
Lactobacillus casei DPCC 6072	2 [32]	32 [64]	16[64]	1 [4]	0.25 [1]	0.12 [1]	4 [4]	4 [4]
Lactobacillus casei DPCC 6073	1[32]	8 [64]	8 [64]	1 [4]	0.12 [1]	0.12 [1]	4 [4]	1 [4]
Lactobacillus casei DPCC 6074	[32]	[64]	[64]	[4]	[1]	[1]	[4]	[4]
Lactobacillus casei DPCC 6077	1[32]	16 [64]	16 [64]	1[4]	0.5 [1]	0.06[1]	2 [4]	0.5[4]
Lactobacillus casei DPCC 6078	2 [32]	32 [64]	16 [64]	**8 [4]**	0.25 [1]	0.06[1]	4 [4]	0.5[4]
Lactobacillus casei DPCC 6079	2 [32]	64[64]	16[64]	1 [4]	0.12 [1]	0.12 [1]	4[4]	1[4]
Lactobacillus casei DPCC 6080	1 [32]	32[64]	16 [64]	2 [4]	0.25 [1]	0.12 [1]	4 [4]	1 [4]
Lactobacillus delbrueckii DPCC 6082	4 [32]	64 [64]	8 [64]	0.5 [4]	0.03 [1]	0.06 [1]	4 [4]	0.5 [4]
Lactobacillus casei DPCC 6083	4 [32]	**128 [64]**	32 [64]	2 [4]	0.5 [1]	0.25 [1]	**8 [4]**	1 [4]

FODMAP carbohydrate utilisation enzymes from genomic data

Whole genomes of the 10 *Lactobacillus* strains were sequenced using Illumina short read sequencing. The ten genomes were manually perused using the Artemis software and queried using BLAST [57]. The genes encoding the enzymes involved in digesting sucrose, lactose, raffinose/stachyose and mannitol are present in all genomes. The entire set of genetic elements required for Fructooligosaccharide (FOS) and inulin degradation were not present within any of the ten genomes. The presence of FODMAP digesting genes are displayed in table 5. For the most part, genes displayed in table 5 are the genes responsible for hydrolysis of the carbohydrate and not the transport and regulatory elements.

Several other genes play an important role in the use of carbohydrates. The lactose operon: LacT, LacE, LacG and LacF, is present in all genomes explored, with the exception of DPC 6072. DPC 6071 shows the Lac operon connected to a Gal operon for galactose utilisation, similar to the reference genome *L casei* BL23. DPC 6071 and DPC 6078 have two copies of the Lac operon. AgaSK, a dual enzyme responsible for alpha galactosidase and sucrose kinase activity, is present in either duplicate or triplicate across all ten genomes. AgaSK appears to be transcribed without accompanying genes within an operon. RafA, another alpha galactosidase that is flanked by mannose family sugar transporters, is also present in DPC 6071. MtlD, the mannitol dehydrogenase gene, is present in all genomes. MtlD is located on an operon including MtlAFR. MtlF and MtlA, mannitol specific phosphotransferase enzymes IIA and IIB respectively, and MtlR, the mannitol operon repressor, are found in all ten genomes analysed. Interestingly, all mannitol operons are arranged identically, except for DPC 6082 (Figure 1). This strain, the only *Lactobacillus delbruekki,* demonstrated significantly worse growth when mannitol was the sole carbon source.

Table 5 The distribution of common genes associated with the metabolism of each FODMAP carbohydrate.

Gene	L *rhamnosus* DPC 6071	*L. casei* DPC 6072	L. *casei* DPC 6073	L. *casei* DPC 6074	L. *casei* DPC 6077	L. *casei* DPC 6078	L. *casei* DPC 6079	L. *casei* DPC 6080	L. delbruekki DPC 6082	L. *casei* DPC 6083	Role of gene
FruA	+	+	+	+	+	+	+	+	+	+	Fructose transport / Extracellular Fructansucrase
ScrB	+	+	+	+	+	+	+	+	+	+	Sucrose hydrolase
LacG	+	+	+	+	+	+	+	+	+	+	Beta galactosidase
agaSK	+	+	+	+	+	+	+	+	+	+	Raffinose/Stachyose degradation
RafA	+	-	-	-	-	-	-	-	-	-	Raffinose/Stachyose degradation
FosE	-	-	-	-	-	-	-	-	-	-	Fos/Inulin degradation
MtlD	+	+	+	+	+	+	+	+	+	+	Mannitol dehydrogenase
gutB	+	-	-	-	-	+	-	+	-	-	Sorbitol dehydrogenase
xdh	-	-	-	-	-	-	-	-	-	-	Xylitol dehydrogenase

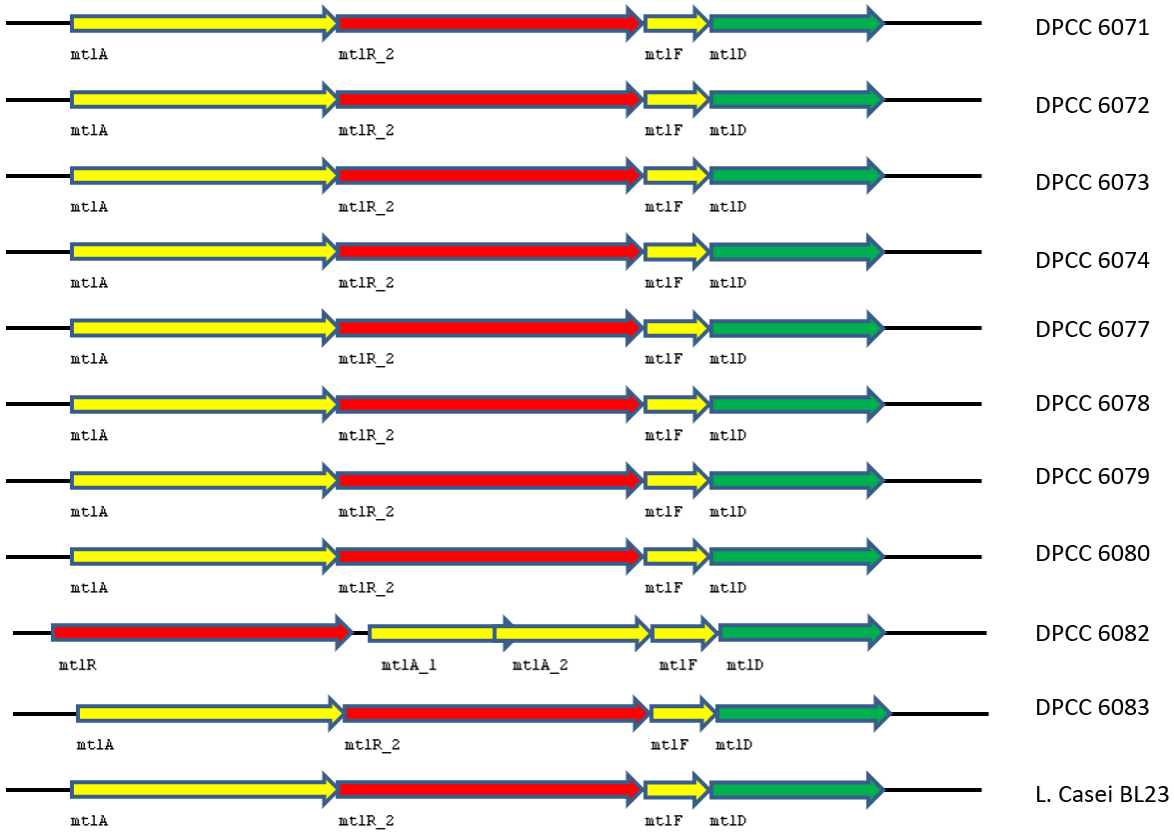

Figure 1. Mannitol dehydrogenase operon. Lactobacillus casei BL23 is used as a reference strain.

FruA, an extracellular fructansucrase or a fructose transport component is present in all genomes analysed with seven copies present in the DPC 6071 genome. SacC, a beta-fructosidase gene, was discovered within just three genomes: DPC 6073, 6078 and 6079. Fructose specific PTS transporters LevE and LevD were on the same operon as SacC in these three genomes. LevE was discovered without an accompanying operon in DPC 6071. Sorbitol dehydrogenase (GutB) was found in *L. rhamnosus DPC* 6071, *L. casei* 6078 and *L. casei* 6080. Sorbitol uptake is mediated by a PTS system encoded by the Srl ABRE operon in the L *casei* W56 genome [58]. SrlB and SrlE encode EIIA and EIIB components of the sorbitol specific PTS system, respectively. SrlR mediates the repression of the operon. These complete operons are present in DPC 6071 and 6078. The maltitol dehydrogenase gene is not present in any of the genomes. Finally, the genomes show no xylitol dehydrogenase or relevant uptake systems present in the genomes.

Primary energy pathways present with the genome

A fully functional Embden-Meyerhof (EM) pathway is expected from all homofermentative and facultative heterofermentative lactobacilli. The EM pathway consists of ten essential genes [31]. As expected, all *Lactobacillus* strains sequenced are equipped with the complete set of genes. This pathway culminates in pyruvate being produced. This pyruvate is primarily converted to lactic acid using the Lactate dehydrogenase (Ldh) enzyme. Three copies of Ldh are present in all genomes indicating a robust ability to convert pyruvate to a final product of lactic acid. The alternative pathway available to heterofermentative LAB is the Phosphoketolase Pathway (PKP). Indeed, all ten strains have the genetic elements necessary to use the Phosphoketolase Pathway. CO_2 is produced during the PKP, however, the production of CO_2 from glucose in anaerobic conditions after 24 hours was ruled out in all 10 strains via the hot loop test (results not shown) [59]. This result confirms a preference for the EMP for all ten strains.

DbCAN2 prediction of Glucoside Hydrolase families relevant to FODMAP degradation

The carbohydrate utilisation potential of the ten strains was predicted by DbCAN2 [60]. DbCAN2 is software that utilises HMMER, DIAMOND and Hotpep to annotate a provided genome for Carbohydrate Active Enzymes from the CaZymes database. Table 6 details the number of predicted genes within Glycoside Hydrolase families relevant to FODMAP degradation. DIAMOND, HMMER and Hotpep must each determine the presence of the gene independently to be included.

Table 6. Prediction of number of genes for FODMAP relevant Glycoside Hydrolase (GH) families within each genome. The three prediction software must agree the gene is present for it to be included.

	GH1	GH2	GH3	GH4	GH31	GH32	GH35	GH36
L. rhamnosus DPCC 6071	8	1	1	2	2	1	0	2
L. casei DPCC 6072	3	1	1	0	2	1	0	2
L. casei DPCC 6073	3	1	1	0	2	1	1	1
L. casei DPCC 6074	4	2	1	1	2	2	1	2
L. casei DPCC 6077	6	0	2	1	1	1	2	0
L. casei DPCC 6078	8	0	2	1	1	2	1	2
L. casei DPCC 6079	4	2	1	1	2	2	1	2
L. casei DPCC 6080	3	1	1	0	2	1	0	2
L. delbruekki DPCC 6082	3	1	0	1	0	0	0	0
L. casei DPCC 6083	3	1	1	0	2	1	0	2
L. GG + Control	3	1	1	0	2	1	0	2

2.4.3 | *In vitro* and *in silico* analysis of three selected strains

L. casei DPC 6072 has significantly greater adherence that the positive control

The three selected strains were chosen after assessing the previous data. DPC 6071, DPC 6072 and DPCC6083 were selected based on their superior FODMAP fermentation capacity while achieving adequate results in other tests. Strains DPC 6071, 6072 and 6083 were tested for their capacity to adhere to human intestinal cells (Figure 2). DPC 6071, 6072 and 6083 displayed varied binding capacity to a differentiated Caco-2 monolayer. DPC 6072 was found to be the most adherent strain tested. DPC 6072 showed significantly more adherence than the positive control *Lactobacillus rhamnosus* GG p-value <0.01 (DPCC 6072 mean % adhered 1.417% SD 0.2123%, LGG mean % adhered 0.8912% SD 0.1486%).

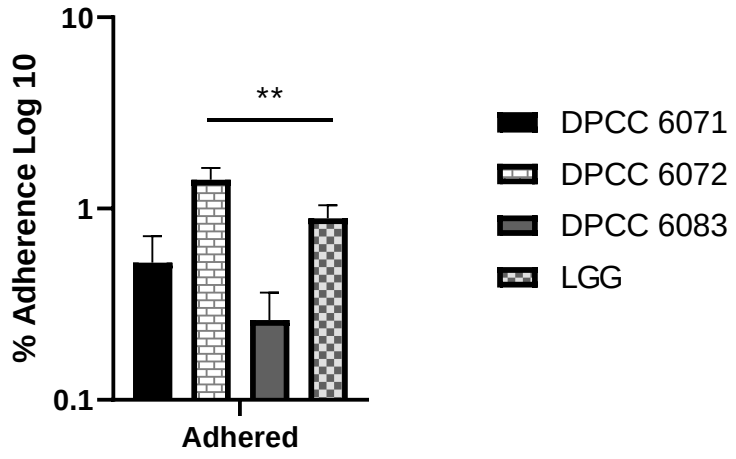

Figure 2. Percentage of adhesion to Caco-2 cells of the three strains included in this study. Lactobacillus rhamnosus GG was used as a reference strain. Graph displays mean ± SEM.

Bacteriocin production for *L. rhamnosus* DPC 6071, *L. casei* 6072 and *L. casei* 6083 assessed *in vitro*

Bacteriocin production was determined using an indicator strain, *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901to determine if *L. rhamnosus* DPC 6071, *L. casei DPC* 6072 and *L. casei* DPC 6083 inhibited growth of the indicator strain via bacteriocin production. No zones of clearance were observed in the *L. delbrueckii* subsp. *bulgaricus*. *L. rhamnosus* DPC 6071, *L. casei* 6072 and *L. casei* 6083 were grown as the indicator strain in separate experiments to observe any antagonistic bacteriocin activity between the three strains. No bacteriocin activity was observed after this experiment.

Survivability after freeze drying and during water suspension assessed *in vitro*

L. rhamnosus DPC 6071, *L. casei DPC* 6072 and *L. casei* DPC 6083 are freeze dried for storage and transport purposes after which they will be suspended in the water supply during murine trials. All three strains demonstrated no loss of viability after freeze drying and 3 months storage at -20°C (Figure 3). Freeze dried samples stored for 3 months were reconstituted in 1ml of PBS at room temperature and added to 9ml of water. No loss of viability was observed after 24 and 48 hours in water (Figure 4).

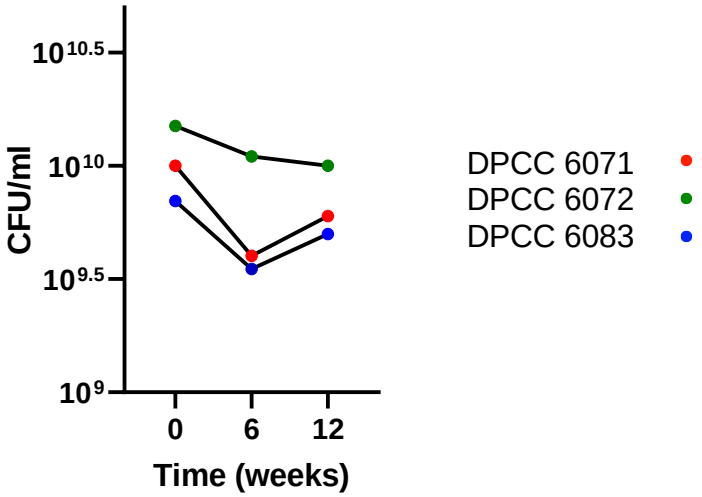

Figure 3. Freeze dried sample survivability illustrated for three selected strains. T-test was used to compare mean viability. No significant differences were observed over a 12 week period.

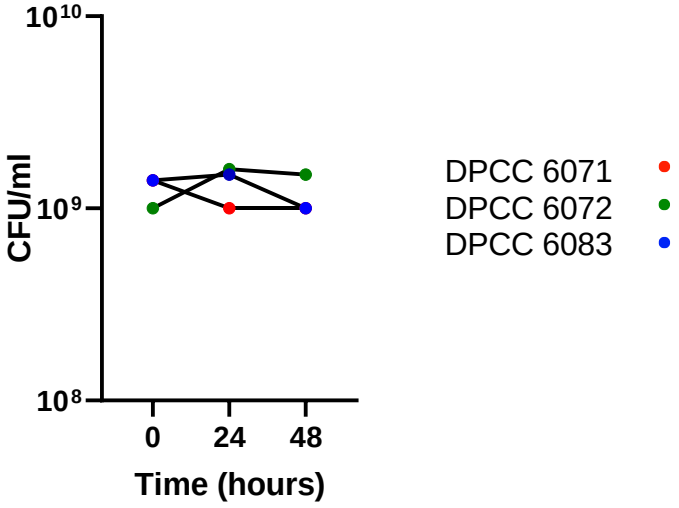

Figure 4. Survivability of *Lactobacillus* strains suspended in water illustrated for three selected strains. T-test was used to compare mean viability. No significant differences were observed over a 48 hour period.

2.4.4 | **Desirable genetic elements present in the three selected strains**

The three chosen strains were manually assessed for the presence of genes related to desirable phenotypes. This was carried out in a manner similar to previous research on potentially probiotic *Lactobacillus* strains [61]. Genes pertaining to gastric transit resistance, antibiotic resistance and virulence were manually examined. The manual search did not reveal many genes present that could explain the phenotypic characteristics of the strains of interest. *L. rhamnosus* DPC 6071, *L. casei* 6072 and *L. casei* 6083 were also subjected to automated genomic analysis through the ProBioPred software (http://210.212.161.142/ProBioPred/index.php). This software uses the Comprehensive Antibiotic Resistance Database (CARD) to determine the presence of antibiotic resistance elements [62] and Virulent Factor DataBase (VFDB) [63] to assess virulence elements. Genetic elements that may confer probiotic traits are also highlighted by this software.

Antibiotic resistance

The CARD database was used to determine the prevalence of antibiotic resistance genes present in the genomes of DPCC6071, DPC 6072 and DPC 6083. A chromosomally encoded lincosamide antibiotic efflux pump was reported within the L. *rhamnosus* DPC 6071 genome. L. *casei* 6072 displays an APH(2") gene; however, the APH(2") element does not translate to greater gentamycin and kanamycin resistance *in vitro*. *L. casei* 6083 harbours two beta-lactamase resistance genes within its genome. Analysis of the position of these antibiotic resistance genes and corresponding structures surrounding these genes shows that they are not located on transferable elements. These three strains present very limited risk for transferring meaningful antibiotic resistance within the host. This should alleviate concerns of contributing to the pool of antibiotic resistance that is present in the microbiota [64].

Cluster of orthologous groups

Cluster of orthologous groups (COG) represents prokaryotic clusters of proteins that are linked to functional characteristics. There are a total of 23 classifications for COGs including RNA processing, carbohydrate metabolism and transport, transcription etc [65]. Comparing genomes based on their COG functions indicates the functional similarities between genomes. The conserved COG between the three selected *Lactobacillus* strains and the default *Lactobacillus casei* strain used in software OrthoVenn2 downloaded from the Ensembl database was analysed [66]. DPC 6071 and DPC 6072 showed considerable conservation in their functional proteins (Figure 5). These two genomes have 614 unique clusters compared to both other genomes. Analysing these 614 unique COGs for overrepresentation compared to the total distribution of COGs between the four genomes reveals that PEP-depended sugar PTS, inositol and rhamnose catabolic processes were significantly enriched

compared to the mean in genomes DPC 6071 and DPC 6072 (p-value = 4.05E-11, p-value = 3.35E-06 and p-value = 0.000146, respectively).

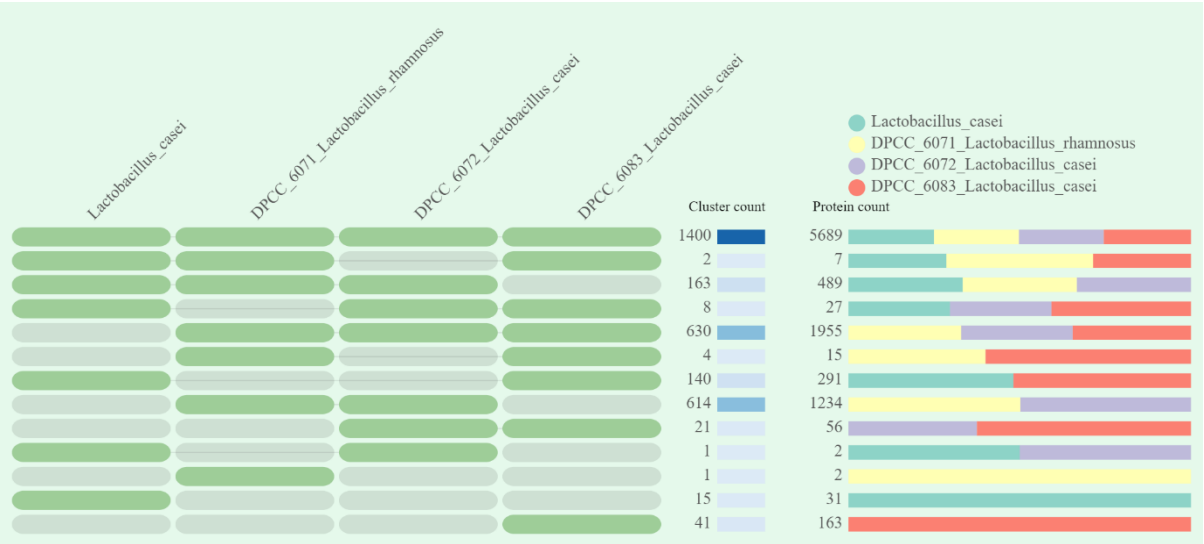

Figure 5. Overlapping COGs between the three selected strains and the default Lactobacillus casei strain using OrthoVenn2 [66]. Each row represents a different combination of genomes and their count of unique clusters and proteins.

Bile acid and gastric juice resistance

Several genes relevant to bile acid and gastric juice resistance were detected within the genomes. The genomes of all strains contained many similar genes encoding resistance elements. ClpE and clpL were identified as mechanisms of bile resistance due to their role in DNA protection, repair and managing environmental stress [67-70]. DltA, part of an operon responsible for maintenance of the cell envelope producing Lipoteichoic Acids (LTAs) and Wall Teichoic Acids (WTAs), was found in all three genomes and confers potential protection to in vivo growth [71]. A putative amino acid permease, LBA0995, capable of active removal of stressors is also located in DPC 6071, 6072 and 6083. Rrp1, encoding 2 CRS regulators conferring bile resistance, is also present. These 5 genes are found within *L. rhamnosus* DPC 6071, *L. casei* 6072 and *L. casei* 6083. A dps gene also present in *L. casei* DPC 6083, acting similarly to clp, encodes a protein responsible for DNA protection and repair [72-75]. No virulence genes were detected with the genomes of *L. rhamnosus* DPC 6071, *L. casei* 6072 and *L. casei* 6083 using the Virulence factor database (VFDB) [76].

Bacteriocin production

Bacteriocin production was not observed after *in vitro* testing, however, *L. rhamnosus* DPC 6071 and *L. casei* 6072 demonstrated genomic capacity to produce at least one bacteriocin. Lactobin A/cerein 7B, a class IIb family bacteriocin, was reported by Bagel 4.0 [77] flanked by an immunity gene and GG-

motif leader sequence (Figure 6). Class IIb family bacteriocins do not to undergo post translational modification apart from cleavage of the GG-motif at the N-terminal end and are relatively common within the *Lactobacillus* genus [78]. This bacteriocin family exert its anti-microbial effect through pore formation in the target cell membrane [79, 80]. The *in silico* presence of bacteriocin genes may not translate to *in vitro* expression of the protein due to lack of dedicated bacteriocin transporters and accessory proteins. The disparity between *in silico* and *in vitro* has been discussed previously and this knowledge may yield potential anti-microbial proteins [78, 81].

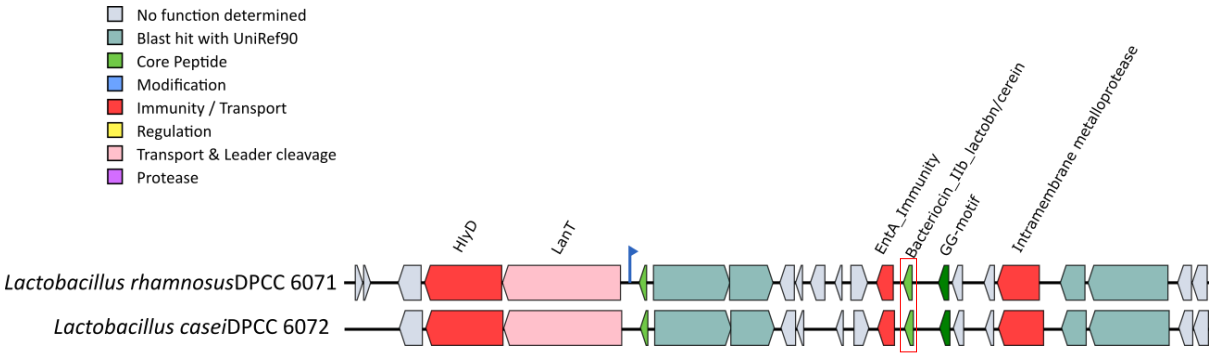

Figure 6. Presence of a gene encoding a class IIb bacteriocin within L.rhamnosus DPC 6071 and L. casei DPC 6072. Gene of interest enclosed by red box.

Conjugated Linoleic acid production

Many LAB have the capacity to produce conjugated linoleic acid (CLA). Considering the many health benefits associated with CLA production, we assessed the genomes of *L. rhamnosus* DPC 6071, *L. casei* 6072 and *L. casei* 6083 for the presence of the myosin cross-reactive antigen (mcra) gene, among others involved in CLA production [82-84]. Interestingly, Peng el al. reported that the presence of linoleic acid in higher concentrations has shown increased adherence of *Lactobacillus casei* to human epithelial cells [82]. The gene encoding myosin cross-reactive antigen was not present within the three genomes, nor was it observed upon after using BLAST to search the genome using a closely related mcra gene.

2.5 | **Discussion**

Targeting the cause of FODMAP induced IBS represents a straightforward method to alleviate the symptoms of this complex multi-factorial disorder. Moderating this IBS mechanism relies on providing an alternative fermentation route that reduces CO_2 and H_2 production. In this study, we have demonstrated the potential for three *Lactobacillus* strains to deliver this therapy. We hypothesise that appropriate use of potential probiotics, such as the strains described above, may reduce the need for a low FODMAP diet in some individuals. The primary trait of interest for each strain was their carbohydrate fermentation capabilities. The various *Lactobacillus* strains demonstrated a broad variety of enzymes capable of fermenting the FODMAP carbohydrates. *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 were superior to the other strains tested in this regard. Collectively these three strains can grow on 10 of the 11 FODMAP carbohydrates tested, with xylitol being the exception.

To exert the beneficial effect on the host, these ten unique strains must reach the gut alive after transit through the GI tract. To determine the potential for these microbes to survive GI transit, this study carried out bile acid and gastric juice resistance tests. Both tests represented particularly severe examinations including 180 minutes in simulated bile acid and 90 minutes in simulated gastric juice. This is in part due to the lack of metabolizable sugars present within the simulated liquids [85]. In similar tests, *Lactobacillus rhamnosus GG*. has demonstrated worst survival performance without the presence of sugar [85]. From ten strains examined, nine maintained a concentration above 1 X 10^7 CFU/ml after simulated gastric juice exposure. Seven of the ten strains had a concentration of 1 X 10^6 CFU/ml after the three-hour incubation in simulated bile acid. These results compare favourably to *Lactobacillus* subjected to similar treatments [61, 85-87]. Increasing the survivability of *Lactobacillus* strains during transit is also possible. Various methods of encapsulation [88] and the inclusion of metabolizable sugars [85] increase viability after GI transit.

Antibiotic resistance among bacterial groups has become a major health concern [89]. The prevalence of these traits within the microbiome has resulted in strict regulations regarding antibiotic resistance profiles of probiotic bacteria [90]. The resistance profiles of the ten strains examined show three strains with values above the EFSAs cut off values for appropriate antibiotic resistance. DPC 6071, 6078 and 6083 displayed moderate resistance to Chloramphenicol, Tetracycline and Kanamycin + Chloramphenicol, respectively. *Lactobacillus* resistance to these antibiotics has been described previously [91]. The CARD database reported several antibiotic resistance elements that partially

explain the phenotypic data received. Moderate to weak resistance may be conferred by mechanisms used to remove substances from the cytosol, such as efflux pumps [92]. DPC 6071 *Lactobacillus rhamnosus* demonstrated lincosamide efflux pumps which may explain its greater resistance to Clindamycin than the other strains assessed. DPC 6072 *L. casei* displays an APH(2") gene; however, it is likely the accessory genes required for antibiotic resistance with this gene are not present. APH(2") confers high level gentamycin and aminoglycoside resistance that is not present in *in vitro* testing of DPC 6072 *L. casei [93, 94]*. Similarly, despite the presence of two beta-lactamase resistance genes in DPC 6083 *L. casei*, increased ampicillin resistance is not reported in phenotypic results [95]. Due to the three strains phenotypic resistance to chloramphenicol, tetracycline and kanamycin only slightly above the cut off values, it is likely these microbes do not present a risk to health. This is further supported by genomic analysis, as the resistance genes are not located on transferable elements in the genome.

Adherence to intestinal structures is a desireable trait for probiotic bacteria. Caco-2 adherence assays are commonly used to assess a strains ability to colonise the gut ecosystem [96]. Caco-2 cells are intestinal epithelial cells that simulate the internal wall of the colon [97]. The three strains of interest were tested individually and as a pooled sample while *Lactobacillus rhamnosus* GG was used as a positive control. DPC 6072 outcompeted L. GG with almost twice the % of cells adhering to the in vitro Caco-2 cells. This is notable considering L. GG is well known for its ability to adhere to intestinal cells [98]. These results suggest that the *Lactobacillus* strains chosen can colonise the gut after administration.

Genomic analysis was carried out to identify the genetic elements responsible for the described desirable phenotypic traits. Initially manual curation of the genomes demonstrated complete Embden Meyerhof and Phosphoketolase pathways available in all genomes. No detectable CO_2 production during growth on glucose *in vitro* demonstrated that these ten strains preferentially use the Embden Meyerhof pathway for primary fermentation. During genomic analysis of carbohydrate enzymes, clear links between genotype and phenotype were determined for some FODMAPs. Efficient mannitol usage was clearly tied to a correctly arranged MtlADFR operon. The lack of appropriate MtlA resulted in reduced growth on a sole mannitol substrate. However, some genetic elements responsible for carbohydrate fermentation could not be determined within the genomes. This may be due to enzymes that are capable of degrading multiple carbohydrates and transport systems capable of the same [99]. The ScrB enzyme degrades sucrose but may also degrade FOS by hydrolysing terminal β-D-fructofuranosides in (phospho-)β-D-fructofuranoside oligosaccharides [100, 101]. Extracellular

fructansucrases, such as FruA (GH32), are able to hydrolyse FOS in environments where the FOS:Sucrose ratio is high [102]. These FruA proteins are extracellular, explaining the lack of Inulin and FOS uptake systems. Similarly, transporters such as MalFGKX, the Cut1 ABC transporters, are often used for raffinose and stachyose transport. These MalFGKX proteins have a broad uptake affinity for many carbohydrate substrates despite their annotation as maltose ABC transporters [99]. The presence of a correct operon for lactose usage was also not located in all genomes. Lactose relies on LacE and LacF for PTS mediated transport through the membrane, LacT as an anti-terminator and LacG as the beta-galactosidase [103]. The lack of Lactose operon in DPC 6072 is most likely an example of incorrect annotation. These unreliable annotations of carbohydrate fermenting elements are frequent in the *Lactobacillus* genus, making the process of linking genotype to phenotype more difficult [104, 105].

An estimation of each genomes' carbohydrate fermenting capacity was carried out by DbCAN2 [60]. DbCAN2 revealed more FODMAP fermenting enzymes than those originally annotated within the genomes. GH1 and GH35 families of beta-galactosidases were present in the highest numbers. This is expected as all ten of these strains were isolated from a dairy environment. Alpha-galactosidases, present within families GH4, GH31 and GH36, were also present in greater numbers than were initially annotated. Finally, the GH32 family represents polysaccharide hydrolases and indicates that a gene present can hydrolyse these longer-chain, inulin like carbohydrates. Analysis of COG functional capacity of the three selected *Lactobacillus* strains, using WebMGA [106], demonstrated that DPC 6071 and DPC 6072 were closest in functional potential [65]. Carbohydrate degradation and PEP PTS transport COG functions were overrepresented in the genomes of DPC 6071 and DPC 6072. This indicates a clear functional capacity to ferment and assimilate carbohydrates within DPC 6071 and DPC 6072.

Analysing the genomic data was not useful in determining a strains ability to survive GI transit. Despite very different in vitro results, the *in silico* analysis reported almost identical genes present in the genomes from the three selected strains. This indicates an inability to correctly determine the genetic components responsible for GI transit. Finally, no virulence factors were present after searching manually and using the ProBioPred software. This result is expected from members of the *Lactobacillus* genus. *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 demonstrate significant potential for use as probiotics. These strains would specifically target individuals that suffer from IBS and have previously seen a benefit from a low FODMAP diet. They have shown potential to survive gastro-intestinal transit and potentially exert their FODMAP degrading ability.

2.6 | **Conclusion**

Lactobacillus genus. *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 have demonstrated probiotic potential for the reduction of FODMAP induced IBS symptoms. These strains are selected based on their ability to degrade FODMAP carbohydrates, survive gastro-intestinal transit and adhere to intestinal epithelial tissue. The three strains present strong carbohydrate fermentation, robust Caco-2 cell adherence, resistance to simulated GI transit and no virulence traits. The *Lactobacillus* strains represent a targeted treatment approach to a specific mechanism of action. Administration of these probiotics may reduce the FODMAP degrading pathways that result in CO_2 and H_2 production within the gut, reducing the bloating and pain associated with IBS. These strains hold potential for IBS sufferers that have found symptom relief in a low FODMAP diet, however, human studies will be required to prove their efficacy.

2.7 | **References**

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Chapter III

Lactobacilli modulate the microbiome to potentially reduce FODMAP-induced IBS *in vivo*

3.1 | **Abstract**

Irritable Bowel Syndrome (IBS) is the most common functional gastrointestinal disorder, often characterised by increased pain and luminal distension. This disorder is only partially understood with many associated factors such as previous infection history, antibiotic use, chronic inflammation, genetic factors and diet/microbe interactions. Symptoms of pain and bloating often originate from the production of gas compounds such as CO_2 and H_2 during the digestion of Fermentable Oligo-, Di-, and Mono-saccharides and Polyols (FODMAPs). Consequently, a diet with reduced FODMAP carbohydrates is recommended for IBS sufferers. Certain probiotic bacteria capable of reducing hydrogen production *in vivo* may reduce the need for this severely limiting diet. In this study, we assessed the efficacy of a *Lactobacillus* mix that is capable of enzymatically digesting FODMAP carbohydrates *in vivo*, without the genesis of these gas compounds. Firstly, we attempted to induce an IBS-like phenotype in wild-type (WT) mice by inducing stress in offspring via maternal separation. The WT mice were then fed a high FODMAP diet (10% fermentable carbohydrates) with or without the lactobacilli strains or a low FODMAP diet (0% fermentable carbohydrates) without lactobacilli. The three *Lactobacillus* strains, *L. rhamnosus* and two *L. casei*, were administered via the water supply. In a second animal model of maternal separation, the impact of an optimal N-6 PUFA to N-3 PUFA ratio was also analysed in terms of the FODMAP diet and *Lactobacillus* administration using the Fat1 mouse model. Maternal separation proved inadequate to induce the IBS-like phenotype in mice. However, our study revealed that the *Lactobacillus* spp. survived gut transit and caused a shift in alpha and beta diversity within the gut microbiome. The administered *Lactobacillus* strains, particularly *L. rhamnosus*, appeared to become dominant species within the gut capable of redirecting microbial metabolism and thus reducing hydrogen gas genesis by increasing propionate production. The high FODMAP diet resulted in an increase in alpha diversity, beta diversity and groups of beneficial microbes. Fat1 animals displayed fewer changes in microbial abundance than WT animals possibly due to increased N-3 PUFA removing the niche for anti-inflammatory species in the gut.

3.2 | **Introduction**

Irritable Bowel Syndrome (IBS) is a chronic functional bowel disorder that is characterised by abdominal pain, bloating, discomfort and a change in symptom severity following defecation [1]. The clinical diagnosis of this condition relies on criteria set by the Rome foundation, called the Rome IV criteria [2]. Diseases with known biochemical factors such as Inflammatory Bowel Disease (IBD) and celiac disease must be ruled out in order to diagnose IBS [3]. IBS is prevalent worldwide and represents one of the primary reasons for visiting general practitioners, taking days off work and reduced workplace productivity [4]. This disorder is estimated to affect approximately 12% of individuals worldwide, disproportionally impacting women and western countries [5]. Multiple factors contribute to epidemiology of IBS including genetic factors, diet, lifestyle and physiological gender differences [6]. Deciphering this complex milieu of potential causes has proven difficult and as such there are limited effective treatments for the condition. Therapies take the form of psychological intervention, dietary alterations, antibiotic intake and probiotic supplementation [7].

Dietary- and microbiota-mediated effects on IBS have gained interest of late. These two factors are intertwined and must be assessed together when possible. The gut microbiome consists of approximately 100 trillion cells that carry out a myriad of functions from regulation of host metabolism, immune function and physiology [8]. It is no surprise that the more than 1000 species of bacteria within the gut interact with the host diet. These microbes produce many compounds that shape the microbiota and the surrounding structures [9]. Diet mediated modulation of the microbiome is constant and has even been observed within periods of time as short as 24 hours [10]. This change is usually maintained within the parameters of the core microbiome initially present [11]. However, the microbiome may shift into a dysbiotic state after significant changes take place [12]. This is often due to antibiotic exposure or pathogenic infections [13, 14]. This shift to a dysbiotic microbial community is viewed as a hallmark of IBS and is frequently considered a precursor to the occurrence of symptoms [15, 16]. The abundance of specific groups of microbes has also been associated with the prevalence of IBS [17, 18].

Non-digestible carbohydrates are often grouped under the acronym FODMAP, or Fermentable Oligo, Di and Mono-saccharides and Polyols. Many carbohydrates that come under the FODMAP umbrella are normally considered prebiotics. Prebiotics are compounds that preferentially promote the growth of beneficial intestinal microbes [19]. However, to many IBS sensitive individuals these FODMAPs cause intestinal distress, abdominal pain and luminal distension [20]. Several studies, including meta-analysis, have shown the beneficial effect of consuming a low FODMAP diet in order to reduce these symptoms [21]. Indeed, this diet holds significant promise for responders. This theory has been

supported by the observation that FODMAP carbohydrates reach the colon of ileostomates ingesting a diet high in FODMAPs [22]. These patients showed a significant volume of fructans and sorbitol reaching the colon undigested [22]. Removing these carbohydrates from the diet appears to be a suitable resolution, however the elimination stage of this diet is severe and difficult to maintain for the recommended 6 weeks. Moreover, many do not attempt the re-introduction stage as they are content with their symptom relief. Subsequently, large swaths of 'healthy' food types are omitted from their regular diet negatively impacting general health as well as the microbial ecosystem in the gut [23, 24].

This trade-off is far from an ideal treatment for the disorder. A more tempting, but less clear, approach is to directly modulate the gut bacteria, using probiotics, to perform this FODMAP reduction *in vivo.* Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" [25-27]. Research has demonstrated that probiotics reduce a variety of symptoms in IBS sufferers [28-34]. The mechanisms of action of these probiotics have been presented in depth previously [29, 35]. One such mechanism is the diet/microbiome interaction. Many members of the microbiome such as *Bacteroides*, lactobacilli and bifidobacteria carry out the primary FODMAP fermentation process releasing carbon for other microbiome members to interact with. However, some of these inhabitants generate a greater volume of hydrogen gas compounds during this fermentation process. This increased gut hydrogen concentration is believed to cause the link between FODMAP carbohydrates and IBS symptoms [36]. The association between IBS and hydrogen levels has gained credence from research demonstrating higher breath hydrogen concentrations in IBS patients [37, 38]. Dear et al. also noted that a reduction in foods known to enhance hydrogen production led to a reduction in symptoms [39]. This mechanism has been linked to a number of gut microbial species. For example, an increased abundance of *Bacteroides fragilis* and *Bilophila wadsworthia* was positively correlated with gas evacuations and gas volume in the gut [40]. Prominent members of the microbiome such as *Clostridium* spp. are also often used in biohydrogen production due to their high H_2 production [41-43]. Conversely, lactic acid bacteria (LAB) are capable of completing this fermentation process without the production of gas [44]. We hypothesise that by introducing LAB to the microbiome, specifically strains of *Lactobacillus*, we may reduce hydrogen production *in vivo* without a low FODMAP diet.

Previous work screened *Lactobacillus* strains for their ability to ferment an array of 11 FODMAP carbohydrates. Strains with the potential to ferment many of these carbs were chosen for further tests to determine their ability to survive gastrointestinal (GI) transit, freeze dry preparation and intestinal cell adherence. In this study, three promising *Lactobacillus* strains were chosen for an animal trial to determine their effect on the microbiome *in vivo*. Initially, we attempted to induce an IBS-like

phenotype in the mice to simulate the conditions in humans where the high FODMAP diet is problematic. The IBS-like phenotype was induced via the maternal separation model [45] and the high FODMAP diet consisted of 10% fermentable carbohydrates modified from Zhou et al. [46].

Furthermore, chronic mucosal inflammation plays a significant role in the aetiology of IBS [47]. Given the strong anti-inflammatory properties of omega-3 polyunsaturated fatty acids (PUFA) we were also interested in determining the effect, if any, high N-3 PUFA has on the induced IBS [48, 49]. Hutchinson et al. (2020) reviewed the potential therapeutic effects of combining N-3 PUFA and probiotics and reported improved insulin sensitivity and reduced systemic inflammation among other beneficial effects [50]. Some research on the dietary impact of omega-3 on IBS has been performed; however, purity of the PUFA, caloric content, and ratios in differing diets may cause inconsistent results between animals. To eliminate the potentially confounding factors of dietary interaction with other compounds and heterologous assimilation of dietary omega-3, the Fat1 mouse model was used [51]. This transgenic mouse model is capable of endogenously converting N-6 PUFA to N-3 PUFA to achieve a ratio near 1:1, making it an ideal method for determining the impact a greater amount of N-3 PUFA may have [52]. As for the previous model, the IBS-like phenotype was induced in Fat1 mice via the maternal separation model [45] and the high FODMAP diet consisted of 10% fermentable carbohydrates. This is the first study utilising this refined technique for studying N-3 PUFA effects on IBS.

3.3 | **Materials and methods**

3.3.1 | Mice and diet

Transgenic *fat-1* mice were generated as previously described [51] followed by backcrossing onto a C57BL/6 background. *Fat-1* phenotype was confirmed by gas chromatography flame-ionization detection (GC-FID) following identification of increased tissue N-3 PUFA compared with Wild Type (WT). Mice were housed in the Massachusetts General Hospital (MGH) animal facility in a biosafety room (level 2) in hard top cages with filtered air. Mice were maintained in a temperature-controlled room (22–24 °C) with a 12-h light/dark diurnal cycle. Food and water were provided ad libitum. *Fat-1* and WT mothers were fed a diet high in N-6 PUFA (AIN-76A with 10% corn oil) from LabDiet in order to maintain *fat-1* and WT phenotypes. At postnatal day (PND) 28, male offspring were weaned onto a high FODMAP diet consisting of 10% w/w FODMAPs, comprising 3.5% w/w fructose, 3% w/w oligofructose, 2% w/w lactose, 0.75% w/w mannitol and 0.75% w/w sorbitol. This diet was altered from a similar high FODMAP diet (HFD) used in a rodent trial [46]. Each gram of HFD contains 3.72 kcal, with 10% of calories provided by fat in the form of high omega-6 corn oil [51], 69% of calories provided by carbohydrate, and 20% of calories provided by protein. Body weight and food intake were measured weekly using an electrical balance. Animals were sacrificed using CO_2. Dissected tissues were flash frozen in liquid nitrogen. All animal procedures in this study were performed in accordance with the guidelines approved by the MGH Subcommittee on Research Animal Care.

3.3.2 | Experimental design

Animals were distributed between the groups described below. C57BL/6 mice were subjected to early life stress by the Maternal Separation Stress (MSS) model [53]. After weaning, on day 28, the mice were placed on either a high FODMAP diet of 10% fermentable carbohydrates (3.5% Inulin, 3% fructose, 2% lactose, 0.75% mannitol, 0.75% sorbitol) or a low FODMAP diet of 0% fermentable carbohydrate. Mice assigned to the *Lactobacillus* groups received three *Lactobacillus* strains from weaning until a 3-month age. The *Lactobacillus* strains were administered through the water supply which was freshly prepared every 48 hours. The viability of the three strains was ensured to last 48 hours in water (Data not shown). All animals were sacrificed at 3 months of age.

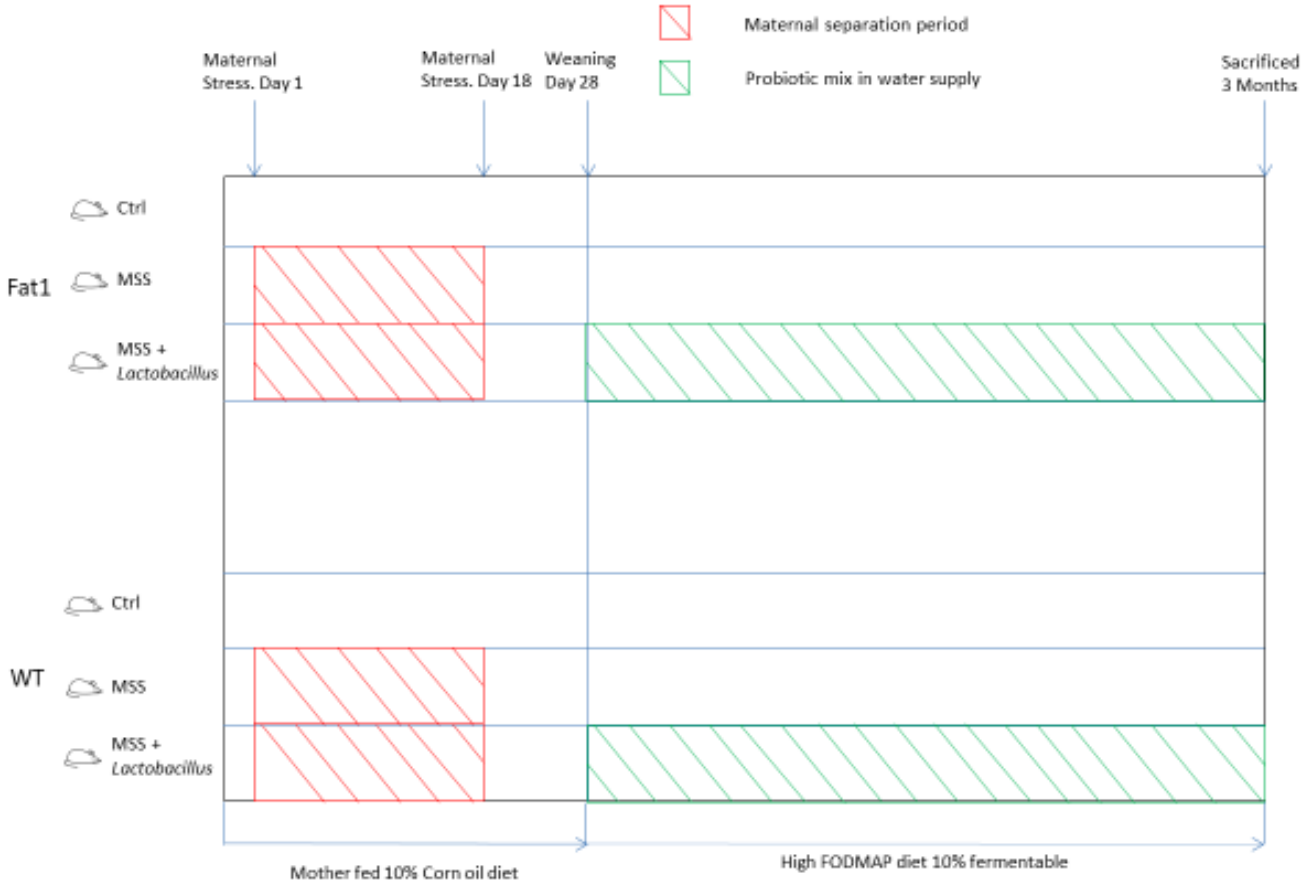

Figure 1. Experimental design for mice fed a high FODMAP diet. Red bars indicate the period of Maternal Separation Stress (MSS) and green bars represent the period of Lactobacillus *administration. Corn oil diet contains 0% fermentable carbohydrates.*

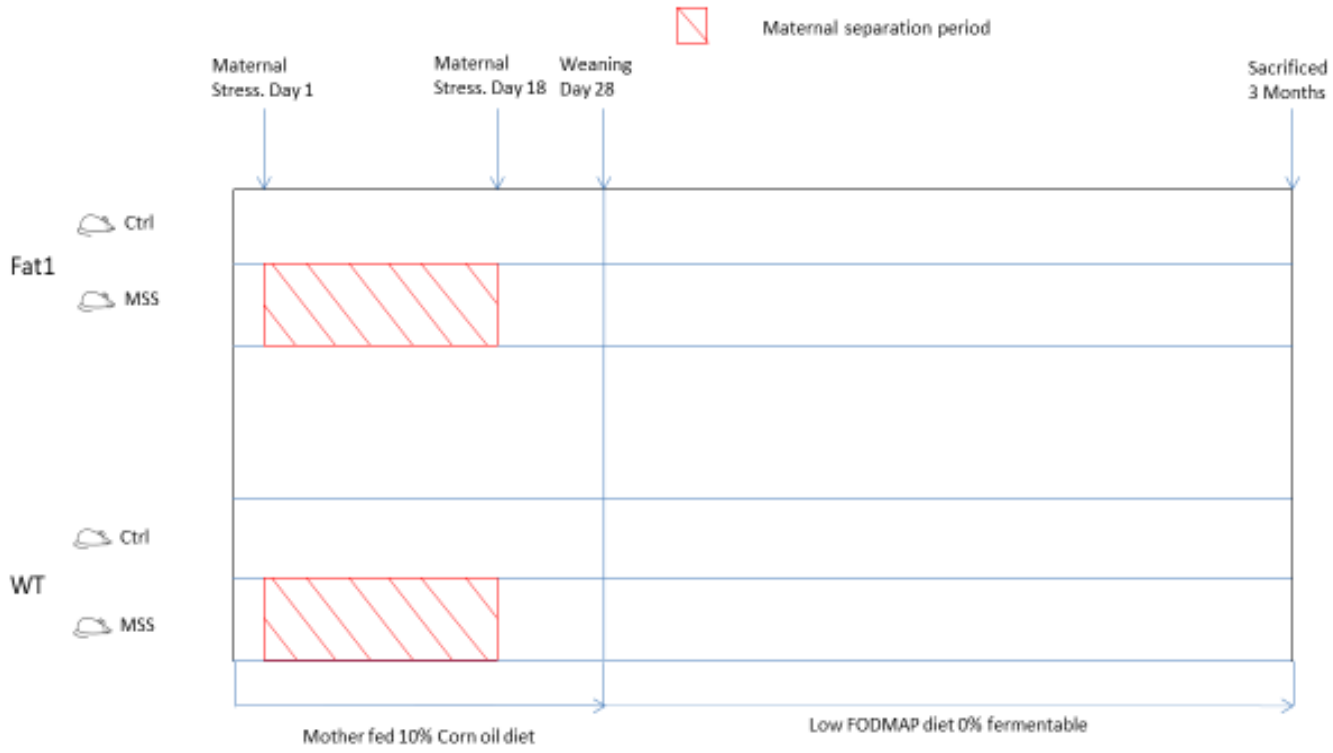

Figure 2. Experimental design for mice fed a low FODMAP diet. Red bars indicate the period of Maternal Separation Stress (MSS). Corn oil diet contains 0% fermentable carbohydrates.

3.3.3 | Maternal Separation Stress (MSS)

Pregnant females were housed individually during the third week of gestation. All maternal separation was carried out during the light phase at irregular times of the day. Pups were exposed to MSS from Postnatal Day (PND) 1 through 18 (Pups were born on PND 0). During the procedure, the dams were removed and placed in a clean cage on their own. The pups were then placed in another clean cage and individually separated by plastic dividers. Each litter was placed into a single cage. Offspring were observed and kept separated. After 3 hours of separation dams and offspring were returned to the original home cage. After PND18 the offspring remained with the dams until weaning prior to PND28. Control pups were not separated from the dams. After weaning the offspring were housed according to the standard housing conditions between 2-4 animals per cage according to group. Experimental groups consisted of 43 males.

3.3.4 | *Lactobacillus* strains

Lactobacillus rhamnosus 6071, *Lactobacillus casei* 6072 and *Lactobacillus casei* 6083 were isolated originally from Sicilian dairy cheese [54]. The three strains were routinely grown in MRS media (Difco) with 0.05% L-cysteine-hydrochloride (Sigma Aldrich) added, using a 1% inoculum at 37 °C for 24 hours [55]. Cultures were concentrated via centrifugation, after which they were resuspended in 15% trehalose solution to a concentration of 1 X 10^{10}. Strains were freeze dried individually at this concentration in 1 ml glass vials. The strains were reconstituted with sterile H_2O and mixed to an appropriate concentration. The concentration administered was such that 1 X 10^{9} bacteria per strain per animal were consumed daily. As such, the concentration of the mixed vials was calculated based on the average volume of water consumed by the mice on a weekly basis. The *Lactobacillus* water supply was replaced every 48 hours. The volume drank by the mice was approximately 3ml per day.

3.3.5 | DNA extraction and 16S rRNA sequencing

Total bacterial DNA was extracted from caecal content samples using a previously described RBB+C method [56]. The amplification of the V3 and V4 region was carried out using the following primers; Forward = TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, Reverse = GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

Amplification of the V3 and V4 region was performed using a HiFi HotStart ReadyMix Kit (Kapabiosystems, USA) according to manufacturer's instructions. Reactions were carried out using a G-storm PCR machine under the following conditions: 95°C for 3 minutes followed by 25 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, the cycler was then held at 72°C for 5 minutes before holding at 4°C.

The PCR reaction products were cleaned using Agentcourt AMPure XP kit (Beckman Coulter Genomics, UK) according to the manufacturer's instructions. The beads were separated from solution using a 96 well magnetic plate stand (Life technologies, Carlsbad, USA). An Index PCR reaction using the Nextera XT Index kit (Illumina, San Diego, USA) and KAPA HiFi HotStart ReadyMix kit on a G-storm PCR machine was performed under the following conditions; 95°C for 3 minutes, followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. This was followed by a 5 minute elongation stage at 72°C. The index PCR reactions underwent another cleaning step using the Agentcourt AMPure XP clean up. The cleaned Index PCR reactions were quantified using a Qubit dsDNA High sensitivity Assay kit and recorded using a Qubit 2.0 Fluorometer according to manufacturer's instructions. The reactions were pooled to produce an equimolar mix.

3.3.6 | Extraction of Mesenteric Lymph Nodes (MLN) and Flow Cytometry immune cell enumeration

MLN from each mouse was dissociated through a 70 µm cell strainer with FACS medium (1x PBS + 1 mM EDTA + 1 % calf serum) using the plunger of a 3 ml syringe. Cells were then collected by centrifugation at 1500 rpm for 5 min. Then, the cells were blocked with Fc receptor antibody in FACS medium on ice for 15 minutes and removed the blocking medium by centrifugation at 1500 rpm for 5 min. To identify different lymphocyte population, the cells were further incubated with anti-CD4, anti-CD8, anti C-19 and anti-CD25 antibodies on ice for 15 minutes and the antibodies were removed using centrifugation at 1500 rpm for 5 min. The cells were fixed with 3% formaldehyde in FACS medium and stored at 4°C. The cells were analyzed using an Attune™ NxT Flow Cytometer (ThermoFisher Scientific). All the antibodies were purchased from Biolegend, San Diego, CA, USA.

3.3.7 | SCFA analysis

Standard Curve and internal standard

Acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, valeric acid, and 2-ethyl-butyric acid (internal standard, IS) were purchased from Sigma Aldrich. A 100mM stock solution of 2-ethylbutyric acid in formic acid was diluted to 10mM in Milli-Q water and was stored at -20^{o}C. An external standard solution of acetate (20mM), propionate (20mM), Iso-butyrate (2mM), butyrate (20Mm), Iso-valerate (2mM) and valerate (20mM) in water was generated and stored at -20^{o}C. A 7 point standard curve was produced (SCFA 0.1mM, 0.5mM, 1mM, 2mM, 4mM, 8mM & 10mM; BCFA 0.01mM, 0.05mM, 0.1mM, 0.2mM, 0.4mM, 0.8mM & 1mM) in Milli-Q water with 1mM IS.

Extraction

Caecal water was prepared by homogenising the murine caecal contents (approx. 150mg) with 1.0mL of MilliQ water using a vortex mixer for 10 minutes before centrifugation at 15,000rpm for 5 minutes. The supernatants were syringe-filtered with 0.22µM filters (Corning) and duplicate aliquots of 270µL of filtrate were mixed with 30µL of 10mM internal standard. The samples were homogenised briefly before a further centrifugation step of 15,000rpm for 3 minutes. The supernatant was transferred into 250µL (Agilent) inserts placed in amber glass 2mL GC vials (Agilent) sealed with silicone/PTFE screw caps (Aglient).

GC-FID analysis

Standards and samples were analysed by gas chromatography flame ionisation detection (GC-FID) using a Varian 3800 GC system, fitted with an Agilent DB-FFAP column (30 m L x 0.32mm ID x 0.25 µm df; Phenomenex) and a flame ionisation detector with a CP-8400 autosampler. Helium was employed as the carrier gas at an initial flow rate of 1.3 mL/min. The initial oven temperature was 50°C, and was maintained for 0.5 min, raised to 140°C at 10°C/minute and held for 0.5 minutes, before being increased to 240°C at 20°C/minute, and held for 5.0 minutes (total run time 20 minutes). The temperatures of the detector and the injection port were 300°C and 240°C, respectively. A splitless injection of 0.2µL was carried out for each sample or standard using a 10uL syringe (Aglient) installed to a CP-8400 autosampler (Varian). Peaks were integrated using Varian Star Chromatography Workstation version 6.0 software. Blank water vials were run between each sample duplicates to check for any potential carryover.

3.3.8 | Data analysis

Data analysis was carried out using IBM SPSS Statistics 24 and GraphPad Prism 7. Physiological data was analysed using student's T test or analysis of variance (ANOVA). ANOVA was corrected for multiple testing via Tukeys multiple comparison test. Significance for caecal mass was determined using student's T test. ANOVA was used to assess the change in body weight and immunological results. Results were considered significant at $p\text{-value} < 0.05$.

Microbiome data was analysed using the web-based program Calypso (version 8.84) [57]. Differential abundance of microbial groups were determined using analysis of composition of microbiomes (ANCOM) [58]. Alpha diversity was determined with raw reads. A variety of metrics were used to analyse alpha diversity. These consisted of Shannon index, Simpson's index, Fischer's alpha and species richness. $P\text{-value} < 0.05$ was considered to be significant.

3.3.9 | Intestinal permeability

Intestinal permeability was conducted as previously described [59]. Initially, mice were fasted for 6 hours after which FITC-dextran (70kDA, Sigma-Aldrich, in PBS solution) was administered by oral gavage. The dose, 600mg/kg body weight, was specified by the mass of the mouse measured directly before gavage. Blood samples were collected via the facial vein 90 minutes after the gavage was administered. PBS was used to dilute the serum in a 1:1 ratio, and fluorescence intensity was measured using a fluorospectrophotometer (Perkin-Elmer) with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. A standard curve generated from a serial dilution of FITC-dextran was used to calculate the serum concentration of FITC- dextran.

3.3.10 | Polyunsaturated Fatty acid analysis

Fatty acid profiles of tail and intestinal tissues were determined using gas chromatography as previously described[60]. Briefly, tail snip tissue samples were ground to powder form under liquid nitrogen and underwent total lipid extraction and fatty acid methylation by 14% boron trifluoride (BF3)-methanol reagent (Sigma-Aldrich) at 100 °C for 1 h. Fatty acid methyl esters were analyzed using a fully automated HP5890 gas chromatography system equipped with a flame-ionization detector (Agilent Technologies, Palo Alto, CA). The fatty acid peaks were identified by comparing their relative retention times with the commercial mixed standards (NuChek Prep, Elysian, MN), and area percentage for all resolved peaks was analyzed by using a PerkinElmer M1 integrator.

3.4 | **Results**

3.4.1 | Caecal content mass altered by fermentable carbohydrate load

Caecal content mass was compared between groups via a three-way ANOVA. Main effect differences were calculated for diet (p-value <0.001). This result showed a significantly higher caecal content mass for high FODMAP fed animals (Figure 3). Groups that were fed *Lactobacillus* did not have an altered caecal mass. The volume of food eaten was similar across all groups.

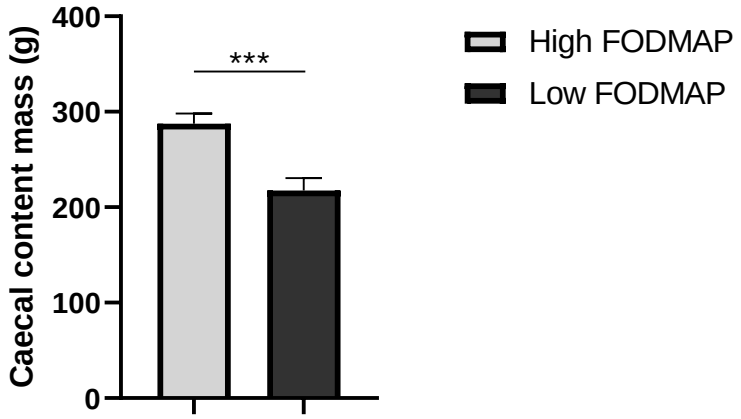

*Figure 3. Weight of caecal contents after extraction from the caecum. Dietary FODMAP content, genotype and maternal separation groups were compared. Dietary FODMAP content revealed a significant difference, whereas genotype and maternal separation did not. High FODMAP N = 25, low FODMAP N = 26. Three way ANOVA was used to compare means. Error bars represent SEM *** p-value <0.001*

3.4.2 | Fat1 mice display altered N-6/N-3 ratio

PUFA N6 and N3 were compared between groups to assess the efficacy of the Fat1 genotype. The mean N6/N3 ratio for mice fed *Lactobacillus* with a Fat1 background was 0.908 (SD 0.131) and Wild Type (WT) mice 20.312 (SD 3.708), p-value < 0.0001. This group was used as an example, all groups showed similar substantial differences between Fat1 and WT N6/N3 ratio (Figure 4).

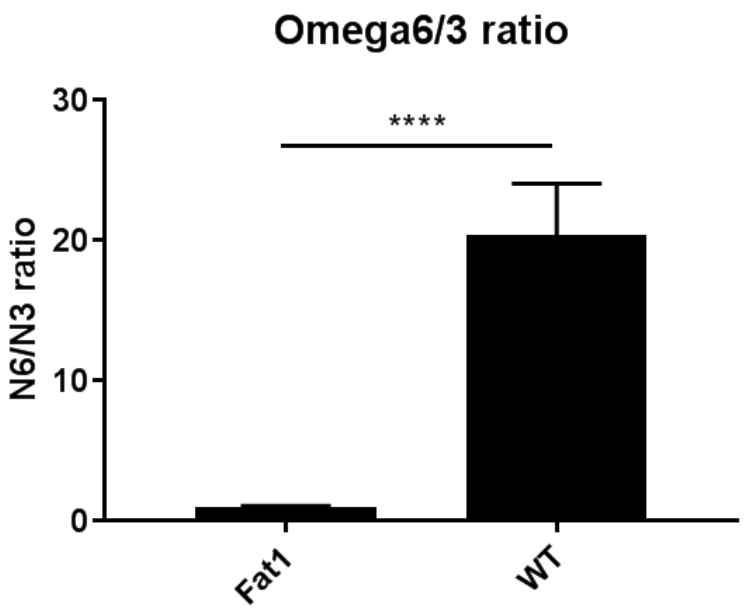

*Figure 4. N6 PUFA to N3 PUFA ratio in Fat1 mice compared to WT mice. N6 and N3 concentration was determined using a tail snip sample prior to weaning.Genotype and maternal separation groups were compared. Genotype revealed a significant difference, whereas maternal separation did not show significant differences. Fat1 N = 9, WT N = 16. Two way ANOVA was used to compare means. Error bars represent SEM **** p-value < 0.0001*

3.4.3 | MSS results in altered mouse body weight at weaning

At 28 days old, the age at which the animals were weaned, the body weight of MSS animals was determined to be higher than their non-separated counterparts by a three-way ANOVA analysing separation status, genotype and *Lactobacillus* status. We further analysed this main effect to determine if specific groups were causing the difference. WT MSS animals (mean 19.7048 SD 2.47739 n=21) were not significantly different in weight compared to Non separated animals (mean 17.7714 SD 1.3659 n=7) p-value = 0.062. The body weight of Fat1 animals show a significant difference between MSS animals (mean 19.7091 SD 2.05059 n=11) compared to non-MSS animals (mean 16.78 SD 2.48938 n=5 p-value = 0.026). No significant weight differences were observed one week after weaning.

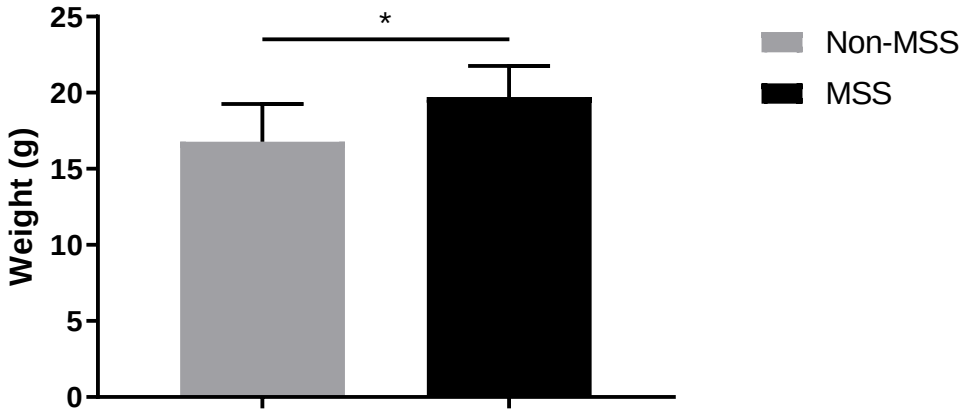

Figure 5. Body weight of MSS *animals shown to be increased at the date of weaning. Genotype,* Lactobacillus *administration and maternal separation groups were compared. Maternal separation revealed a significant difference, whereas genotype did not show significant differences. MSS N = 32, Non-MSS = 11. Three way ANOVA was used to compare means. Error bars represent SEM * p-value <0.05*

3.4.4 | *Lactobacillus* and maternal stress cause changes to immunological profiles in Fat1 mice

CD4+, CD8+, CD19+ and CD25+ cells were extracted from Mesenteric Lymph Nodes (MLN) located on the wall of the colon. The concentrations of these important immunological mediators were compared by group.

CD19+

A three way-ANOVA was carried out and showed significantly increased CD19+ cells in groups that were fed *Lactobacillus* (p-value < 0.05). To determine more about this main effect, we interrogated the CD19 distribution within the high FODMAP groups. This significant increase in CD19 was confined to the Fat1 genotype. The Fat1 *Lactobacillus* groups showed significantly higher CD19 cells than the Fat1 MSS group (p-value < 0.01) (Figure 6).

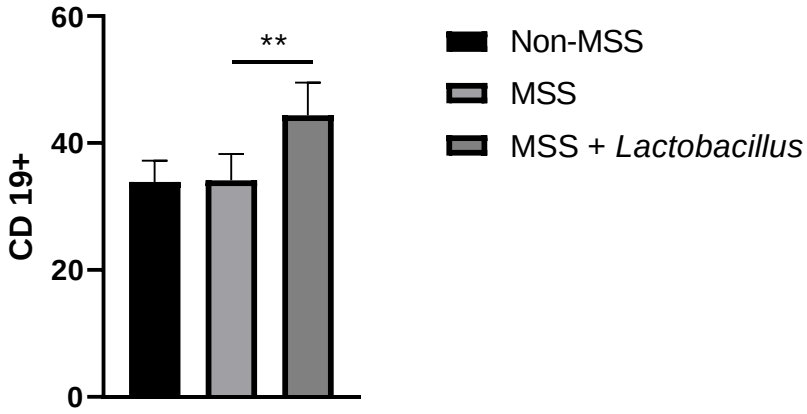

Figure 6. Fat1 Lactobacillus *fed animals demonstrated a higher concentration of CD19+ cells present in the Mesenteric Lymph Nodes. Genotype,* Lactobacillus *fed and maternal separation groups were compared. Maternally separated mice with a Fat1 genotype fed* Lactobacillus *revealed a significant difference compared to maternally separated Fat1 mice. Significant differences were not observed between other groups. Non-MSS N = 4, MSS N = 5, MSS + Lactobacillus N = 6. Three way ANOVA was used to comapre means. Error bars represent SEM ** p-value <0.01*

CD25+

A three-way ANOVA determined that MSS significantly reduces CD25+ cells in the MLN. Analysing the main effect of this significant three-way ANOVA shows that CD25 is significantly different in Fat1 mice. Fat1 non-MSS mice demonstrate higher CD25+ cells (mean 1.654; SD 0.459) in comparison to Fat1 MSS mice (mean 1.03; SD 0.216) p-value < 0.05 (Figure 7).

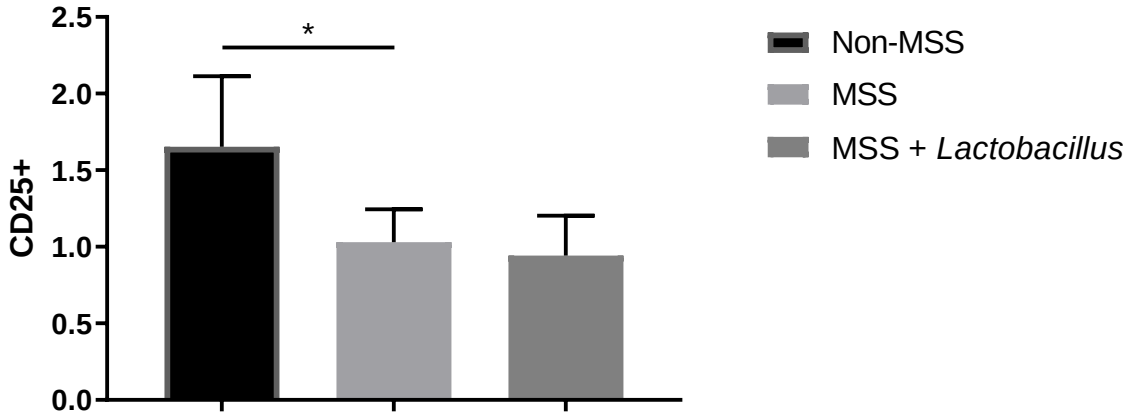

*Figure 7. Fat1 Non-*MSS *mice demonstrated higher CD25+ immune cells present in the Mesenteric Lymph Nodes. Genotype,* Lactobacillus *fed and maternal separation groups were compared. Non maternally separated mice with a Fat1 genotype revealed a significant difference compared to maternally separated Fat1 mice. Significant differences were not observed between other groups. Non-MSS N = 4, MSS N = 5, MSS + Lactobacillus N = 6. Three way ANOVA was used to comapre means Error bars represent SEM * p-value <0.05*

CD4+8+

Similarly to the CD25+ response, a significant increase is observed in the Fat1 non-MSS group (Mean 4.192 SD 1.221 N=5) in comparison to the Fat1 MSS group (Mean 2.0575 SD .363 N=4) p-value<0.01.

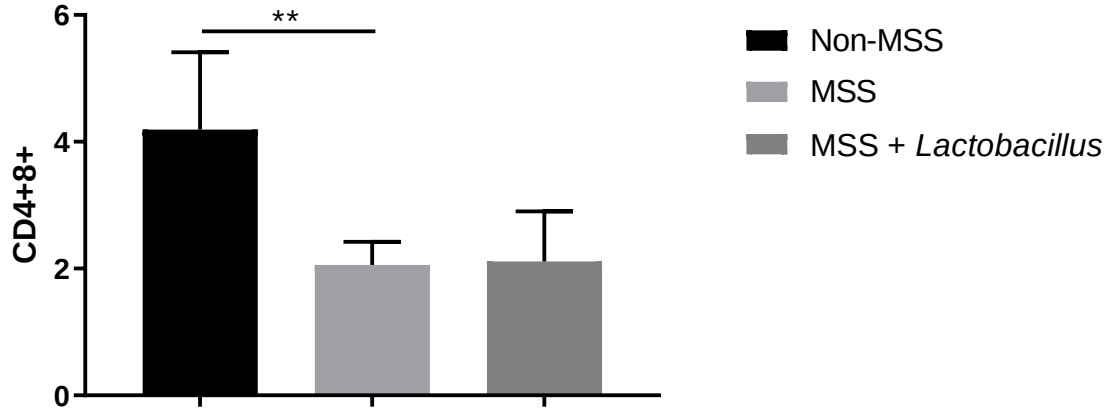

*Figure 8. Non-*MSS Fat1 *mice report an increased number of CD4+CD8+ cells present in the Mesenteric Lymph Nodes in comparison to* MSS *Fat1 animals. Genotype,* Lactobacillus *fed and maternal separation groups were compared. Non maternally separated mice with a Fat1 genotype revealed a significant difference compared to maternally separated Fat1 mice. Significant differences were not observed between other groups. Non-MSS N = 4, MSS N = 5, MSS + Lactobacillus N = 6. Three way ANOVA was used to compare means Error bars represent SEM ** p-value <0.01*

3.4.5 | *Lactobacillus* and diet cause substantial shifts in microbiome diversity and composition

Beta diversity

Unweighted UniFrac analysis of Beta diversity presented a clear separation of samples based on their diet and *Lactobacillus* consumption. WT, MSS animals showed a distinct separation based on both these parameters. *Lactobacillus* consumption separates the samples along the PCoA1 axis, accounting for 32% of total variability between the samples.

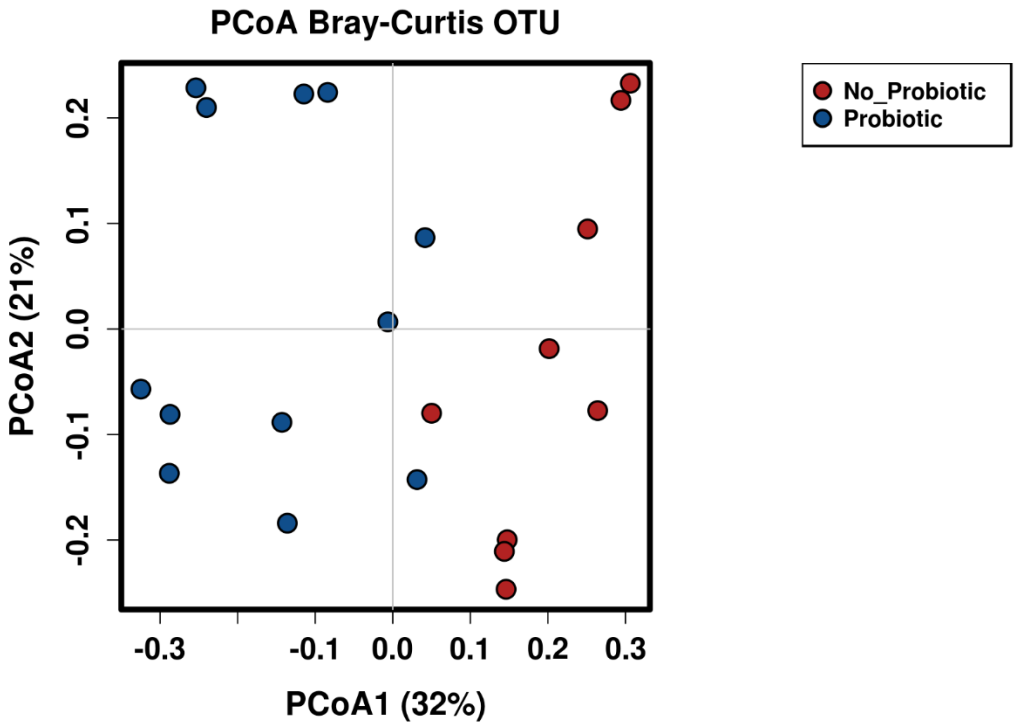

Figure 9. PCoA using the Bray-Curtis distance metric. Blue represents Lactobacillus *fed mice whereas red represents non-*Lactobacillus *fed animals.*

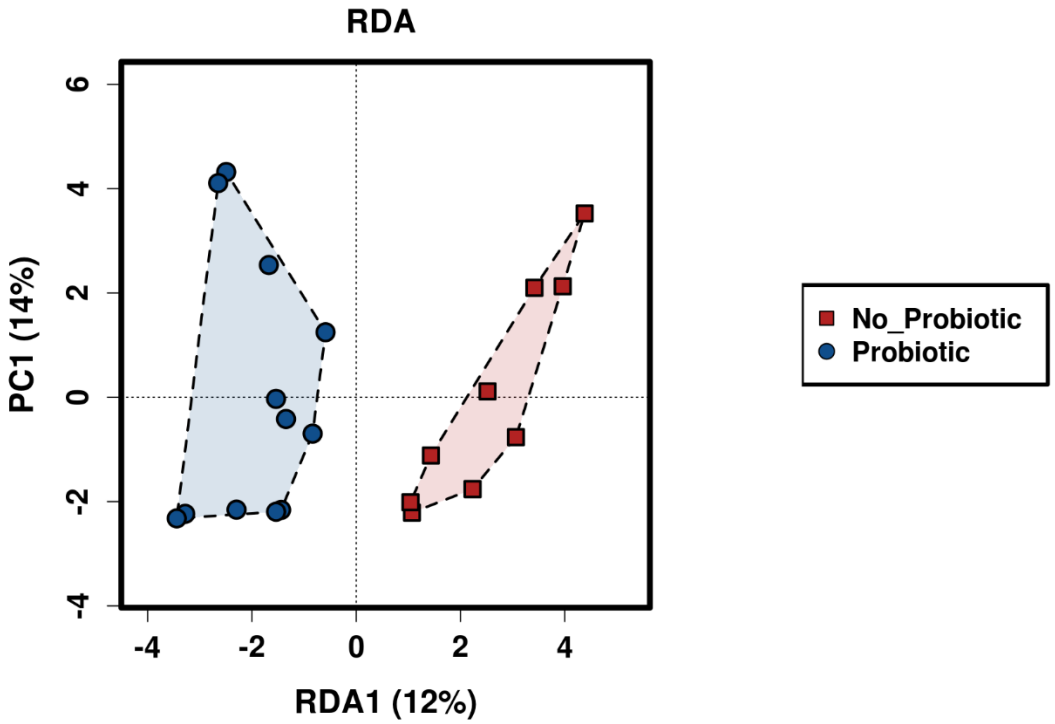

Figure 10. Redundancy Analysis (RDA) based on Bray-Curtis distances demonstrates two completely separated groups based on Lactobacillus *consumption. This separation was signficant with a p-value = 0.001 and variance of 65.62*

The high FODMAP MSS groups were compared to the low FODMAP MSS group in order to maintain an accurate comparison; however, this separation is indicative of all comparisons across diet. PCoA1, accounting for 43% of total variability using a Bray-Curtis distance measurement, cleanly separates the samples.

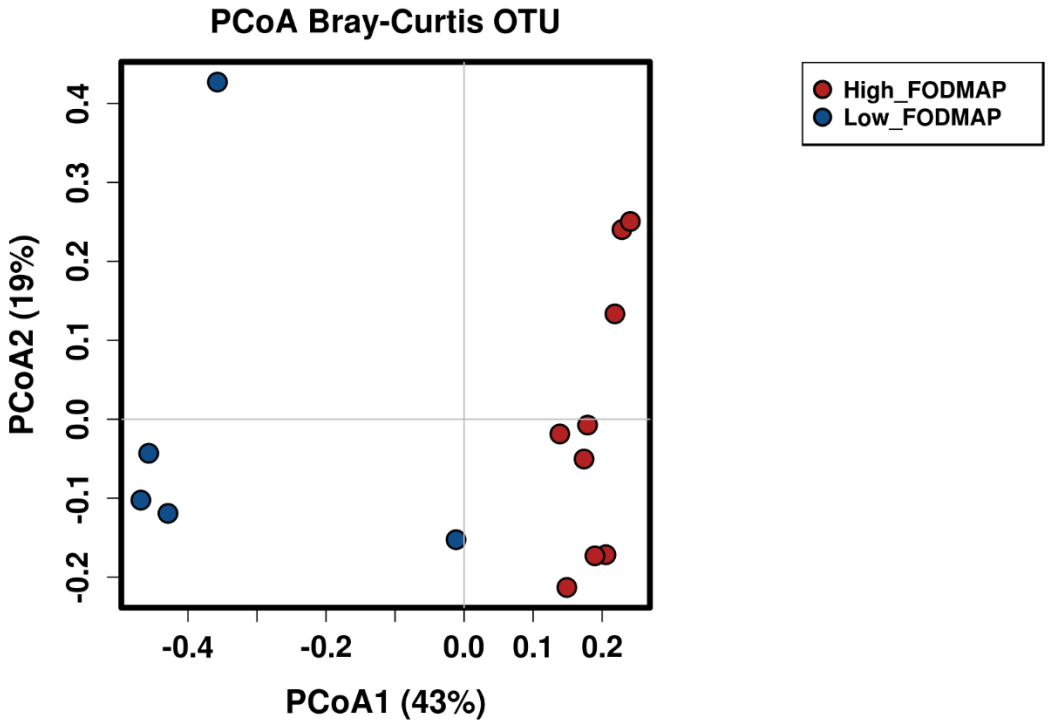

Figure 11. MSS *high FODMAP fed mice (Red) were compared to their low FODMAP fed counterparts (Blue) using a PCoA plot with Bray-Curtis to determine the distance between points.*

Beta-diversity illustrates that diet and *Lactobacillus* are causing a consistent change to the murine microbiome.

Alpha diversity

Alpha diversity was assessed via four separate metrics, namely Shannon index, Simpson index, Chao1 and species richness. *Lactobacillus* supplementation caused a reduction in alpha diversity in WT animals for Shannon (p-value = 0.014), Richness (p-value = 0.013) and Chao1 (p-value = 0.034) whereas Simpson was non-significant (p-value = 0.1).

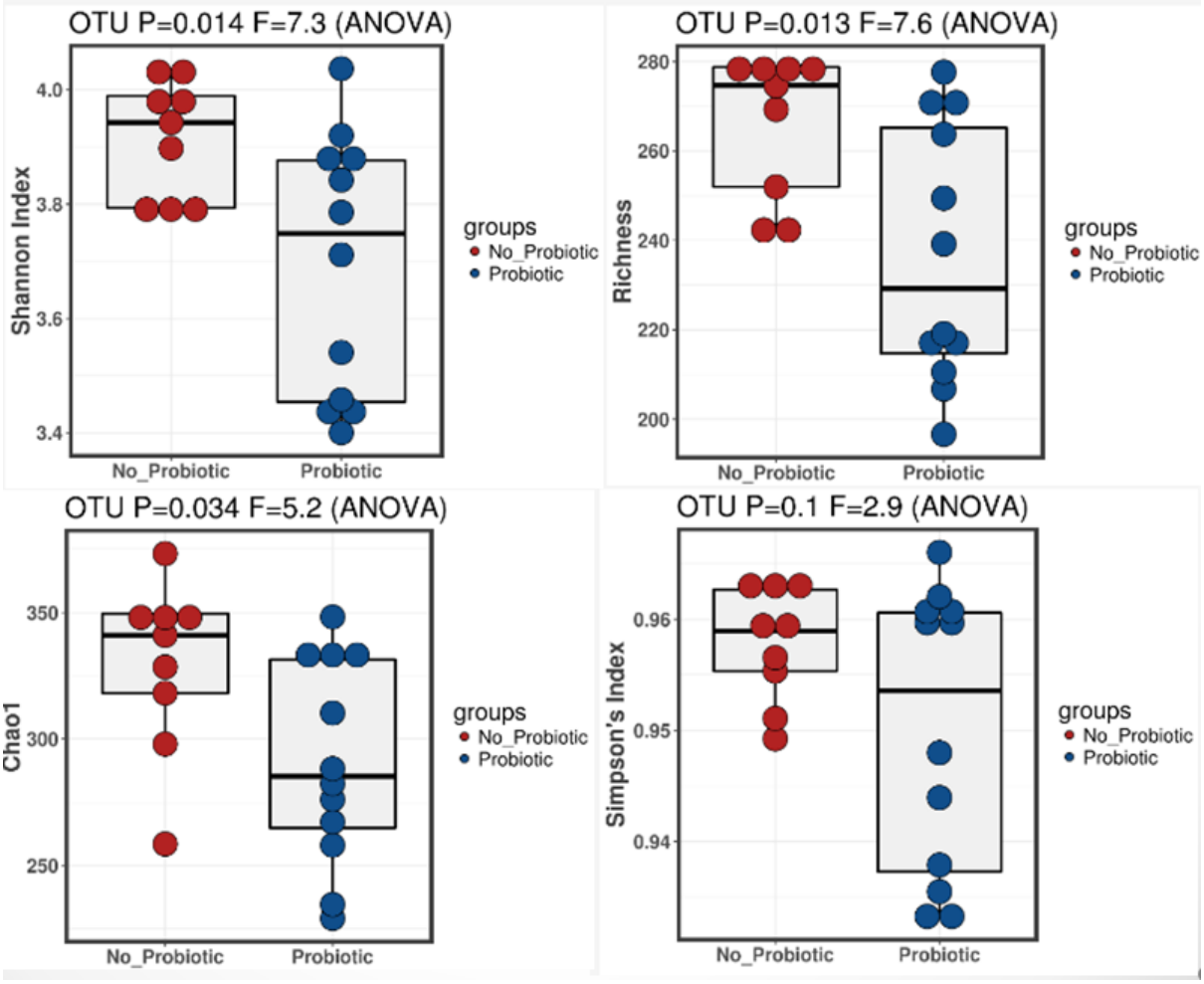

Figure 12. Alpha diversity was determined at the OTU level using the four distance metrics displayed above.

The high FODMAP diet resulted in an increase in alpha diversity metrics across 3 of the 4 tests. The groups analysed were WT MSS animals fed a high FODMAP diet compared to the same low FODMAP fed counterparts. The same trend in diversity was seen across all compared groups looking at high FODMAP versus low FODMAP fed animals. The same diversity metrics were used as above, Shannon (p-value=0.0084), Simpson (p-value=0.057), Chao1 (p-value=0.015) and Richness (p-value=0.0099). These results indicate a significant increase in the diversity of species in animals fed a fermentable fibre diet.

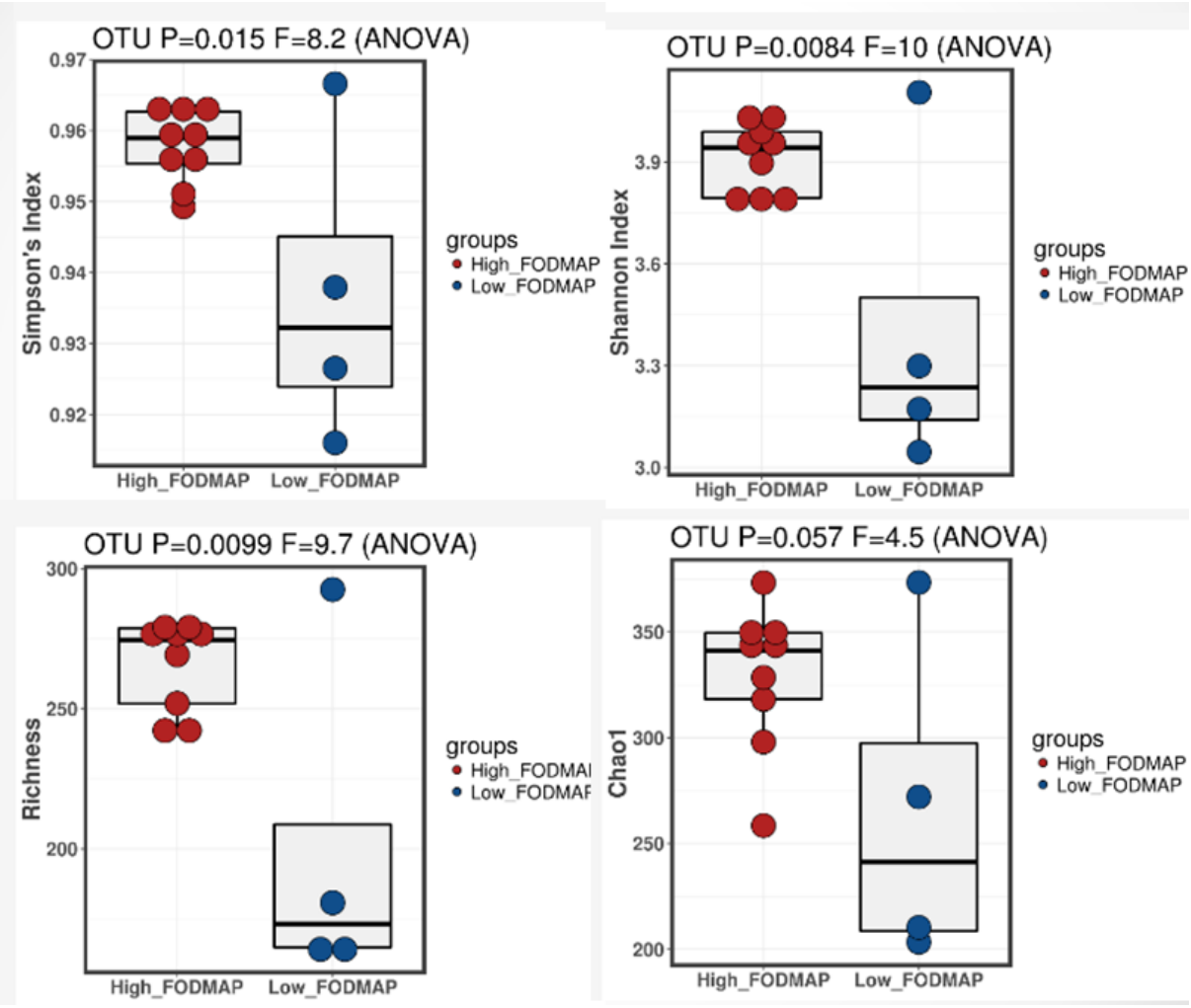

Figure 13. Alpha diversity was determined at the OTU level using the same four distance metrics.

Microbiome composition

To analyse the differential abundance between groups we employed a statistical method designed for microbiome analysis, ANCOM [61]. ANCOM, or Analysis of Composition of Microbiomes, has been reported as the most sensitive method for analysis while effectively controlling the false discovery rate [62]. Unless stated otherwise, analysis of abundance of microbial taxa between groups was carried out using this approach. The web-based Calypso was the interface used to analyse the microbiome samples [57]. This software provides a user-friendly front to R-based analysis.

Wild Type animals administered *Lactobacillus* strains via their water supply experienced a substantial rearrangement in their microbiota. Administration of *Lactobacillus rhamnosus* 6071, *Lactobacillus casei* 6072 and *Lactobacillus casei* 6083 resulted in an increased abundance of *Lactobacillus* species in the *Lactobacillus* fed animals (3800 fold p-value<0.001). Increased abundance of genera *Roseburia* (4.33 fold p-value<0.001) and *Akkermansia* (3.06 fold p-value<0.001) was also observed with *Lactobacillus* administration. *Lachnospiraceae* NK4A136 group and *Odoribacter* were significantly reduced in abundance in *Lactobacillus* fed animals (4.47 fold p-value<0.01 and 5 fold p-value<0.001 respectively). *Mucispirillum* was also significantly decreased (3.5 fold p-value 0.01).

Fat1 animals saw a substantially different profile to their WT counterparts. Despite similar data illustrating the increase in *L. rhamnosus* (7-million fold increase, p-value<0.001), limited microbial changes were observed using ANCOM. A reduction in *Mucispirillum* was the only significant change (p-value<0.001).

The high FODMAP and low FODMAP fed animals demonstrated profoundly different microbiome compositions. WT MSS animals were compared to determine the changes that fermentable fibres had on the composition of the microbiome. The relative abundance of *Bifidobacteria* (169 fold change, p-value <0.05), *Desulfovibrio* (6.8 fold, p-value<0.01), *Lachnospiraceae* FCS020 group (10 fold, p-value<0.001), *Mucispirilum* (6.3 fold p-value<0.05), *Muribaculum* (13 fold change p-value<0.05), *Lactobacillus* (18 fold change p-value<0.01), *Roseburia* (22 fold change p-value<0.001) and *Peptococcus* (33000 fold change p-value< 0.01) were increased on the high FODMAP diet. Interestingly, *Christensenelaceae* R7 group and *Parabacteroides* were decreased in the high FODMAP group (12 fold p-value <0.001 and 13 fold change p-value <0.001, respectively).

Despite significant changes in immunological data, Fat1 MSS and non-MSS animals displayed only minor changes in microbial groups. *Ruminococcus_1*, *Blautia* and *Lachnospiraceae UCG008* all increased in abundance in the MSS animals (7.7 fold change, p-value <0.001; 6.7 fold change, p-

value<0.01 and 13.5 fold change, p-value<0.001 respectively). Whereas *Rikenellaceae* RC9 group was more abundant in the non-MSS animals (5.2 fold change p-value <0.05).

3.4.6 | Diet, MSS and *Lactobacillus* modulate the SCFA metabolic output

SCFA extracted from the caecum indicates the level of FODMAP fermentation carried out by the microbes present and the metabolic pathways they may have used. A three-way ANOVA was carried out to assess the interactions between diet, genotype and MSS. The results from this ANOVA demonstrated a significant main effect of diet on the volume of total SCFAs generated (p-value <0.001). An increased amount of total SCFA was observed for high FODMAP groups. The high FODMAP groups also demonstrated increased specific SCFA's; acetate (p-value<0.001), butyrate (p-value<0.001), propionate (p-value <0.001), isovalerate (p-value<0.001) and isobutyrate (p-value<0.001).

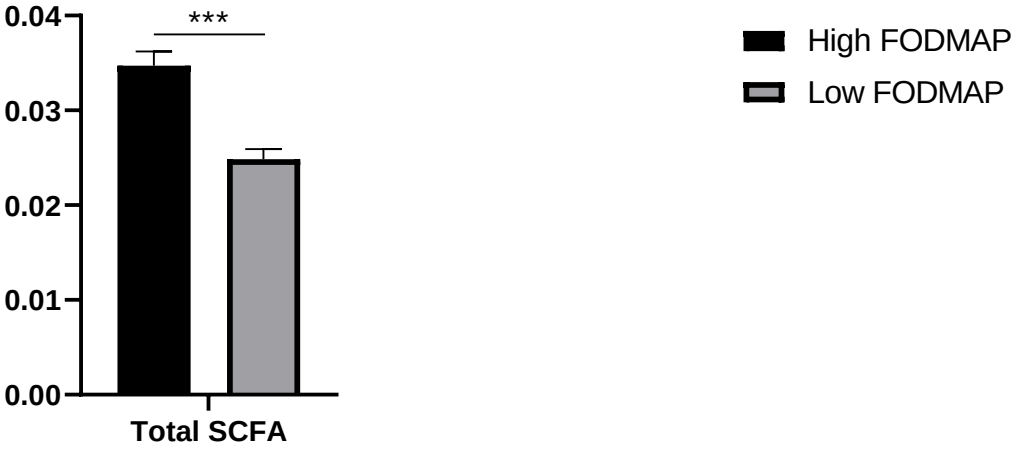

*Figure 14. Combined concentration of all SCFA's extracted from the caecum of sacrificed mice. Three way ANOVA was used to compare means from groups differing by diet, genotype and maternal separation. High FODMAP N = 25, Low FODMAP N = 26.Error bars represent SEM *** p-value <0.001*

Diet and MSS interactions were also notable in valerate (p-value = 0.011), isobutyrate (p-value = 0.014) and butyrate (p-value = 0.025).

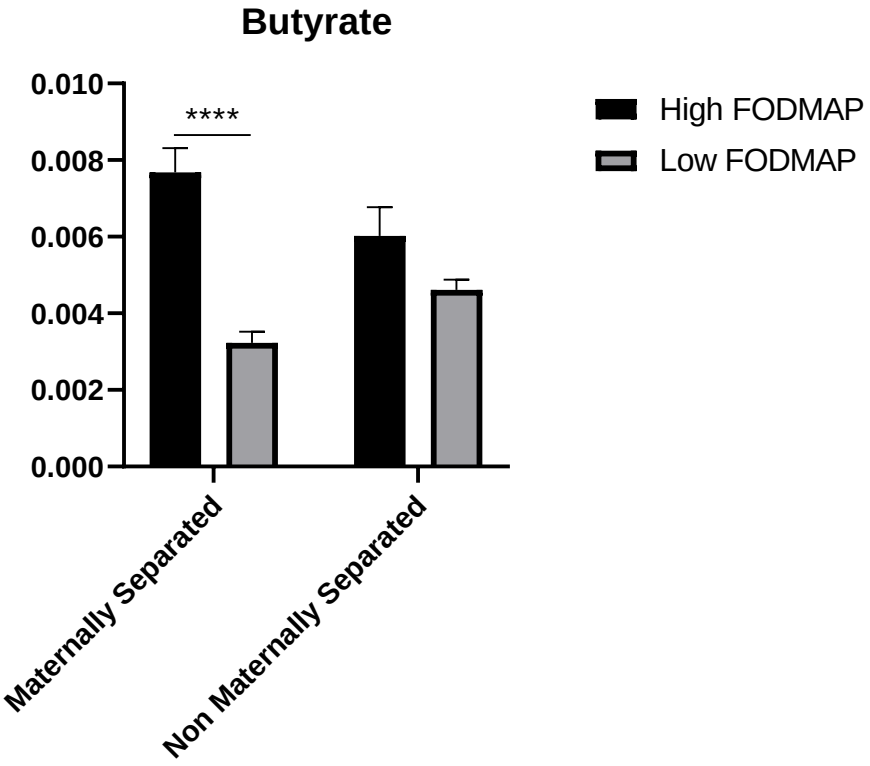

*Figure 15. Interaction between dietary content and MSS. Three way ANOVA was used to compare means from groups differing by diet, genotype and maternal separation. High FODMAP MSS N = 14, High FODMAP Non-MSS N = 11, Low FODMAP MSS N = 13, Low FODMAP Non-MSS N = 13. Error bars represent SEM **** p-value <0.0001*

Lactobacillus administration was analysed in a two-way ANOVA including *Lactobacillus* consumption and genotype. This comparison showed a significant increase in propionate due to *Lactobacillus* consumption (p-value 0.021). When a multi-factorial ANOVA was performed with all four groups it appears that *Lactobacillus* consumption has the same impact with slightly more power. Propionate is increased due to *Lactobacillus* consumption with a p-value of 0.009. Other SCFAs do not see any increase.

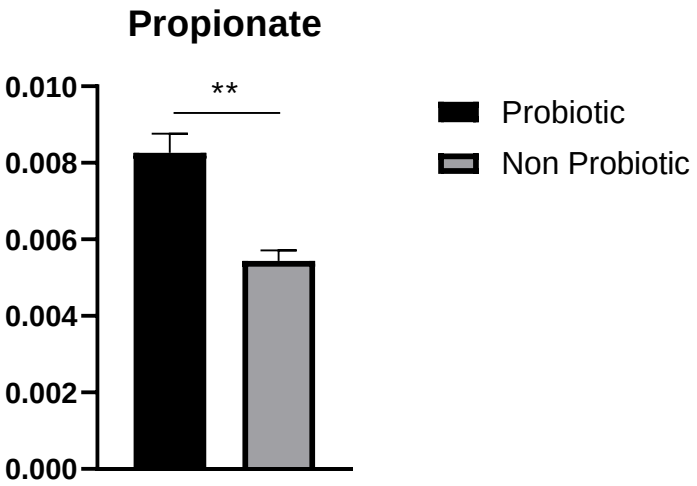

Figure 16. The impact of Lactobacillus *on propionate levels within the caecum. All SCFAs were compared to understand the impact of* Lactobacillus *strains. Two way ANOVA was used to compare means from groups differing by genotype and maternal separation. Probiotic N = 18, Non Probiotic N = 25. Acetate, butyrate and valerate were not reduced after* Lactobacillus *adminstration. Error bars represent SEM ** p-value <0.01*

3.4.7 | FITC intestinal permeability analysis

FITC was used to assess whether the intestinal barrier function was compromised or improved after the mice were subjected to the various experimental parameters. Analysis of the intestinal permeability using FITC dextran revealed no changes between groups. Genotype, diet, maternal separation and probiotic supplementation did not have a significant impact on intestinal permeability as measured by FITC dextran.

3.5 | **Discussion**

3.5.1 | Body weight changes

MSS WT animals did not show a significant change in body weight at the weaning period compared to their non-MSS counterparts (p-value=0.062). However, the same comparison in Fat1 animals revealed a significant change in weaning weight after MSS (p-value = 0.02). Interestingly, this change represented an increased mass for animals that were subjected to MSS. It is possible that the separation of cubs from the dam resulted in an "overmothering" effect when the mother was reunited with the cubs. Despite relevant research indicating the efficacy of maternal separation [53, 63-67], Own et. al. describes increased maternal care as a function of separation and resilience of c57bl/6 offspring to MSS [68]. This research corroborates our findings of an increased level of maternal care immediately after maternal separation and a less anxiety-like immunological profile in offspring [68]. The presence of an optimal N6/N3 ratio may be further mediating this anti-inflammatory effect when compared to WT animals. This altered body weight was not present between groups one week after weaning. The results of this research suggest that maternal separation is not an appropriate mechanism for inducing stress in C57bl/6 mice.

3.5.2 | Immune cells in the MLN respond to *Lactobacilli* and MSS

The CD19+ cell population can be seen as an anti-inflammatory immune cell through its secretion in skin and cancer disorders [69-71]. A reduction in CD19+ cells has been observed in post infectious IBS patients in comparison to healthy controls [72] Indeed, Shulman et al. describes a lower percentage of CD19+ cells present in IBS patients in comparison to healthy controls [73]. A strong inverse association was also reported between CD19+ cells and number of pain episodes, pain severity and mean maximum pain severity [73]. This develops a strong link between low CD19+ cells and a more severe IBS phenotype. For this reason, it is interesting to observe a significant increase in CD19+ cells within the Mesenteric Lymph Nodes (MLNs) when Fat1 mice were treated with *Lactobacillus*. From these data it is inviting to suggest that the *Lactobacillus* strains are regulating inflammation within the colon via CD19+ B immune cells, in an N-3 dependent manner.

CD25+ cells are regulatory T-cells considered important for protection from auto-immunity and mediating the inflammation process in the gut [74]. An increase in CD25+ cells has been observed in colonic mucosa of IBS patients [75]. This marker was reduced in MSS animals and seems to contradict the finding that maternal separation induces IBS. CD4+ 8+ cells have been linked to increased visceral sensitivity and mast cell degranulation [76]. Similar to the result observed for the concentration of CD25+ cells present, we see a reversal of the expected result. This may further indicate that the MSS model is not inducing the IBS-like phenotype that was expected. Increased maternal care likely causes

the reduction in CD25+ cells and CD4+/8+ cells reported in the MLNs of Fat1 mice [68]. Own et. al. describes this phenomenon in C57bl/6, indicating that offspring exposed to MSS have a modest shift to a less anxiety-like immune profile than those treated regularly [68]. This indicates an unsuccessful induction of IBS using the MSS model.

3.5.3 | *Lactobacilli* and fermentable carbohydrates restructure microbial composition

Lactobacillus consumption in WT animals resulted in a significant rise in abundance in several groups known for exerting anti-inflammatory effects. Primarily, *Akkermansia* and *Roseburia* were increased in *Lactobacillus* fed WT MSS animals. *Akkermansiaceae* members have been negatively correlated with obesity and inflammation [77, 78]. *Akkermansia muciniphila* has been researched extensively and is a known producer of acetate and propionate and relies on feeding off mucin surrounding the gut lumen [79]. Similarly, *Roseburia* spp. are known for their anti-inflammatory role [80, 81]. This is largely due to their ability to produce butyrate, and to a lesser extent, propionate [82]. The increase in propionate concentration was borne out in the SCFA results. It is clear that *Lactobacillus* supplementation caused a notable and detectable change in the microbiome composition and metabolic output of these animals.

While *Akkermansia* and *Roseburia* increased in abundance in the WT animals, this increase was not observed in Fat1 animals that underwent similar treatment. This observation is an interesting one due to the nature of the Fat1 animals and their high N-3 PUFA concentration, a known anti-inflammatory [83]. It is possible that an anti-inflammatory niche has been fulfilled by the high N-3 PUFA concentration in Fat1 animals, which gut microbes *Roseburia* and *Akkermancia* fulfil in WT animals. In this manner, microbes with known anti-inflammatory properties may induce a localised anti-inflammatory response, resulting in a reduced host immune reaction to these microbial groups [84].

Mice that consumed a highly fermentable diet showed a substantial rearrangement in microbial composition compared to those that consumed no fermentable carbohydrates (low FODMAP diet). MSS WT mice fed a high FODMAP diet were compared to their low FODMAP counterparts to understand this change. Well known genera such as Bifidobacterium, *Roseburia*, *Lactobacillus* and a *Lacnnospiraceae* group were substantially increased in response to a highly fermentable diet. This represents a significant shift in abundance of primary fermenters, or taxonomic groups that use products from primary fermentation, within the microbiome [85-87]. Many of these genera are considered beneficial microbes and promote a healthy phenotype [88]. This increase correlates with the increase in almost all SCFAs and infers a significant up-regulation in metabolic activity [89]. This response highlights the issues with removing FODMAP carbohydrates from the diet to manage IBS symptoms, as many of the positive effects of these dietary fibres are also removed [90-92]. The

products of this fermentation, SCFAs, have a role in many health promoting pathways and the loss of these benefits may have a negative impact on the underlying IBS causative factors [93]. The data discussed above demonstrates the widespread restructuring of the microbiome that occurs in the presence of increased fermentable carbohydrates.

Interestingly, high FODMAP-fed WT animals also revealed a reduction in the *Christensenellaceae* group and *Parabacteroides* genus. Members of the *Christensenellaceae* family are inversely associated with BMI in many obesity studies to date [94, 95]. It is likely that these microbes are capable of proliferation in low calorie, low carbon environments. These less rich conditions are likely to be present in lower BMI individuals when compared to their obese counterparts. For this reason, a reduced abundance of this group in animals on a high FODMAP diet is expected. *Parabacteroides* are possibly capable of digesting the cellulose within the low FODMAP diet, causing an increase in abundance [96]. This may explain the increased abundance of this genus in low FODMAP fed mice.

MSS Fat1 animals also demonstrated significant changes in microbial abundance. These results may be influenced by the increased immune activity in non-MSS animals. Interestingly, Fat1 separated mice showed a greater abundance of mucin degrading bacteria. It is possible that the increased immune response in the non-separated group caused more bactericidal activity against mucin degrading groups such as *Ruminococcus* and *Lachnospiraceae* [97]. The abundance of the genus *Blautia*, also reduced in non-separated mice, has previously been linked to *Ruminococcus* in a cross feeding structure after transcriptomics analysis [98]. The reduction in *Ruminococus* is likely having a knock-on impact in reducing *Blautia* spp. by disrupting this cross-feeding mechanism. These data illustrate the interlinked nature of the diverse genera present and their reliance on each other for survival [99].

The high FODMAP diet had a significant impact on the diversity of the murine microbiome compared with the low FODMAP diet. Beta-diversity measures between-subject diversity, whereas alpha diversity measures the within-subject diversity [100-102]. PCoA plots illustrating beta-diversity clearly separate samples by diet, indicating that the intricate cross-feeding structure is enhanced by the introduction of fermentable carbohydrate substrates. Similarly, alpha diversity was increased in high FODMAP diet fed mice compared with those on the low FODMAP diet. Interestingly, human trials have reported no reduction in alpha and beta-diversity after a low FODMAP diet compared with a 'Sham' low FODMAP diet [103-106]. The discrepancy between our findings and the cited research is likely due to the contrasting diets possible in animal trials. A low FODMAP human diet will still contain a certain volume of fermentable carbohydrates, whereas the low FODMAP feed used in this research contained 0% fermentable carbohydrates. Likewise, the 'Sham' diets, used as a control diets in these studies, may not provide the consistent and varied high concentration of fermentable carbohydrates that the

10% FODMAP diet in this research did. The high contrast between the two diets fed to the mice may explain the substantial re-arrangement observed in beta-diversity. This finding may also suggest that limited fermentable carbohydrates may be sufficient to maintain diversity within a microbiome [103, 104].

Lactobacillus consumption status also appears to separate the samples based on PCoA plots illustrating beta-diversity. Previous research has determined that microbial shifts are not guaranteed by probiotic supplementation and that diversity is often unchanged [107-111]. However, other probiotic strains cause substantial change and induce a shift in diversity [112-114]. The decreased diversity observed indicates the *Lactobacillus* mix is reducing competitor strains in carbohydrate fermentation and as such is reducing the number of other OTUs present within the microbiome. Interestingly, Tankou et. al. describes dominant *Lactobacillus* strains that reduce alpha diversity and demonstrate a significant shift in beta diversity in healthy human subjects, but not in multiple sclerosis patients [113]. These findings suggest that the impact probiotic strains have on microbiome diversity are strain- and environment-specific. Despite the negative association with reduced diversity [115], some dominant strains are necessary to maintain a robust microbiome [116]. Coyte et al. hypothesised that many cooperating species require many species to survive for microbiome functionality to remain the same. However, dominant species that do not rely on others add structure to these environments. This increases the chance that they will persist through perturbations that might otherwise destabilise cooperative relationships between members of the microbiome. The data presented above indicates that *Lactobacillus* strains added to the microbiome reduce diversity and may increase stability. It is possible that the FODMAP-degrading *Lactobacillus* strains had the ideal environment to colonise and dominate the murine microbiome due to the presence of a high FODMAP diet.

3.5.4 | Significant changes in metabolic output observed due to diet and *Lactobacillus*

SCFAs have been implicated in the improvement of various disorders through mediation of tight junctions, immune responses and cell signalling [117-119]. These results and others demonstrate the importance of increasing total SCFA concentrations within the gut. Our study showed that mice fed a high FODMAP diet showed substantially higher SCFA production than their low FODMAP counterparts. This result is to be expected as many of these FODMAP carbohydrates are precursors to SCFA production in microbial metabolic pathways [120]. An increase in gut derived SCFA's may confer beneficial effects on the host [121, 122]. A low FODMAP diet removes the precursors to these pathways resulting in lower concentrations of SCFA [123]. This reduction in SCFAs is often cited as a concern for the long term consumption of a low FODMAP diet [90, 124, 125]. Butyrate, valerate and iso-butyrate showed an interaction between MSS animals and the dietary FODMAP content. Interestingly, the MSS mice appeared to have more extreme levels of these SCFAs than the non-MSS animals. This was illustrated using butyrate as an example and manifested in a significant difference in butyrate between high and low FODMAP MSS animals (p-value < 0.0001) and no difference between non separated animals. There is no clear observable difference in microbial composition that may explain these differences. More research is required to determine the causative effect behind this interaction.

A multi-factorial ANOVA assessing the impact of *Lactobacillus* on SCFA production determined a substantial increase in propionate when animals were administered *Lactobacillus*. Reduced propionate levels have been associated with an IBS phenotype and have been suggested as a disease biomarker for IBS [126]. This correlation is likely connected to the hydrogen cycle within an anaerobic environment such as the gut. Hydrogen gas is often produced in anaerobic environments to regenerate NAD+ molecules [127]. During the production of SCFAs, hydrogen in the microenvironment is produced or consumed depending on the SCFA generated. Acetate produces 4 moles of H_2 per mole of glucose, butyrate produces 2 moles of H_2 per mole of glucose and propionate consumes 2 moles of H_2 per mole of glucose [82, 128-130]. Propionate production from carbohydrates is in part carried out by members such as *Roseburia inulinivorans*, *Eubacterium hallii*, *Blautia obeum* and *Lactobaicllus reuteri* [82]. These microbes incorporate free Hydrogen during intermediate steps of propionate production. An increased concentration of propionate indicates a reduction in hydrogen levels and may indicate a mechanism of action for the inverse relationship between IBS severity and propionate levels [126]. The three *Lactobacillus* strains added were capable of moderating the murine gut microbiome, rearranging important immune analytes and altering the metabolic output of the

microbiome. Our data show that *Lactobacillus* strains added to the gut modulate the microbiome metabolism, potentially reducing hydrogen concentration through increased propionate production.

3.6 | **Conclusion**

Lactobacillus species can reach the murine gut microbiome and exert significant changes. These include causing the proliferation of anti-inflammatory genera and increasing SCFA production. An increased concentration of propionate after lactobacilli were administered demonstrates a clear mechanism for reduction of H_2 due to the consumption of H_2 during propionate production. The high FODMAP diet causes widespread changes to the microbiome and metabolic end products. This can be observed through the differential abundances in many microbial groups and an increase in almost all SCFAs measured in comparison to the low FODMAP-fed animals. Fat1 animals displayed fewer changes in microbial abundance than WT animals. This is possibly due to the increase in anti-inflammatory N-3 PUFA removing the niche for anti-inflammatory species in the gut. Body weight of MSS animals was increased at weaning stage and the immune response reported was indicative of a less stressed phenotype. This study demonstrates a potential case for *Lactobacillus* strains in reducing hydrogen gas generation from FODMAP fermentation. Further analysis is needed to assess their direct impact on IBS symptoms in human subjects.

3.7 | **Acknowledgements**

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3.6 | **References**

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Chapter IV

Lactobacilli and N-3 PUFA modulate the murine immune system, providing protection against Dextran Sulfate Sodium (DSS) induced colitis.

4.1 | **Abstract**

Inflammatory Bowel Disease (IBD) is a complex disorder impacted by several factors including immune response, psychological stress and host-microbe interactions. Dextran Sulfate Sodium (DSS) induced colitis is a robust murine model for examining therapeutic approaches for IBD onset. This study examines the impact of a *Lactobacillus* mix on the colonic health and gut microbial community of mice with DSS induced colitis. Firstly, we attempted to induce a chronic stress phenotype in offspring via a maternal separation stress model. After weaning, wild-type (WT) mice were administered two *Lactobacillus casei* and one *Lactobacillus rhamnosus* via drinking water until DSS challenge commenced at 8 weeks old. Mice were fed a diet high in fermentable carbohydrates to aid the *Lactobacillus* mix in colonising the murine gut microbiome. A second animal model of DSS induced colitis examined the impact that an optimal N-6 PUFA to N-3 PUFA ratio may have on disease onset. This optimum ratio was analysed in terms of its complimentary effects with *Lactobacillus* administration using a Fat 1 mouse model. Maternal separation proved inadequate to provoke a stress like phenotype, inducing 'overmothering' like effects. Significant changes in the blood serum immune profiles were observed after DSS treatment began, indicating a robust onset of colitis. *Lactobacillus* strains persisted throughout DSS challenge and were observed in increased abundance 14 days after supplementation ceased. *Lactobacillus* strains modulated the murine immune response in Fat1 animals, reducing the concentration of IFN-gamma and MCP-1, and trending towards a reduction in TNF-alpha, five days after DSS treatment began. *Lactobacillus* fed DSS treated mice demonstrated reduced weight loss and increased colon length in comparison to their non-*Lactobacillus* fed counterparts, coinciding with improved blood serum immune profiles. Microbiota diversity within all groups was severely reduced after DSS challenge and also reduced after *Lactobacillus* supplementation. These data demonstrate potential for *Lactobacillus* strains to modulate the murine immune response, in a N-3 PUFA dependent manner, as a preventative measure to reduce the severity of colitis.

4.2 | **Introduction**

Inflammatory Bowel Disease (IBD) is a chronic inflammatory bowel disorder that is divided into two major forms – Ulcerative colitis (UC) and Crohn's disease (CD). The aetiology of IBD is broad and includes genetic, microbiological, immunological and environmental factors [1]. These components interact in all forms of IBD, which is generally diagnosed by assessing clinical, histological, endoscopic and radiological presentations [2]. UC and CD are differentiated by the site of inflammation and type of inflammation within the gastrointestinal tract (GIT). Inflammation in UC progresses proximally from the rectum and its damage is located solely in the colon, whereas CD may affect any GIT site and may be discontinuous [3]. The high concentration of microbes present in the gut environment and the strong immune response associated with IBD has led to research analysing the interaction between host and microbiological factors [4, 5]. This work has uncovered an aberrant immune response to commensal bacteria in genetically susceptible hosts. This indicates a breakdown of the intestinal epithelial barrier that mediates the interactions between host and microbe [6]. The current therapies for IBD include high dose steroids, immunomodulators, restrictive diet and, in severe cases, surgery [7-9]. These therapies are often considered unpalatable and result in a need for new treatments that can provide relief or prevent IBD recurrence.

The gut microbiota are an ecosystem of approximately 100 trillion microbes and over 1000 species that reside within the human gut [10]. These microbes have a profound effect on the health of the host, influencing many disorders such as type II diabetes, colorectal cancer, irritable bowel syndrome and IBD [11-15]. Host-microbe and microbe-microbe interactions moderate the gut environment causing potentially deleterious effects; however, beneficial microbes are also capable of providing a protective effect in the gut. Given the robust interaction between gut microbes and host and the substantial rearrangement in the microbiota of IBD patients, the microbiota may be a potential therapeutic target in the near future [15-18]. Probiotic bacteria may be capable of reducing the aberrant immune response between host and microbe while restoring a structure more closely resembling that of healthy individuals. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" [19, 20]. The term probiotics encompass a number of microbial Genera, however, *Bifidobacteria* and *Lactobacillus* represent the majority of probiotics currently available [21]. Members of the *Lactobacillus* group were chosen for this study due to their generally recognised as safe (GRAS) status, formidable enzymatic capacity and robust ability to survive gastrointestinal transit. Probiotic candidates should meet specific criteria in order to be selected [22], including ability to survive bile acid exposure and low pH present in the upper GIT and non-transferable resistance to a pre-defined range of antibiotics set by the European Food Safety Authority (EFSA) [23].

The microbiota present within the gut generate energy from metabolising carbohydrates that reach the gut undigested. These carbohydrates are often called prebiotics or Fermentable oligo-, di-, mono-saccharides and polyols (FODMAPs) [24, 25]. FODMAPs are common dietary carbohydrates that are not digested by host enzymes, and that reach the gut microbiota [26, 27]. Three *Lactobacillus* strains administered to the mice were previously selected based on their ability to ferment FODMAP carbohydrates. The combination of a high FODMAP diet and administration of these three *Lactobacillus* strains will investigate whether FODMAP carbohydrates facilitate the colonisation of *Lactobacillus* within the murine gut. A robust colonisation of the murine gut may exert a protective effect against the onset of colitis, as previously reported [28, 29].

Understanding the potentially therapeutic effect of *Lactobacillus* strains requires an appropriate animal model to analyse the interaction between IBD onset and microbial consortia present in the colon. Dextran Sulfate Sodium (DSS) induced colitis is a commonly used proxy for human ulcerative colitis in murine models [30]. This chemically induced colitis provides a reproducible phenotype similar to human UC. DSS induced colitis creates an appropriate experimental model to analyse the potential preventative effect that probiotics may have on the initiation or recurrence of the disease. Many probiotics have been analysed using this approach with varied results [29, 31-34]. These results suggest that although there are potential protective effects, they are specific to the strain level. The severity of DSS induced colitis is assessed by measuring the loss of body weight, mortality, the reduction in colon length, the activation of immune cells, destruction of the intestinal wall and alteration in stool consistency [30, 35].

Early life stress in humans is a known risk factor in human anxiety and mood disorders [36-38]. This early life stress model has been used in murine trials to elicit anxiety and depressive like phenotypes [39-41]. Maternal separation is also employed to induce an IBS like phenotype in rats and mice [42-44]. Immunological response, behavioral examinations and response to physical stimuli are used to determine the efficacy of the Maternal Separation Stress (MSS) [45-50]. Investigating the role that early life stress may have on DSS treated mice may indicate the role of psychological trauma on IBD pathology.

Genetically modified Fat1 mice were assessed for their potentially protective effect on DSS induced colitis. Fat1 mice convert N-6 Polyunsaturated Fatty Acids (PUFA) to N-3 PUFA in order to maintain an optimum ratio between these PUFAs [51, 52]. A high N-6 PUFA to N-3 PUFA ratio has been associated with cardiovascular disease [53], obesity [54] and metabolic syndrome [55] among many others [56, 57]. Research investigating the link between the PUFA ratio and IBD severity have reported inconclusive evidence [58-60]. Hutchinson et. al. (2020), recently reviewed the impact of a

combination approach of N-3 PUFA and probiotics to reduce inflammation [61]. Distinct forms of N-3 PUFA, Eicosapentanoic acid (EPA) and Docasahexanoic acid (DHA), possess specific beneficial health effects [62, 63]. Dietary supplementation of EPA and DHA have previously demonstrated a lower inflammatory response to DSS induced colitis [64, 65]. The fat1 model provides a more refined method for interrogating the impact N-3 PUFAs and *Lactobacillus* may have on DSS induced colitis in mice [52].

This aims of this study are to assess the potentially protective effect of three *Lactobacillus* strains and an optimum N-3 PUFA concentration on DSS induced colitis using a murine model. The study also investigates the impact that DSS induced colitis has on the gut microbial ecosystem present.

4.3 | **Materials and methods**

4.3.1 | Mice and diet

Transgenic *fat-1* mice were generated as previously described [51] followed by backcrossing onto a C57BL/6 background. *Fat-1* phenotype was confirmed by gas chromatography flame-ionization detection (GC-FID) following identification of increased tissue N-3 PUFA compared with Wild Type (WT). Mice were housed in the Massachusetts General Hospital (MGH) animal facility in a biosafety room (level 2) in hard top cages with filtered air. Mice were maintained in a temperature-controlled room (22–24 °C) with a 12-h light/dark diurnal cycle. Food and water were provided ad libitum. *Fat-1* and WT mothers were fed a diet high in N-6 PUFA (AIN-76A with 10% corn oil) from LabDiet in order to maintain *fat-1* and WT phenotypes. At postnatal day (PND) 28, female offspring were weaned onto a high FODMAP diet consisting of 10% w/w FODMAPs, comprising 3.5% w/w fructose, 3% w/w oligofructose, 2% w/w lactose, 0.75% w/w mannitol and 0.75% w/w sorbitol. This diet was altered from a similar high FODMAP diet used in a rodent trial [66]. Each gram of high FODMAP diet contains 3.72 kcal, with 10% of calories provided by fat in the form of high N-6 PUFA corn oil [51], 69% of calories provided by carbohydrate, and 20% of calories provided by protein. Body weight and food intake were measured weekly using an electrical balance. Animals were sacrificed using CO_2 and the dissected tissues were flash frozen in liquid nitrogen. After sacrifice, colon samples were tested to determine the N-3 PUFA concentrations within the colonic tissues. Colon tissues were analysed in the same manner as tissue samples, using GC-FID to determine increased N-3 PUFA concentration. All animal procedures in this study were performed in accordance with the guidelines approved by the MGH Subcommittee on Research Animal Care.

4.3.2 | Maternal Separation

Pregnant females were house individually during the third week of gestation. All maternal separation was carried out during the light phase at irregular times of the day. Pups were exposed to maternal separation from Postnatal Day (PND) 1 through 18 (Pups were born on PND 0). During the procedure, the dams were removed and placed in a clean cage on their own. The pups were then placed in another clean cage and individually separated by plastic dividers. Each litter was placed into a single cage. Offspring were observed and kept separated. After 3 hours of separation dams and offspring were returned to the original home cage. After PND18 the offspring remained with the dams until weaning prior to PND28. Control pups were not separated from the dams. After weaning the offspring were housed according the standard housing conditions between 2-4 animals per cage according to group.

4.3.3 | Administration of *Lactobacillus* strains

Lactobacillus rhamnosus DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 were isolated originally from Sicilian dairy cheese. The three strains were freeze dried individually at a concentration of 1 X 10^{10} CFU in 1ml of 15% trehalose (Merck, Germany) solution. The mix of three strains was performed after reconstitution with sterile H_2O. The volume of water consumed by the mice was used to determine the concentration of *Lactobacillus* added to the water supply. 1 X 10^{9} CFU of bacteria per strain was consumed daily. The *Lactobacillus* seeded water supply was replaced every 48 hours. The volume drank by the mice was approximately 3ml per day. Animals within the *Lactobacillus* group stopped receiving *Lactobacillus* in the water supply when the DSS challenge began. The animals also did not receive any *Lactobacillus* after DSS challenge during the seven day recovery period.

4.3.4 | Dextran Sulfate Sodium induced colitis

Dextran Sulfate Sodium (DSS, MP Biomedicals; MW=36,000-50,000) was administered in the water supply to mice for 4 weeks post weaning (8 weeks old) to chemically induce a colitis phenotype. The concentration of DSS in the water supply was 2.5% (w/v). Animals consumed the DSS treated water supply *ad libitum* for 7 days. The animals were returned to non DSS supplemented water for 7 more days, after which they were sacrificed. The volume of water consumed was measured daily and control animals received water with no DSS. The body weight of all animals was measured daily. Animal weight was used to evaluate physiological changes after maternal separation and DSS challenge. Mice were prematurely sacrificed if they displayed significant weight loss and very poor physiological condition.

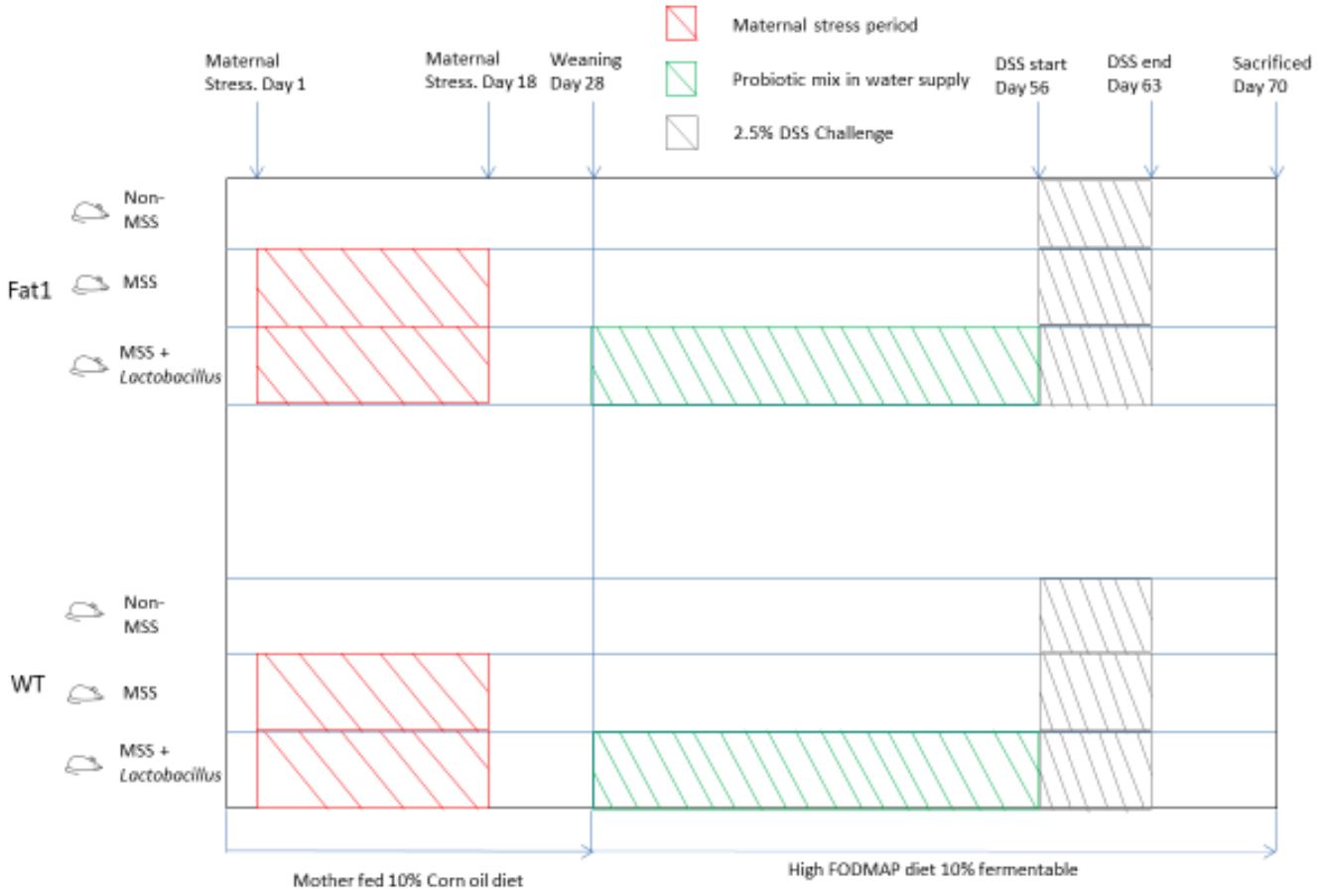

Figure 12 Experimental design for DSS induced colitis.

4.3.5 | Analysis of caecum microbiota

The contents of the caecum were thawed, extracted and weighed. DNA was extracted using the DNA Fast Stool DNA extraction kit (Qiagen, Hilton, Germany) using the protocol for Gram positive bacteria and including an additional bead beating step at the beginning of the procedure [67]. DNA was quantified using the Qubit High Sensitivity Kit (Life Technologies, Carlsbad, CA, USA), standardised and then used as a template for PCR. 16S metagenomic libraries were prepared using primers to amplify the V3–V4 region of the bacterial 16S rRNA gene, with Illumina adaptors incorporated as described in the Illumina 16S Metagenomic Library Preparation guide. Following index PCR and purification, the products were quantified using the Qubit high sensitivity DNA kit (Life Technologies) and pooled equimolarly. Libraries were then sequenced (2 × 300 bp) on the Illumina MiSeq platform.

4.3.6 | Extraction of Mesenteric Lymph Nodes (MLN) and immune cell enumeration

MLN from each mouse was dissociated through a 70 µm cell strainer with FACS medium (1x PBS + 1 mM EDTA + 1 % calf serum) using the plunger of a 3 ml syringe. Cells were then collected by centrifugation at 1500 rpm for 5 min. Then, the cells were blocked with Fc receptor antibody in FACS medium on ice for 15 minutes and removed the blocking medium by centrifugation at 1500 rpm for 5 min. To identify different lymphocyte population, the cells were further incubated with anti-mouse CD19, anti-mouse CD25, anti-mouse CD4 and anti-mouse CD8 antibodies on ice for 15 minutes and the antibodies were removed using centrifugation at 1500 rpm for 5 minutes. The cells were fixed with 3% formaldehyde in FACS medium and stored at 4°C. The cells were analyzed using an Attune™ NxT Flow Cytometer (ThermoFisher Scientific). All the antibodies were purchased from Biolegend, San Diego, CA, USA.

4.3.7 | Blood serum immune cell quantification

The determination of the count of multiple cytokines in mice pre DSS treatment and mice 5 days after DSS treatment began was performed using Human Magnetic Luminex Assay from R&D Systems (Minneapolis, MN, United States) according to the manufacturer's instructions. Levels of GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, LIX 53, IL-17A, KC, MCP-1, MIP-2 and TNF-α were analysed using a MHSTCMAG-70KPMX (Merck) plate incorporating these analytes. Bio-Plex MAGPIX Multiplex Reader and Bio-Plex Manager software from Bio-Rad (Bio-Rad Laboratory, Hercules, CA, United States) were used to perform the analysis.

4.3.8 | Data Analysis

Microbiota compositional changes were analysed using the web server, R based software Calypso (version 8.84) [68]. Statistical changes in composition were detected using Analysis of Composition of Microbiomes (ANCOM) [69]. This statistical method accounts for multiple testing and the many zero's in compositional data. ANCOM has been demonstrated as the most sensitive method for analysis while effectively controlling the false discovery rate (FDR) [70]. Alpha and beta diversity were determined at the OTU level using raw reads, rarefied to a read depth of 22,817. Alpha diversity was compared using Simpson's index, Shannon's index, Chao1 and Richness. Beta diversity was measured using PCoA plots to illustrate the per centage of variability explained by variables. Linear discriminant analysis effect size (LEfSe) was used to identify the signature species that were associated with each variable. All data analysis was carried out using R through the Calypso platform.

4.4 | **Results**

4.4.1 | Mice excluded from subsequent analysis after DSS treatment

Three non-*Lactobacillus* fed DSS challenged mice, two fat1 and one Wild Type, were sacrificed prematurely due to poor condition (Figure 2). All *Lactobacillus* fed animals maintained an appropriate body condition throughout the experiment and none were prematurely sacrificed. The two fat1 mice were sacrificed on day 9 and day 10 after DSS challenge began, and the WT mouse was also sacrificed on day 10 after the challenge began. These sacrificed animals were not included in any subsequent analysis.

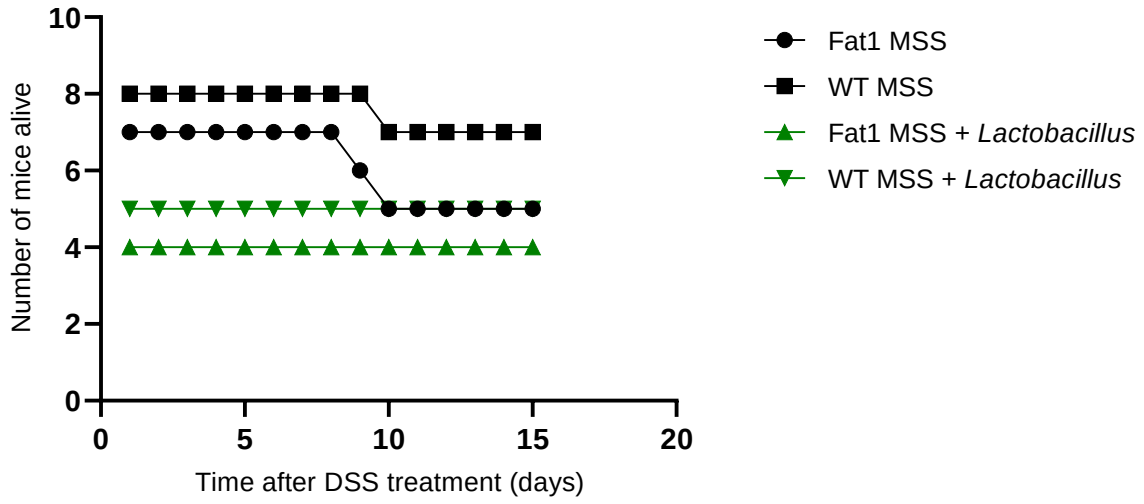

Figure 2 Mortality rate of mice exposed to DSS induced colitis. Murine groups displayed were all fed high FODMAP diet. Animals before DSS treatment N = 24.

4.4.2 | Maternal Separation Stress (MSS) alters murine body weight.

Maternal Separation Stress (MSS) mice were compared to animals that did not experience MSS. Behavioural and immunological parameters studied showed no notable differences between the two groups (data not shown). However, MSS mice had a higher body weight at weaning (28 days old).

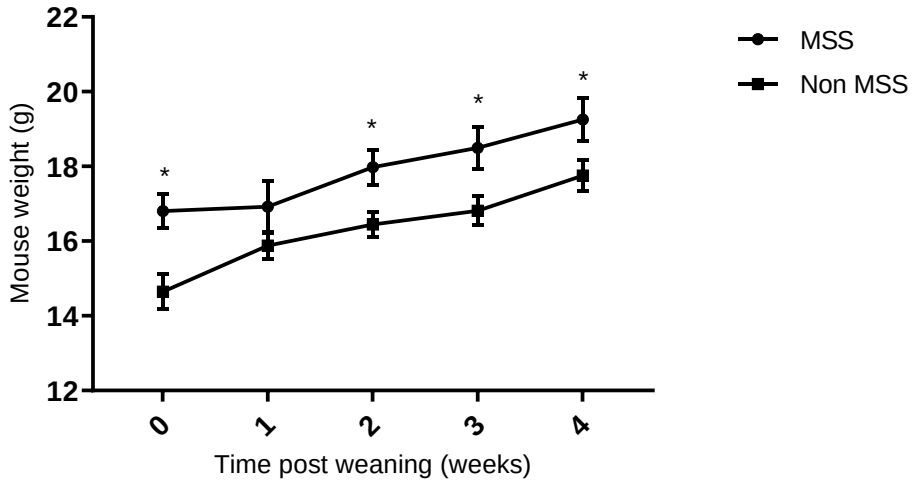

Figure 3 The impact of maternal separation on mouse weight post weaning and pre DSS challenge. Time 0 represents the first day of weaning at approximately 28 days of age. All mice presented were fed a high FODMAP diet. MSS groups and Non-MSS groups consist of both WT and Fat1 genotype. Mice that differed by genotype were not significantly different in weight. MSS N = 24, Non-MSS N = 9.T-test was used to compare means. Values plotted as mean ± SEM (p-value <0.05).*

This body weight difference was significant at every week, except week 1 post weaning, at a 95% confidence interval (Figure 3). This change in body weight persisted until the beginning of the DSS challenge.

4.4.3 | Significantly altered N-6/N-3 ratio observed in the colon of Fat1 mice

The PUFA profiles from colon tissues were analysed to understand the N-6/N-3 ratio at the site of interest. A significant difference was observable between the fat1 and WT groups. Fat1 N6/N3 ratio (mean 5.83; SD 2.458) is significantly different to the WT counterpart (mean 23.03; SD 8.516) with a p-value of <0.0001. This significant difference was observed for each fat1 and WT group (Figure 4). Similarly, the eicosapentanoic acid (EPA) availability in the colonic tissue is significantly different between the fat1 (mean 24.54; SD 8.316) animals and their WT (mean 0.00; SD 0.00) counterparts with a p value of <0.0001. This displays a clearly functioning transgenic model and confirms that the N3 PUFAs are at an optimum level within the colonic tissue.

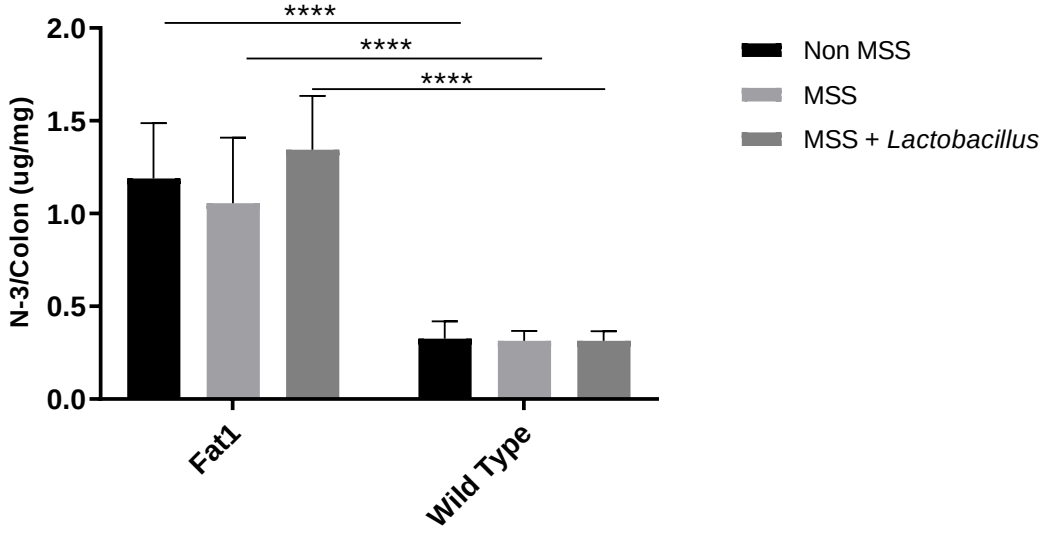

*Figure 4. The concentration of N-3 PUFA within the colon in fat1 and Wild Type (WT) mice. All mice presented here were fed a high FODMAP diet. Non-MSS N =9, MSS N = 15, MSS + Lactobacillus N = 9. Values plotted as mean ± SEM (**** p-value <0.0001).*

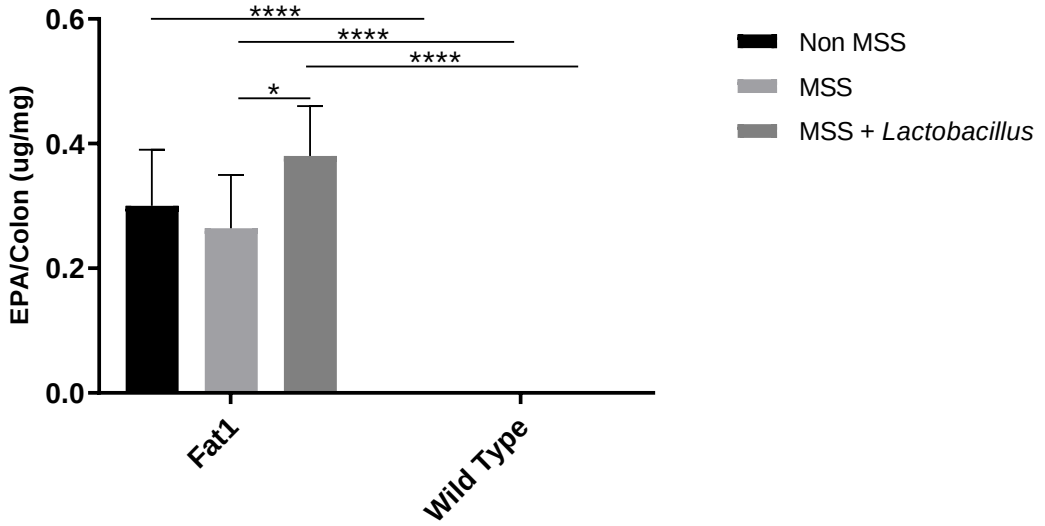

Figure 5. The concentration of eicosapentaenoic acid (EPA) present within the colon in fat1 and WT mice. All mice presented here were fed a high FODMAP diet. Non-MSS N =9, MSS N = 15, MSS + Lactobacillus N = 9. Values plotted as mean ± SEM (p-value < 0.05, **** p-value < 0.0001).*

No EPA was detectable in the colonic tissue of WT mice (Figure 5). Interestingly, *Lactobacillus* fed fat1 groups appear to have greater EPA available within the colon compared to their non-Lactobacillus fed counterparts (Figure 5). This may indicate an interaction between the *Lactobacillus* fed animals and the high N3 PUFA concentration within the gut.

4.4.4 | *Lactobacillus* fed fat1 mice demonstrate an altered response to DSS induced colitis

Weight loss during DSS treatment

MSS fat1 mice were compared to MSS fat1 *Lactobacillus* fed mice. During the DSS period, the body weight of all animals challenged reduced substantially. This is a characteristic feature of DSS challenge in mice. Our results show a protective effect to weight loss in fat1 mice that had consumed the *Lactobacillus* mix. This protective effect was calculated using the Area under the Curve (AUC) analysis. AUC for maternally separated animals had a mean of 230.7, 95% confidence interval 226.6 to 234.7, SE 2.07. Maternally separated animals administered with *Lactobacillus* had an AUC mean of 246.6, 95% confidence interval of 237.2 to 256.0 and Standard error of 4.799. The two AUC scores of MSS and MSS animals fed *Lactobacillus* are significantly different at a confidence interval of 95% (p value < 0.05) (Figure 6).

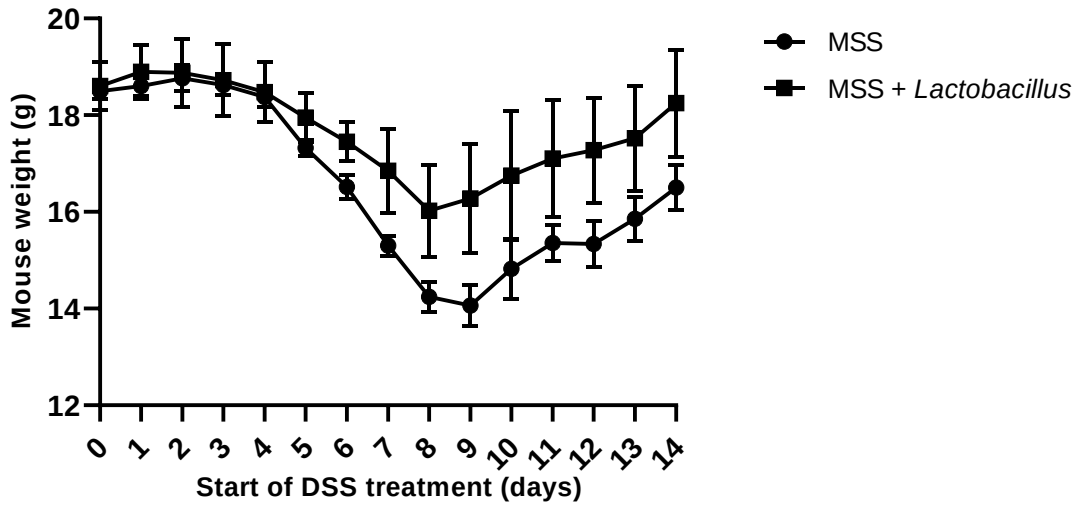

Figure 613. Change in weight during DSS challenge and recovery period. Day0 to Day7 animals were given DSS in their water supply. Day 7 to Day 14 animals were given only water to recover. Day 14 animals were sacrificed. All mice presented were fed a high FODMAP diet and were of Fat1 genotype. MSS N = 7, MSS + Lactobacillus N = 4. No significant differences were observed after Lactobacillus *supplementation in WT mice. Area under the curve was used to compare groups. Values plotted as mean ± SEM.*

Colon length after DSS treatment

Colon length is a characteristic measurement used to understand the severity of DSS treatment. The colon length of DSS treated mice in our study was significantly reduced compared to non-DSS treated mice. Gross images of the murine colon were captured after sacrifice (Figure 7).

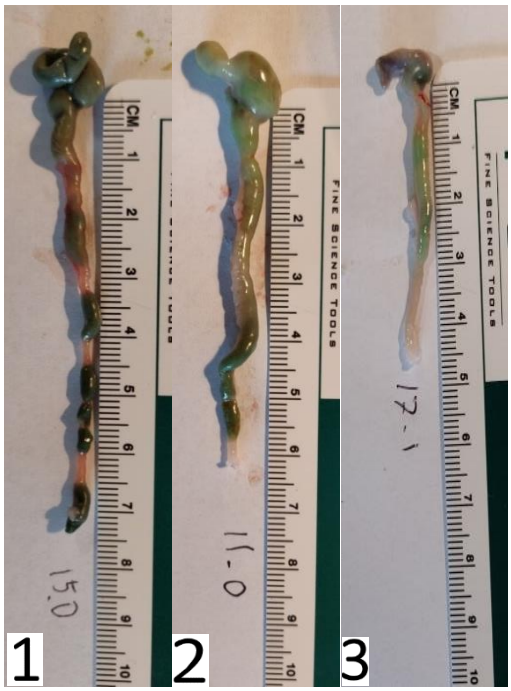

Figure 7. Gross images of mice colon. 1 – Non-DSS treated mouse that drank water with no DSS. 2 – DSS treated mouse that consumed Lactobacillus *strains prior to DSS treatment. 3 – DSS treated mouse that did not consume* Lactobacillus *prior to DSS treatment. Images taken 7 days after DSS treatment ended.*

A one-way ANOVA of colon length showed a significant difference between animals that consumed *Lactobacillus* prior to DSS treatment and those that did not (p-value <0.05) (Figure 8). We assessed this main effect between the groups to report the simple effects. This analysis resulted in no significant differences between individual groups.

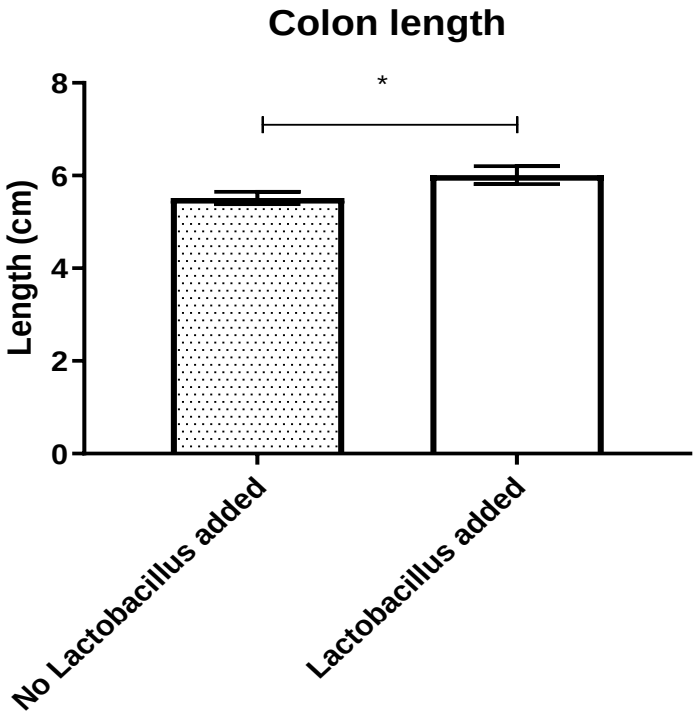

Figure 8. Mice receiving the Lactobacillus mix of strains are compared to all mice not receiving Lactobacillus*. Lactobacillus N = 9, No Lactobacillus N = 24.Values plotted as mean ± SEM (* p-value < 0.05)*

4.4.5 | Blood serum immune analytes confirm the onset of severe colitis

DSS treatment causes blood serum immune response

The immune profile of maternally separated, Fat1, *Lactobacillus* fed mice were of particular interest due to the unknown mechanism of the protective effects. Immunological analytes within blood serum of all maternally separated Fat1 mice before DSS treatment were compared to their counterparts five days after DSS treatment began. This analysis demonstrated the impact of DSS on the mice immune profile. After DSS treatment in fat1 mice, levels of IFN-γ (F value 23.529, p value < 0.001), IL-6 (F value 11.872, p value < 0.01), IL-13 (F value 5.068, p value < 0.05), IL-17A (F value 23.544, p value < 0.001), KC (F value 29.480, p value < 0.001), MCP-1 (F value 25.927, p value < 0.001), MIP-2 (F value 16.668, p value < 0.01) and TNF-α (F value 26.798, p value < 0.001) were significantly increased. These pro-inflammatory cytokines clearly demonstrate that DSS has evoked an inflammatory response within the DSS treated mice five days after treatment began.

Lactobacillus strains appear to modulate the severity of many immune analytes

Fat1, maternally separated animals compared using a two-way ANOVA between *Lactobacillus* and DSS treatment illustrated the effect of *Lactobacillus* strains on the mice. *Lactobacillus* consumption reduced IFN-γ (F value 13.442, p value < 0.01) and trended towards reduced MCP-1 (F value 4.496, p value = 0.055), whereas consumption of the strains increased IL-12 (p70) (F value 13.763, p value < 0.01). This result represents the analytes concentration in *Lactobacillus* administered mice in both pre DSS and during DSS. More notably, *Lactobacillus* consumption reduced the concentration of many pro-inflammatory analytes as the strains interacted with DSS treatment, as measured by the DSS treatment X *Lactobacillus* interaction within the 2-way ANOVA. The DSS x *Lactobacillus* interaction reported a significant difference in IFN-γ (F value 5.906, p value < 0.05) and MCP-1 (F value 9.238, p value < 0.01) and a trend towards a difference in TNF-α (F value 4.415, p value = 0.056). This manifests as a change from neutral or higher levels of these analytes before DSS to lower levels of these pro-inflammatory analytes after DSS due to *Lactobacillus* consumption. This reduction in pro-inflammatory cytokines may indicate the mechanism by which *Lactobacillus* is providing a protective effect against DSS induced colitis in a fat1 background.

4.4.6 | DSS treatment reduces microbiome diversity

The contents of the caecum were extracted and sequenced to determine the changes in microbial composition after DSS treatment. Alpha diversity metrics such as Simpson, Shannon, Chao1 and richness were used to determine the OTU diversity between samples. These metrics determined clear differences in the OTU diversity between DSS treated animals and non-DSS treated (DSS control) animals.

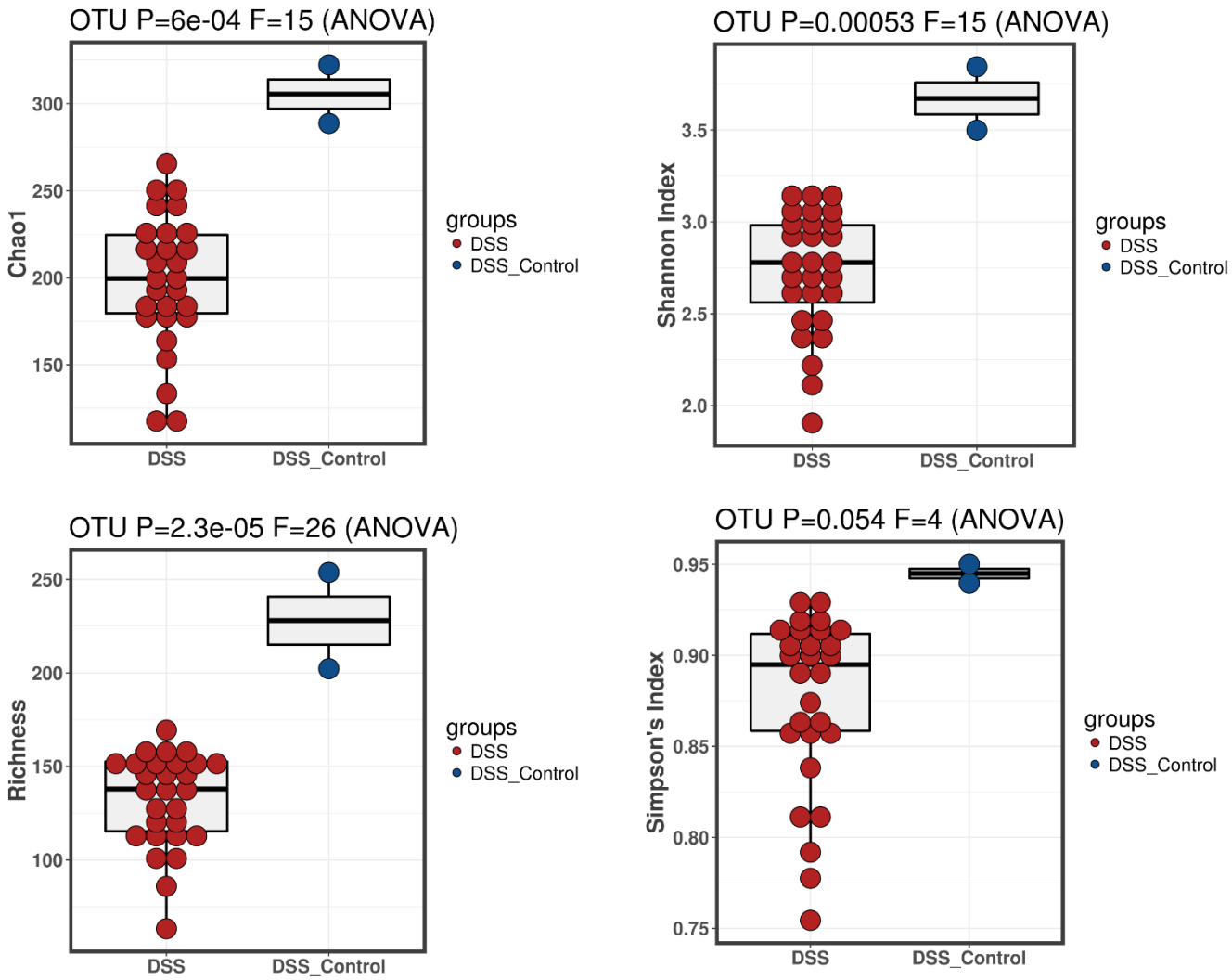

Figure 9. Four separate alpha diversity tests are displayed. Two mice were not treated with DSS in order to facilitate the microbiota comparison.

Three of the four alpha diversity metrics measured demonstrate a significant reduction in diversity after DSS treatment (Figure 9). Chao1 and richness display the most significant change, indicating that rarer OTUs and number of distinct OTUs are most severely impacted. It is apparent that the DSS challenge is damaging the Microbiome residing in the gut.

4.4.7 | *Lactobacillus* administration further alters the diversity of DSS treated mice

Lactobacillus fed animals demonstrated reduced diversity within their gut microbiota in comparison to their non-*Lactobacillus* fed counterparts (Figure 10).

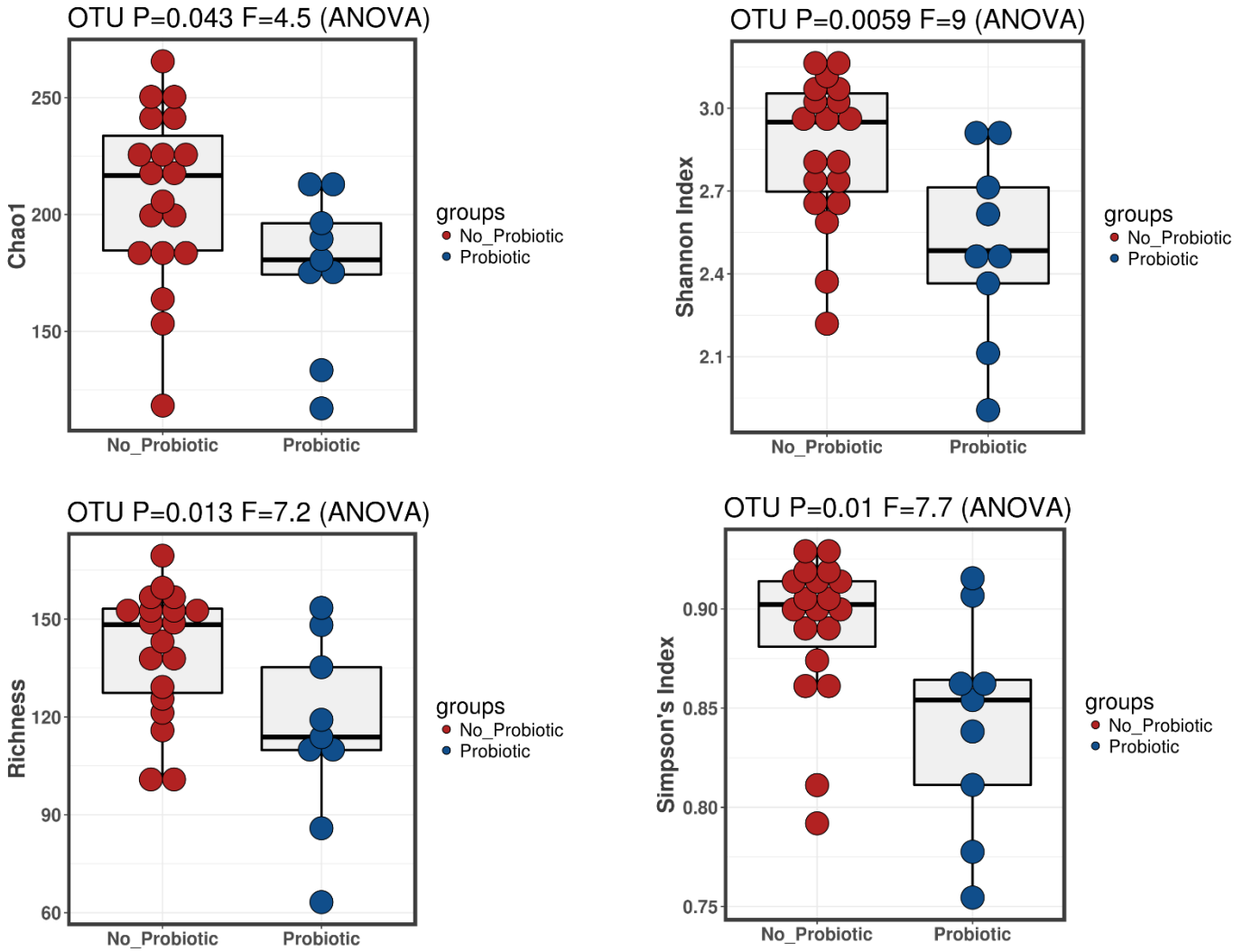

Figure 10. All DSS treated mice microbiota were based on whether they were Lactobacillus *or not* Lactobacillus *fed. Alpha diversity metrics illustrate the variety of microbial OTUs present within the microbiota.*

The supplemented *Lactobacillus* strains appear to have occupied a niche within the murine microbiome, reducing the prevalence of other OTUs. These *Lactobacillus* strains have previously been reported to competitively exclude other members of the microbiota by competition over substrate metabolism and occupation of niches (Chapter 3).

Analysis of composition of microbiomes (ANCOM) was used to compare microbiomes for varied abundance of microbial taxa [69]. Using this methodology, we determined that animals that received *Lactobacillus* prior to the DSS treatment had lower abundance of Order Clostridiales (2.8 fold change,

p-value < 0.05), Genus *Lachnospiraceae_UCG006* (9.1 fold change, p-value < 0.01) and Genus *Desulfovibrio* (9.7 fold change, p-value < 0.05). We used uncorrected ANOVA to assess the change in *Lactobacillus* in animals administered the *Lactobacillus* mix. This analysis revealed an increased abundance of *Lactobacillus* in *Lactobacillus* fed animals (Figure 11).

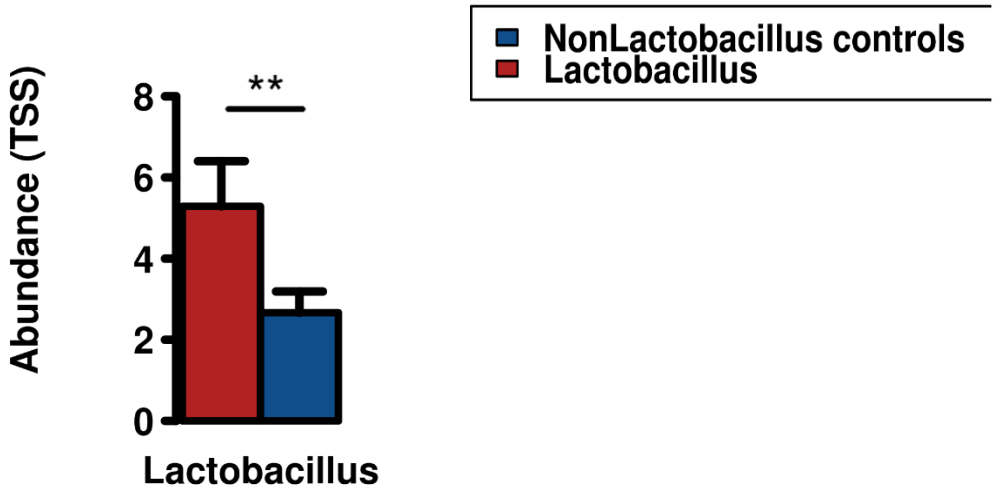

Figure 11. ANOVA compared the abundance (%) of Lactobacillus *Genus between* Lactobacillus *fed animals and Non* Lactobacillus *control animals. Values plotted as mean ± SEM (** p-value <0.01).*

We used Linear discriminant analysis effect size (LEfSe) to analyse the microbial taxa that identify the *Lactobacillus* and non-*Lactobacillus* supplemented groups. LEfSe shows *Lactobacillus* as the top indicator taxa for the *Lactobacillus* group (Figure 12). Many carbohydrate fermenting taxa were more abundant in non-*Lactobacillus* control animals This indicates that the *Lactobacillus* strains are dominating the carbohydrate fermenting niches and persisting in these niches even through DSS challenge.

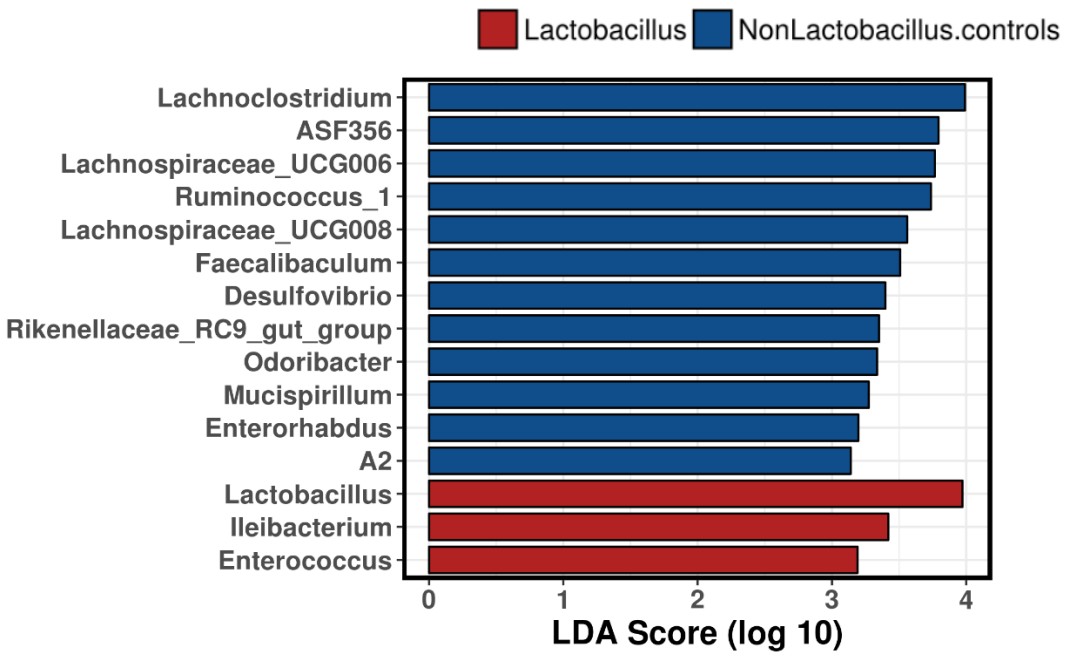

Figure 12. Linear discriminant analysis (LDA) effect size (LEfSe) was used to determine taxa that are markers for the groups in question. LDA score is used to discriminate between the two groups. Lactobacillus *represents the most prevalent distinct Genus between both groups.*

These results indicate that the genus *Lactobacillus* was increased in abundance in *Lactobacillus* administered mice at the time of sacrifice. Animals in the *Lactobacillus* groups received their last dose of *Lactobacillus* prior to DSS treatment, 14 days before sacrifice. This suggests that at least one of the three strains added, *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083, persisted in the murine gut microbiome throughout DSS challenge and recovery. Due to limitations in 16S sequencing, it is not possible to determine which strain is more prevalent.

4.5 | **Discussion**

The results presented above illustrate the impact that three *Lactobacillus* strains, *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083, and an optimum N-3 status may have on DSS induced colitis. Mice that consumed DSS in their water supply demonstrated a severe immune response, reduced body weight and a shortened colon. Fat1 animals that consumed *Lactobacillus* strains prior to DSS challenge reported improved measurements on these three parameters. Notably, *Lactobacillus* strains survived gastric transit and persisted in the gut microbiome throughout DSS challenge and the recovery period. These data indicate *Lactobacillus* supplementation may provide protection against colitis via immune modulation.

4.5.1 | Body weight changes due to maternal separation

The maternal separation model resulted in an unexpected increase in body weight for separated animals. This increased body weight persisted through the post weaning stage. It is possible that an 'overmothering' effect caused this discrepancy i.e. mothers separated from their pups would feed them more upon being reunited. The maternal separation model does not appear effective in inducing a stressed phenotype. Despite relevant research indicating its efficacy [39, 44, 71-74], Own et. al. describes increased maternal care as a function of separation and resilience of c57bl/6 offspring to MSS [75]. This research corroborates our findings of an increased level of maternal care immediately after maternal separation. The results of this research would indicate that maternal separation stress is entirely inappropriate for inducing stress in c57bl/6 mice.

4.5.2 | Fat1 mouse model improves polyunsaturated fatty acid profile within the colon

The N-6 to N-3 ratio was substantially different in fat1 animals compared to the wild type. The mean ratio of N-6 to N-3 in the colon for fat1 animals was 5.83 compared to WT 23.03. Improved N-6/N-3 ratio has been implicated in broad reaching health benefits [76-78]. Research has reported attenuation in colonic inflammation, colitis and tumorigenesis [79-81]. Indeed, Camuesco et al. have reported an improvement in DSS induced colitis after supplementation with dietary N-3 PUFA [64, 65]. Interestingly, our results show the protective effects of increased N-3 on DSS induced colitis appears to be contingent on the concurrent administration of *Lactobacillus* strains. Addition of *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 altered the PUFA profile, increasing EPA concentration within the colonic tissue. EPA acts to supress arachidonic acid derived eicosanoids and is also a substrate for an anti-inflammatory eicosanoid family [82]. Lactobacilli have been reported to incorporate unsaturated fatty acids into their cellular membrane to strengthen membrane stability during GI transit [83]. Lye et. al. postulates that LAB may incorporate EPA into

their cell membranes in this manner as a protective effect against bile acid [84]. This mechanism may increase retention of EPA within the colon, increasing EPA concentration within colonic tissue while acting synergistically to protect the *Lactobacillus* strains [84]. These results verify the efficacy of the fat1 transgenic mouse model and indicate potential synergistic effects with *Lactobacilli*.

4.5.3 | *Lactobacillus* strains provide a protective effect against DSS induced colitis

Probiotic strains are recognised as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" [19]. *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 provided a protective effect against the DSS induced colitis in three important metrics. Three mice were prematurely sacrificed due to very poor physical condition, all three of these mice were in non-*Lactobacillus* fed groups. The body weight loss of fat1 mice fed *Lactobacillus* was reduced in comparison to fat1 mice not administered *Lactobacillus*. A significantly different result was observed in animals of a fat1 background only and suggests an interaction between the optimum N-3 status and the *Lactobacillus* strains present. The protective effect against weight loss during DSS induced colitis has been observed in a number of studies to date [31-33]. Similarly, the colon length has been measured to infer the physical damage caused by DSS [35]. When the colon is shorter in length, colonic oedema, inflammation and mucosal damage are also more severe [85-87]. We observed that colon length was significantly preserved in *Lactobacillus* fed animals in comparison to non-*Lactobacillus* fed animals. This observation suggests that the *Lactobacillus* strains are disrupting the chemical damage to the colon sufficiently to result in a more positive physiology condition. The effect of probiotics and N-3 PUFAs together on inflammation has recently been reviewed [61]. Hutchinson et. al. postulates that specific probiotics and high N-3 PUFAs attenuates chronic inflammation within the gut, reducing the severity of gut disorders [61]. Certain studies suggest that the synergistic effect of these two approaches is due to the antibiotic like effects N-3 PUFAs exert on pathogenic bacteria and increased adherence experienced by *Lactobacilli* in high N-3 PUFA environments [88-90]. These findings may indicate that *Lactobacillus* and high N-3 PUFAs are acting synergistically through a number of mechanisms to provide the protective effect observed.

4.5.4 | Blood serum analytes demonstrate substantial change due to DSS induced colitis

Blood serum analytes, determined five days after DSS treatment began, report a substantially altered profile to the immune analyte profile present before DSS treatment. Eight pro-inflammatory cytokines were increased after five days of DSS treatment. This demonstrates a substantial and robust immune reaction to DSS. The immune analytes that change after DSS treatment are all involved in IBD pathology. IFN-gamma [91], IL-6 [92, 93] IL-13 [30, 94], IL-17A [95, 96], KC [97] MCP-1 [98, 99] MIP-2 [100, 101] and TNF-α [102-104] are significantly increased in the data reported in this experiment, and

are intimately linked to the immune response to IBD and DSS induced colitis. These data illustrate a successful induction of DSS induced colitis consistent with previous models [105].

4.5.5 | *Lactobacillus* consumption reduces several pro-inflammatory cytokines in Fat1 mice, coinciding with improved outcomes

Fat1 maternally separated mice that received *Lactobacillus* strains reported improved outcomes during and after DSS induced colitis. These improved outcomes manifested in reduced weight loss during DSS treatment and increased colon length. Interestingly, *Lactobacillus* consumption in Fat1 maternally separated animals resulted in substantially altered immune profile. IFN-γ significantly decreased while IL-12 (p70) reported a concurrent increase due to *Lactobacillus* consumption. MCP-1 also trended towards a reduction due to consumption of the strains. IFN-γ is a prominent pro-inflammatory cytokine involved in Th1-driven immune responses [106]. DSS induced colitis demonstrates reduced severity after IFN-γ suppression [107, 108]. Probiotic bacteria have previously demonstrated their ability to induce beneficial health outcomes via modulation of IFN-γ [109, 110]. The probiotic stimulated protective effect may be due to increased barrier function due to decreased IFN-γ [110]. IL-12 (p70) is generally considered a pro-inflammatory cytokine [111], however, depending on physiological condition may signal anti-inflammatory responses [112, 113]. *Lactobacillus casei* strains are known to induce host production of IL-12 [114]. MCP-1 is potentially reduced by the consumption of *Lactobacillus* strains in this murine model. MCP-1 plays a critical role in IBD pathogenesis [115-117]. Reducing MCP-1 reduces leukocyte migration to sites of inflammation, ultimately reducing tissues damage and destruction [116]. The flexible communications between *Lactobacilli* and the host immune system may result in beneficial modulation of the host response, resulting in a more appropriate reaction to commensal bacteria [118].

The interaction between DSS induced colitis and *Lactobacillus* consumption proved most interesting. IFN-γ and MCP-1 were significantly different in their interaction between *Lactobacillus* and DSS, whereas TNF-α trended towards a significant interaction. These three powerful pro-inflammatory agents reported a greater reduction in *Lactobacillus* fed animals after DSS treatment began. This result highlights a protective anti-inflammatory effect during colitis onset, as opposed to during a healthy state. Reduction of TNF-α during DSS induced colitis has yielded better health outcomes in IBD, including studies successfully using anti-TNF-alpha antibodies as therapeutics for IBD [119, 120]. Probiotic bacteria have previously demonstrated protective effects via immune modulation, reducing the severity of DSS induced colitis [121-123]. *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei*

DPC 6072 and *Lactobacillus casei* DPC 6083 appear to modulate the immune system similarly, albeit in a N-3 dependent manner.

4.5.6 | *Lactobacillus* strains administered before DSS challenge persisted within the microbiome

Greater diversity is frequently considered a sign of a healthier and more robust microbiome [124-126]. DSS treatment has a clear negative impact on the diversity of microbes within the treated animals, severely reducing species diversity. This is clearly observed using the alpha diversity metrics; Shannon index, Simpson index, Richness and Chao1 [127]. Interestingly, microbiome analysis indicates that the *Lactobacillus* Genus was significantly more abundant at the time of sacrifice. This indicates that the mix of *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 survived at least 14 days during DSS treatment and subsequent recovery period. This coincided with *Lactobacillus* fed animals demonstrating reduced alpha diversity compared to their non-*Lactobacillus* counterparts. This result suggests the *Lactobacillus* strains are dominating the microbiome and reducing the variety of other strains. Despite diversity indicating a more robust microbiome, research also suggests that a greater number of dominant species creates a more stable microbiome [128]. Coyte et. al. postulates that cooperating networks of microbes can be efficient but are inherently unstable as they rely on the survival of many species to maintain [128]. This research from Coyte et. al. suggests that dominant species, often referred to as keystone species, may maintain the structure of the gut microbiota during more stressful periods [129]. ANCOM and LEfSe analysis indicates the specific genera that the three *Lactobacillus* strains reduced. Many of these genera, such as the Lachnospiraceae groups, are known carbohydrate fermenters [130-132]. The three *Lactobacillus* strains administered are competent fermenters of carbohydrates and likely provided direct competition to other microbes in this niche. The occupation of this carbohydrate fermentation niche may explain the persistence of these microbes during the 14-day period. These results combined indicate that a reduction in diversity from DSS induced colitis appears more unstructured and may also reduce the ecosystems ability to return to normal function [133]. However, as per Coyte et. al (2015), *Lactobacillus* induced reduction in diversity may maintain a robust and functioning ecosystem [134]. These two responses may indicate that the diversity metric is inaccurately capturing the changing microbiome dynamic.

The interaction between the altered microbiome after *Lactobacillus* administration and the fat1 background is one that requires more detailed research to understand further. The optimum N-3 ratio may work in conjunction with the *Lactobacillus* strains to reduce DSS induced weight loss. Research in this area has described an important role for these long chain fatty acids in IBD aetiology [135-137].

The link between the colonisation of probiotic bacteria and N-3 status has been studied using high N-3 dietary components. Castel et al. reported a positive effect of N-3 PUFAs on the colonisation of *Lactobacillus* strains and their metabolism in the digestive tracts of germ free pigs [138]. Bomba et al. also reported similar results, administration of PUFAs increased the adherence of *L. paracasei* to the jejunal mucosa [139]. N-3 supplementation has also been shown to increase the efficacy of probiotic strains [140]. A review on the subject of atopy concluded that long chain PUFAs promote the action of probiotics and should be used together when possible [89]. This research suggests that higher N-3 status may facilitate greater colonisation of *Lactobacillus*, possibly through increased adherence. This amplified ability to colonise intestinal structures may allow the probiotics to exert their beneficial effects more effectively and survive environmental assaults. Our data indicate that *Lactobacillus* were capable of surviving gastric transit for at least 14 days and exerting a positive impact on the condition of the mice. *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 worked synergistically with an optimum N-3 ratio to modulate the host immune response to DSS induced colitis, reducing weight loss and maintaining colon length in the fat1 *Lactobacillus* fed group.

4.6 | **Conclusion**

C57bl/6 mice underwent a DSS challenge to understand the potential protective effect of administration of *Lactobacillus* and/or optimum N-3 PUFA status while assessing the change to the microbiota. Fat1 animals administered with *Lactobacillus* strains demonstrated the beneficial effects of reduced weight loss and increased colon length after DSS challenge. The *Lactobacillus* genus was significantly increased after *Lactobacillus* supplementation, however, the colonisation of *Lactobacillus* reduced diversity compared to non-*Lactobacillus* fed control animals. The blood serum immune profile of *Lactobacillus* fed Fat1 mice demonstrated an anti-inflammatory response after DSS was administered. A reduction in pro-inflammatory cytokines indicates a potential mechanism by which the *Lactobacillus* strains exert their influence. These results indicate that *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 are surviving gastric transit and colonising the gut for at least 14 days, aided by an optimum N-3 PUFA status, while modulating the host immune response and exerting a beneficial effect on DSS challenged mice. Our data indicate that the combination of *Lactobacillus* strains and an optimum N-3 PUFA status may aid in the amelioration of colitis. This research requires human studies to confirm these results.

4.7 | **Acknowledgements**

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4.8 | **References**

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Chapter V

Impact of low FODMAP food prototypes on the healthy microbiome during simulated ex vivo fermentation

5.1 | **Abstract**

FODMAP-induced IBS is the term used to describe Irritable Bowel Syndrome that is caused by the consumption of fermentable oligo-, di-, and mono- saccharides and polyols (FODMAPs). This form of IBS manifests primarily in symptoms of bloating and pain caused by gut microbial fermentation of FODMAP carbohydrates. A low FODMAP diet is currently recommended for reducing the severity of FODMAP-induced IBS. In this study, a range of foods potentially suitable as part of a low FODMAP diet were examined for their interaction with the human gut microbiota, in order to broaden the range of foods that individuals suffering from FODMAP-induced IBS can consume. Four food types of bread, pasta, lentils and buckwheat in various forms were included. Raw lentil, malted lentil, enzymatically degraded lentils, raw buckwheat, malted buckwheat, high FODMAP bread, low FODMAP bread, psyllium added bread, inulinase treated bread, high FODMAP pasta and low FODMAP pasta were analysed. These foods were selected in a way that the original food: raw lentil, high FODMAP bread, high FODMAP pasta and raw buckwheat, acted as the high FODMAP positive control for each lower FODMAP altered version. The microbiome after fermentation of each lower FODMAP substrate, such as malted lentils, was compared to the microbiome after fermentation of its higher FODMAP counterpart, i.e. raw lentils, to understand if the microbiome was negatively impacted by the reduced FODMAP content. *Ex vivo* fermentation of raw lentils (3.89 g FODMAP / 100 g) resulted in a similar microbiome profile to the that observed after fermentation in the presence of malted lentils (0.84 g FODMAP / 100 g) and lentils processed with FODMAP degrading enzymes (<0.005 g FODMAP / 100 g). Fermentation in the presence of malted buckwheat (<0.005 g FODMAP / 100 g) resulted in an increased abundance of butyrate producers compared to fermentation with raw buckwheat (0.33 g fagopyritol / 100 g). There were no changes in alpha and beta diversity, and limited compositional changes following fermentation in the presence of low FODMAP bread and low FODMAP pasta compared to their high FODMAP counterparts. These results demonstrate potential to use malted lentils, low FODMAP bread and low FODMAP pasta in a low FODMAP diet without compromising microbiome diversity and composition. Finally, *Lactobacillus rhamnosus* DPC6071, *Lactobacillus casei* DPC6072 and *Lactobacillus casei* DPC6083, selected based on their ability to degraded FODMAPs (Chapter 2), were seeded prior to the fermentation of several substrates. These strains demonstrated the ability to dominate the fermentation reaction, reducing other carbohydrate fermenters, hydrogen

producers and acetate levels. The results of this study demonstrate the potential of a range of new food types that are appropriate for use by individuals suffering from FODMAP-induced IBS.

5.2 | **Introduction**

Irritable Bowel Syndrome (IBS) is a chronic functional bowel disorder characterised by abdominal pain, bloating, discomfort and a change in symptom severity following defecation [1]. The clinical diagnosis of this condition relies on the Rome IV criteria set by the Rome foundation [2]. Diseases such as Inflammatory Bowel Disease (IBD) and celiac disease must be ruled out in order to diagnose IBS [3]. IBS affects approximately 10% of the global population and causes a significantly reduced quality of life for sufferers [4]. In recent years, many therapies have been tested for managing chronic IBS symptoms. These treatments include pharmacological agents, psychological treatment and dietary modifications to avoid particular food components [5-8]. The low FODMAP diet, a diet aimed at reducing the volume of fermentable carbohydrates ingested, is currently the most effective method for dealing with IBS [9]. Despite the success of this diet in reducing symptoms, it has two major limitations. The low FODMAP diet must avoid a broad variety of foods to reduce the volume of FODMAP carbohydrates ingested. This makes the diet difficult to implement and is unappealing unless the IBS symptoms are severe. Due to this, individuals that implement this diet incorrectly and without professional guidance also risk nutritional deficits [10]. Secondly, the gut microbiota use these fermentable carbohydrates to proliferate and generate beneficial compounds such as Short Chain Fatty Acids (SCFA) [11]. Long term dietary reduction of these carbohydrates may have a significant negative impact on the health of the host and the gut microbiota [12]. By developing new low FODMAP prototypes that interact favourably with the gut microbiota, this study aims to increase the variety of foods that can be consumed during a low FODMAP diet and maintain a healthy host gut microbiome.

The gut microbiota is made up of over 1000 different species of bacteria and 100 trillion microbes [13]. This diverse ecosystem is in constant contact with the host and has been implicated in numerous disease states [14-17]. A healthy microbiome is capable of maintaining homeostasis, regulating host processes and carrying out beneficial functions [18, 19]. Fermentation of non-digestible carbohydrates is one function of the microbiota. The end products of this fermentation, SCFAs, are compounds capable of mediating immune regulation, positively influencing cardiovascular health and maintaining homeostasis with the gut environment [11, 20-22]. However, FODMAP fermentation may also cause undesirable effects such as IBS symptoms of bloating and pain. These undesirable effects are caused by the gaseous products that are generated during anaerobic fermentation. Hydrogen gas (H_2) is the primary culprit in causing these symptoms. H_2 gas is often produced in anaerobic systems as a mechanism to regenerate NAD^+ and continue generating energy through hydrolysis [23]. The level of H_2 production can be indirectly observed by analysing the concentration of metabolic products such as SCFAs [24]. Acetate, butyrate and propionate are the three primary SCFAs produced in the gut. The

net hydrogen release during the production of each individual SCFA varies considerably. Acetate production releases four moles of H_2 per mole of glucose, butyrate releases two moles of H_2 per mole of glucose whereas propionate production consumes two moles of H_2 per mole of glucose [25]. Due to this disparity, an increase in propionate and decrease in acetate may indirectly indicate a reduction in hydrogen levels within the gut. Moderating the hydrogen levels within the gut may be achieved through altering the metabolic pathways used or reducing the intake of FODMAP carbohydrates. The latter approach results in a severely restricted diet that is difficult to maintain. The low FODMAP prototypes produced for this study aim to make this diet more manageable while increasing adherence rates.

Low FODMAP alternatives to high FODMAP foods are being developed to fulfil a market similar to gluten free foods [26]. Gluten free foods provide a broader range of choice to individuals with celiac disease while eliminating the negative implications of ingesting gluten. Bread, pasta, lentil and buckwheat foods were included in this study to understand their impact on the human microbiome. High and low FODMAP varieties of these food types were fermented, after which the microbiomes were compared to understand if low FODMAP foods can replace high FODMAP foods without negatively affecting the microbiome. Raw lentils (3.89 g FODMAP / 100 g) are naturally high in FODMAPs, however, research preceding this study indicates that malting lentils reduces their primary FODMAP, galactooligosaccharides (GOS), to 0.84 g FODMAP / 100 g [27], suggesting a potential mechanism for generating low FODMAP lentils. Lentils were exposed to α-galactosidase to reduce their FODMAP content, α-galactosidases were previously observed to reduce lentils primary FODMAP GOS to undetectable levels (<0.005 g FODMAP / 100 g), to generate FODMAP degraded lentils [28]. Raw buckwheat contains a potentially fermentable carbohydrate, fagopyritol, at a concentration of 0.33 g / 100 g dry matter (DM) [27]. As buckwheat is a common component of a low FODMAP diet, it is important to clarify if these fagopyritols may result in carbohydrate fermentation. Malting buckwheat reduces these fagopyritols to an undetectable level (<0.005 g FODMAP / 100 g). High FODMAP bread (1.56 g / 100 g DM) and high FODMAP pasta (undetermined FODMAP content) are not used in a low FODMAP diet due to their FODMAP content. Low FODMAP ingredients of wheat starch and gluten were used to develop the low FODMAP bread (<0.005 g / 100 g DM) examined in this study as a potential replacement for its high FODMAP counterpart (Atzler et al, data unpublished). High FODMAP bread and high FODMAP pasta were treated with inulinase, previously shown to degrade fructans in wheat extract to undetectable levels [28], to create low FODMAP pasta (undetermined FODMAP content) and inulinase treated low FODMAP bread (0.19 g / 100 g DM). Finally, bread made with low FODMAP ingredients of wheat starch and gluten was supplemented with psyllium to provide increased fiber content (0.056 g / 100 g DM) (Atzler et al, data unpublished).

The study aims to select the most suitable low FODMAP foods, based on their effect on the human microbiome, that may replace their high FODMAP counterparts as part of a low FODMAP diet [29]. To understand the impact fermentation of these low FODMAP substrates have on the microbiome we compare them to the microbiome after fermentation of their high FODMAP counterparts. The microbiome after fermentation of raw lentil is used as the high FODMAP control and is compared to malted lentil fermentation and fermentation of enzymatically degraded lentil. Similarly, the microbiome after fermentation of raw buckwheat acts as a control for the fermentation of malted buckwheat. High FODMAP bread fermentation results in a microbiome from which to compare fermentation of low FODMAP bread, inulinase treated bread and psyllium added bread. Finally, high FODMAP pasta is used as the positive control for low FODMAP pasta. Understanding the difference in the microbiome after fermentation of these prototypes compared to their high FODMAP counterparts may help determine suitable food replacements that complement a low FODMAP diet.

Using the *ex vivo* fermentation system, we assessed the impact three *Lactobacillus* strains, *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083, previously chosen for their ability to ferment FODMAP carbohydrates (Chapter 2), might have on the composition of the microbial community present and the SCFA metabolites produced. For this reason, a selection of high and low FODMAP food types, developed in University College Cork (Ispiryan et al, data unpublished), were fermented using the microMatrix with and without the added *Lactobacillus* strains.

5.3 | **Materials and methods**

5.3.1 | Food prototype digestions and preparation for fermentation

The food prototypes were subjected to simulated digestion prior to faecal fermentation. *In vitro* digestion of the food prototypes generates food similar to that which reaches the human gut microbiota. The updated INFOGEST *in vitro* digestion method was used to simulate digestion *in vivo* [30]. Briefly, food prototypes were exposed to oral, gastric and intestinal phase digestion. Simulated salivary fluid, simulated gastric fluid and simulated intestinal fluid were prepared at a stock concentration of 1.25x for the three distinct phases. For the oral phase, α-amylase (1500 U mL^{-1}, Sigma, A0521) was added to simulated salivary fluid electrolyte solution and incubated at 37 °C for each prototype for 2 minutes. The gastric phase consisted of the liquid samples from the oral phase, simulated gastric fluid electrolyte solution and pepsin solution (25 000 U ml^{-1}, Sigma, P6887) reduced to pH 3.0 and placed in a shaking incubator at 37 °C for 2 hours. Intestinal phase included the gastric chyme from the gastric phase, simulated intestinal fluid electrolyte solution, pancreatin (800 U ml^{-1}, Sigma, P7545) and fresh bile (10mM) raised to pH 7.0 and placed in a shaking incubator at 37 °C for 2 hours. Finally, post intestinal phase liquids are transferred to dialysis tubes and placed in beakers with water used as the dialysate for 26 hours. The water was changed at 24 hours, 25 hours and 26 hours after which the retentate was removed from dialysis tubes.

5.3.2 | Food prototypes

Substrates added to MicroMatrix fermentation plates were randomised throughout multiple plates and areas to reduce batch fermentation issues. Each substrate represents the average of six reactions (N=6). Each substrate and their abbreviations are as follows: LM = Lentil malt; BM = Buckwheat malt; RL = Raw lentil; BR =Buckwheat raw; LFD = Lentil FODMAPs enzymatically degraded; LFB = Low FODMAP bread; HFB = High FODMAP Bread; PsyB = Psyllium added bread; LFP = Low FODMAP pasta; HFP = High FODMAP pasta; I2uB = Inulinase treated bread. Raw lentil, malted lentil, malted buckwheat, high FODMAP bread and high FODMAP pasta include separate fermentation reactions where *Lactobacillus* strains are seeded with the reaction prior to fermentation.

Table 1 Foods high in FODMAP content and their low FODMAP counterparts. Each low FODMAP food in the right column was compared to its similar high FODMAP food type in the left column. DM refers to dry matter. FODMAP content refers to the concentration of FODMAPs prior to in vitro digestion and ex vivo fermentation took place.

Foods higher in FODMAP content	Total FODMAP content	Lower FODMAP replacement foods	Total FODMAP content
High FODMAP bread	1.56 g / 100 g DM	Low FODMAP bread Psyllium added bread Inulinase treated bread	<0.005 g / 100 g DM 0.056 g / 100 g DM 0.19 g / 100 g DM
High FODMAP pasta	Undetermined	Low FODMAP pasta	Undetermined
Raw lentil	3.89 g / 100 g DM	Malted lentil Enzymatically degraded lentil	0.84 g / 100 g DM
Raw Buckwheat	0.33 g / 100 g GM	Malted buckwheat	<0.005 g / 100 g

5.3.3 | Growth of *Lactobacillus* strains

Lactobacillus rhamnosus DPC6071, *Lactobacillus casei* DPC6072 and *Lactobacillus casei* DPC6083 were isolated and cultured from dairy cheese samples originating in Sicily [31]. These three strains effectively fermented eleven FODMAP carbohydrates from a group of 32 strains isolated from GIT of neonates, adults [32] and other dairy cheese strains [31]. The three strains were freeze dried individually at a concentration of 1 X 10^{10} in 1ml of 15% trehalose solution. The strains were mixed after reconstitution with sterile H_2O. These three strains exhibited resistance to simulated gastric fluids, no virulence factors, adherence to intestinal cells, viability after freeze drying and no gas production *in vitro* (Chapter 2), properties that are desirable as potentially probiotic traits. These *Lactobacillus* strains were routinely cultured in an anaerobic environment (10% H2, 10% CO2 and 80% N2) for 24 hours at 37 ° C.

5.3.4 | Donor Recruitment

Donor recruitment for donation of stool samples and enrolment were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. All donors completed a questionnaire demonstrating their willingness to participate in the study. All donors were healthy adults (23–28 years) who had not taken any antibiotic or probiotics for 6 months prior to donating a faecal sample.

5.3.5 | Preparation of Frozen Standardised Inoculum (FSI) and fermentation media

The Frozen Standardised Inoculum (FSI) was prepared as previously described [33]. Six donor faecal samples were prepared in an anaerobic environment. This process included combining a fixed amount of each sample and filtering them through a stomacher bag after the addition of phosphate buffered saline (PBS). Glycerol was added to produce a 25% glycerol concentration and the FSI sample was stored at -80 ° C. Fermentation media was prepared according to Fooks et al. [34], however, the food

digestate replaced the glucose carbohydrate source. This food retentate was added at 2% (w/v) directly to the bioreactor instead of the media.

5.3.6 | MicroMatrix Cassette Preparation

Faecal fermentation attempts to simulate the environment within the large intestine where gut microbes ferment the compounds present within the lumen. The Micro-Matrix (Applikon Biotechnology, Heertjeslaan 2, 2629 JG Delft, Netherlands) cassette was set up as described [29] with minor alterations. Each sample had six replicates (n=6) and the initial medium volume was 5ml per well. Digested and dialysed food prototype retentates were added to each well at a concentration of 2% (w/v). The run parameters and sampling schedule previously described [29] were also used with minor changes. The low limit for pH (the limit that induces the addition of NH_3) was changed to pH 5.5 from pH 6.6 in the original protocol. *Lactobacillus* strains were added to a number of wells at an average concentration of 3.67×10^8. This concentration equated to approximately 5% of total bacterial abundance present in the well prior to fermentation. The fermentation reaction was carried out for 12 hours.

5.3.7 | Repeated bead beating DNA extraction

DNA was extracted and purified according to the RBB+C method developed by Yu et al. [35]. Briefly, samples were added to a 2ml screw cap tube with zirconium beads of size 0.1mm and 1.0mm (Biospec products), 1ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl (pH 8), 50 mM EDTA, 4 % (w/v) SDS) and prepared according to the protocol described and homogenised at max speed on a Mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA) for 3 minutes. DNA was extracted using the DNA Fast Stool DNA extraction kit (Qiagen, Hilton, Germany). The DNA was eluted in 100µl of molecular grade water (Sigma, Germany).

5.3.8 | MiSeq Compositional DNA sequencing

DNA was quantified using the Qubit High Sensitivity Kit (Life Technologies, Carlsbad, CA, USA), standardised and then used as a template for PCR. 16S metagenomic libraries were prepared using primers to amplify the V3–V4 region of the bacterial 16S rRNA gene, with Illumina adaptors incorporated as described in the Illumina 16S Metagenomic Library Preparation guide. Following index PCR and purification using AMPure XP magnetic beads (Beckman Coulter), the products were quantified using the Qubit high sensitivity DNA kit (Life Technologies) and pooled equimolarly. Libraries were then sequenced (2 × 300 bp) on the Illumina MiSeq platform.

5.3.9 | SCFA Standard Curve and internal standard

Acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, valeric acid, and 2-ethyl-butyric acid (internal standard, IS) were purchased from Sigma Aldrich (Sigma, Germany). A 100mM stock solution of 2-ethylbutyric acid in formic acid was diluted to 10mM in Milli-Q water and was stored at -20°C. An external standard solution of acetate (20mM), propionate (20mM), Iso-butyrate (2mM), butyrate (20Mm), Iso-valerate (2mM) and valerate (20mM) in water was generated and stored at -20°C. A 7 point standard curve was produced (SCFA 0.1mM, 0.5mM, 1mM, 2mM, 4mM, 8mM & 10mM; BCFA 0.01mM, 0.05mM, 0.1mM, 0.2mM, 0.4mM, 0.8mM & 1mM) in Milli-Q water with 1mM IS.

5.3.10 | GC-FID analysis

Standards and samples were analysed by gas chromatography flame ionisation detection (GC-FID) using a Varian 3800 GC system, fitted with an Agilent DB-FFAP column (30 m L x 0.32mm ID x 0.25µm df; Phenomenex) and a flame ionisation detector with a CP-8400 autosampler. Helium was employed as the carrier gas at an initial flow rate of 1.3 mL/min. The initial oven temperature was 50°C, and was maintained for 0.5 min, raised to 140°C at 10°C/minute and held for 0.5 min, before being increased to 240°C at 20°C/min, and held for 5.0 minutes (total run time 20 minutes). The temperatures of the detector and the injection port were 300°C and 240°C, respectively. A splitless injection of 0.2µL was carried out for each sample or standard using a 10µL syringe (Aglient) installed to a CP-8400 autosampler (Varian). Peaks were integrated using Varian Star Chromatography Workstation version 6.0 software. Blank water vials were placed between each sample duplicates to detect potential carryover.

5.3.11 | Data Analysis

Microbiota compositional changes were analysed using the web-server and R based software, Calypso (version 8.84) [36]. Linear discriminant analysis effect size (LEfSe) was used to identify the signature taxa that were associated with each substrate [37]. Statistical changes in composition were also assessed using Analysis of Composition of Microbiomes (ANCOM) [38]. Alpha and beta diversity were determined at the OTU level using raw reads. Alpha diversity was compared using Simpson's index, Shannon's index, Chao1 and Richness. Beta diversity was measured using RDA, CCA and PCoA plots to illustrate the percentage of variability explained by variables. All data analysis was performed using R through the Calypso platform.

5.4 | **Results**

5.4.1 | Impact of lentil and buckwheat fermentation on the human gut microbiome

Raw lentil (3.89 g FODMAP / 100 g) was compared to its lower FODMAP counterparts of malted lentil (0.84 g FODMAP / 100 g) and FODMAP degraded lentils following fermentation in the presence of a human microbiome. Similarly, raw buckwheat (0.33 g FODMAP / 100 g) and malted buckwheat (<0.005 g FODMAP / 100 g) were compared after fermentation in the presence of the microbiome to understand if fagopyritols elicited microbial fermentation. These comparisons explore the changes in alpha diversity, beta diversity and the composition of the microbiome after fermentation of low and high FODMAP foods of lentil or buckwheat.

Microbiome alpha diversity after fermentation in the presence of lentils and buckwheat.

The microbiome alpha diversity in each reaction was determined using four alpha diversity metrics following faecal fermentation in the presence of raw lentils, malted lentils, enzymatically degraded lentils, raw buckwheat and malted buckwheat. Fermentation of raw lentil showed a similar microbiome alpha diversity compared to the microbiome after fermentation of both malted lentil and FODMAP degraded lentils. No significant differences were observed in microbiome alpha diversity after fermentation of raw buckwheat and malted buckwheat. Despite a difference of 3 g FODMAP / 100 g dry matter between raw and malted lentil, and of 0.3 g FODMAP / 100g dry matter between raw and malted buckwheat, no significant difference in alpha diversity were observed (Figure 1).

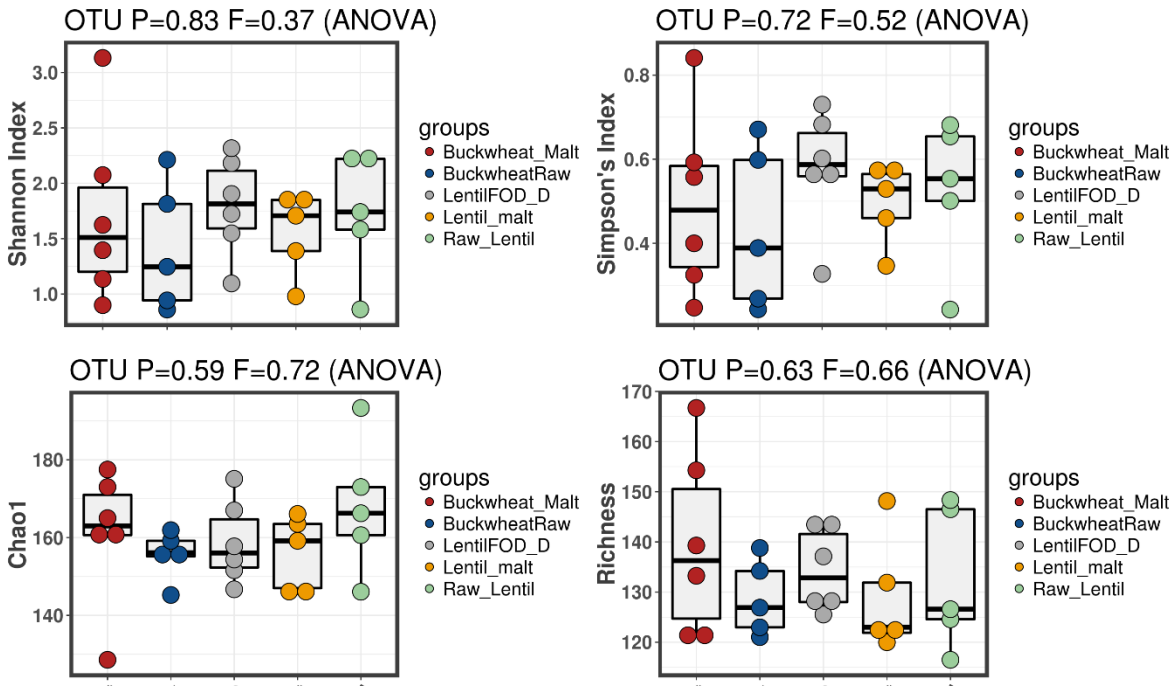

Figure 1 Four measurements of alpha diversity were used to determine the impact of substrate fermentation on microbial diversity. Fermentation of raw lentil, malted lentil and enzymatically degraded lentils [27] show no difference in microbiome diversity. Similarly, reactions fermented in the presence of raw and malted buckwheat result in no significant change in alpha diversity.

Microbiome beta diversity is unaffected after fermentation of lentil and buckwheat foods

Redundancy analysis (RDA) is a measure of beta diversity that attempts to explain the raw compositional data that describes the OTU abundance in each sample [39]. Analysis using RDA revealed no significant differences after the fermentation of raw lentil compared to the two lower FODMAP lentils. Similarly, raw buckwheat and malted buckwheat fermentation did not appear to affect RDA measured beta diversity (Figure 2).

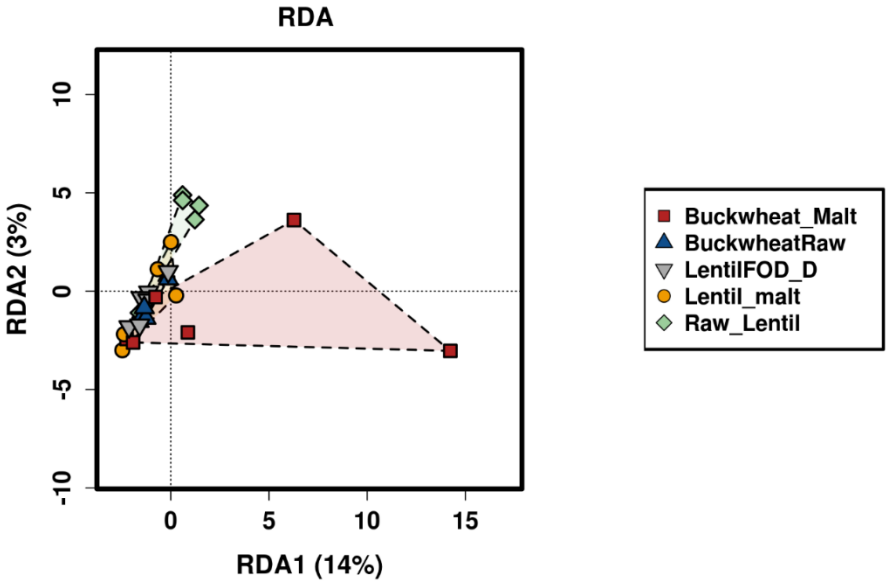

Figure 2 Beta diversity as measured by RDA. Analysis of fermentation reactions with three lentil varieties and two buckwheat varieties reported no significant differences in beta diversity.

Canonical correspondence analysis (CCA), similarly to RDA, explains the diversity in OTU composition between samples [39]. CCA analysis revealed no significant changes in microbiome beta diversity after fermentation of raw lentil compared to fermentation of both malted lentil and FODMAP degraded lentils (Figure 3). Fermentation of raw buckwheat does not cause a significant shift in beta diversity compared to malted buckwheat fermentation.

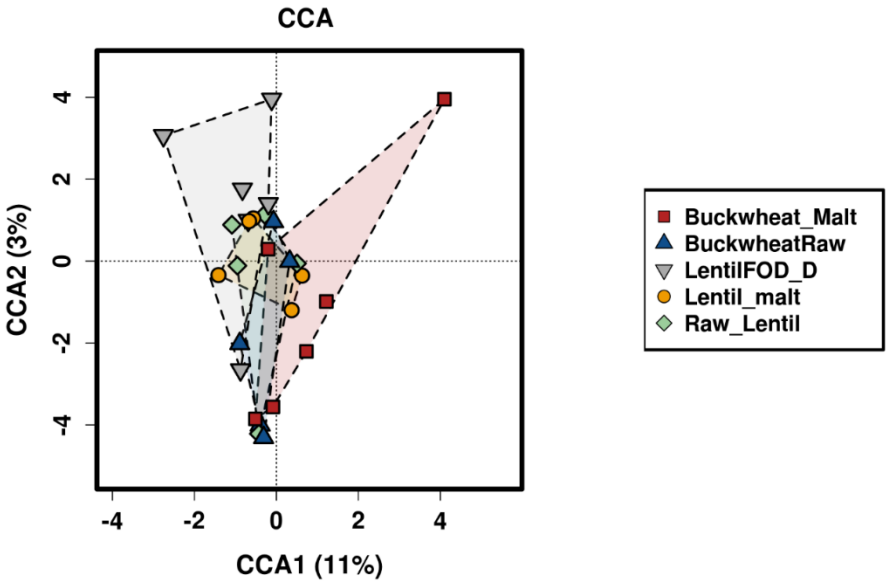

Figure 3 Beta diversity as measured by CCA. Fermentation reactions with three lentil and two buckwheat varieties show no significant differences in beta diversity in the microbiome.

Principal Component Analysis (PCoA) explains the variation of the raw compositional data, rather than the raw data itself [39]. Based on PCoA analysis, the variation in the raw compositional data is not explained by the FODMAP content of any substrate (Figure 4).

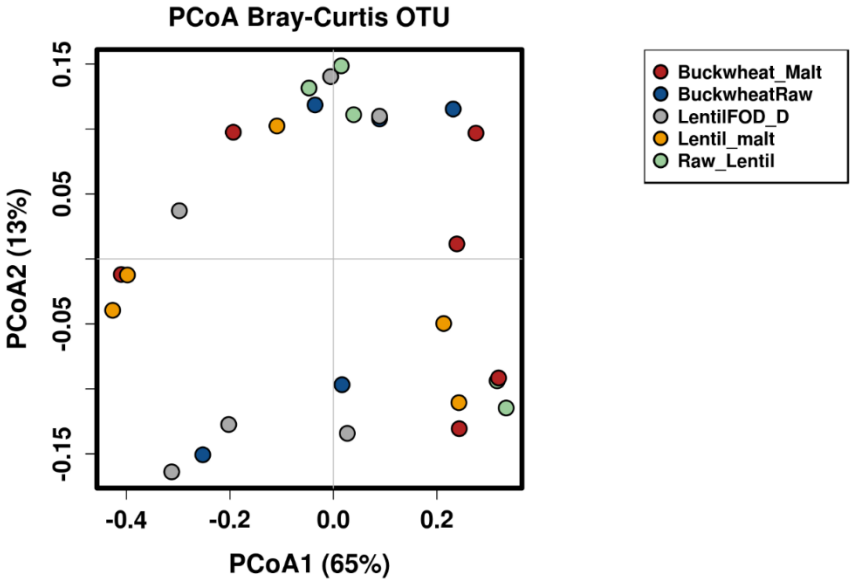

Figure 4 PCoA illustrates the variability of beta diversity between samples. Sample from three lentil and two buckwheat food types are randomly distributed throughout the plot, indicating that the substrate is not modulating beta diversity.

Microbiome composition was altered as a result of substrate fermentation

LDA effect size (LEfSe) analysis was used to determine the genera that were significantly associated with the fermentation of each of the five substrates. Fermentation of malted buckwheat and FODMAP degraded lentil were the only two substrates that were associated with significantly increased genera. *Eubacterium ventriosum* and *Ruminiclostridium 9* were increased in abundance following the 12-hour fermentation in the presence of malted buckwheat (LDA scores of 4.037 and 3.916, respectively). Fermentation of FODMAP degraded lentils was associated with an increase in Megasphaera and Enterococcus (LDA score 4.402 and 3.322 score, respectively) following fermentation.

ANCOM analysis describes the changes in taxonomic groups while statistically accounting for the many zeroes present in compositional data [38]. ANCOM reported differences between groups in the genus Ocilobacter (Figure 5). However, the difference in abundance is between fermentation of raw buckwheat and raw lentil or raw buckwheat and FODMAP degraded lentils (mean abundance 0.014%, 0.0021% and 0.0011%, respectively). Fermentation of malted lentil did not result in any changes in microbial group compared to fermentation of raw lentil, despite its reduced FODMAP content.

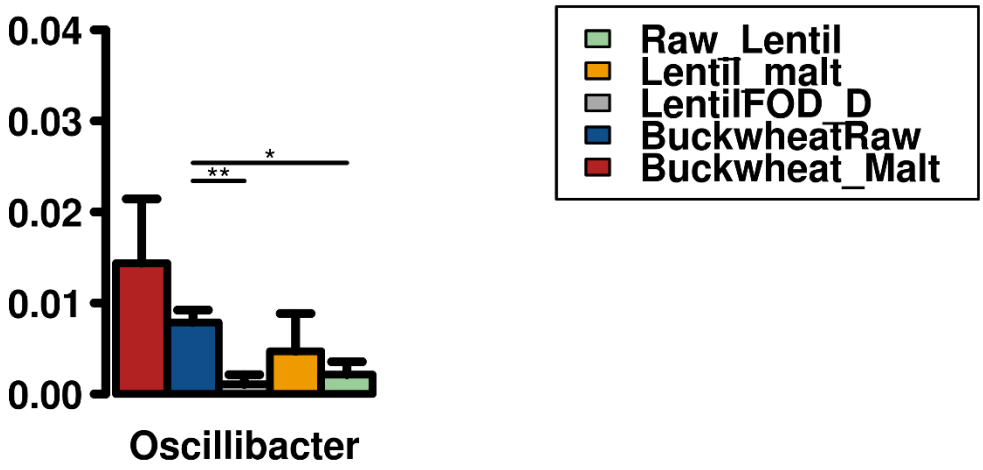

Figure 5 Changes in Oscillibacter abundance after fermentation in the presence of lentil and buckwheat food varieties. Raw buckwheat fermentations show a significantly increased abundance of Oscillibacter compared to both lentil FODMAP degraded and raw lentil fermentations, as determined by ANCOM.

Fermentation of lentil and buckwheat varieties resulted in no changes in SCFA production

SCFAs are often the end products of carbohydrate fermentation within the microbiome. Following fermentation in the presence of raw compared with malted lentil substrates, no significant differences in SCFAs were observed after fermentation, despite the increased FODMAP content in raw lentil compared to malted lentils. Furthermore, fermentation of raw buckwheat did not cause a change in SCFA production compared to fermentation of malted buckwheat (Figure 6). These results suggest that none of the substrates altered the hydrogen production profile following fermentation.

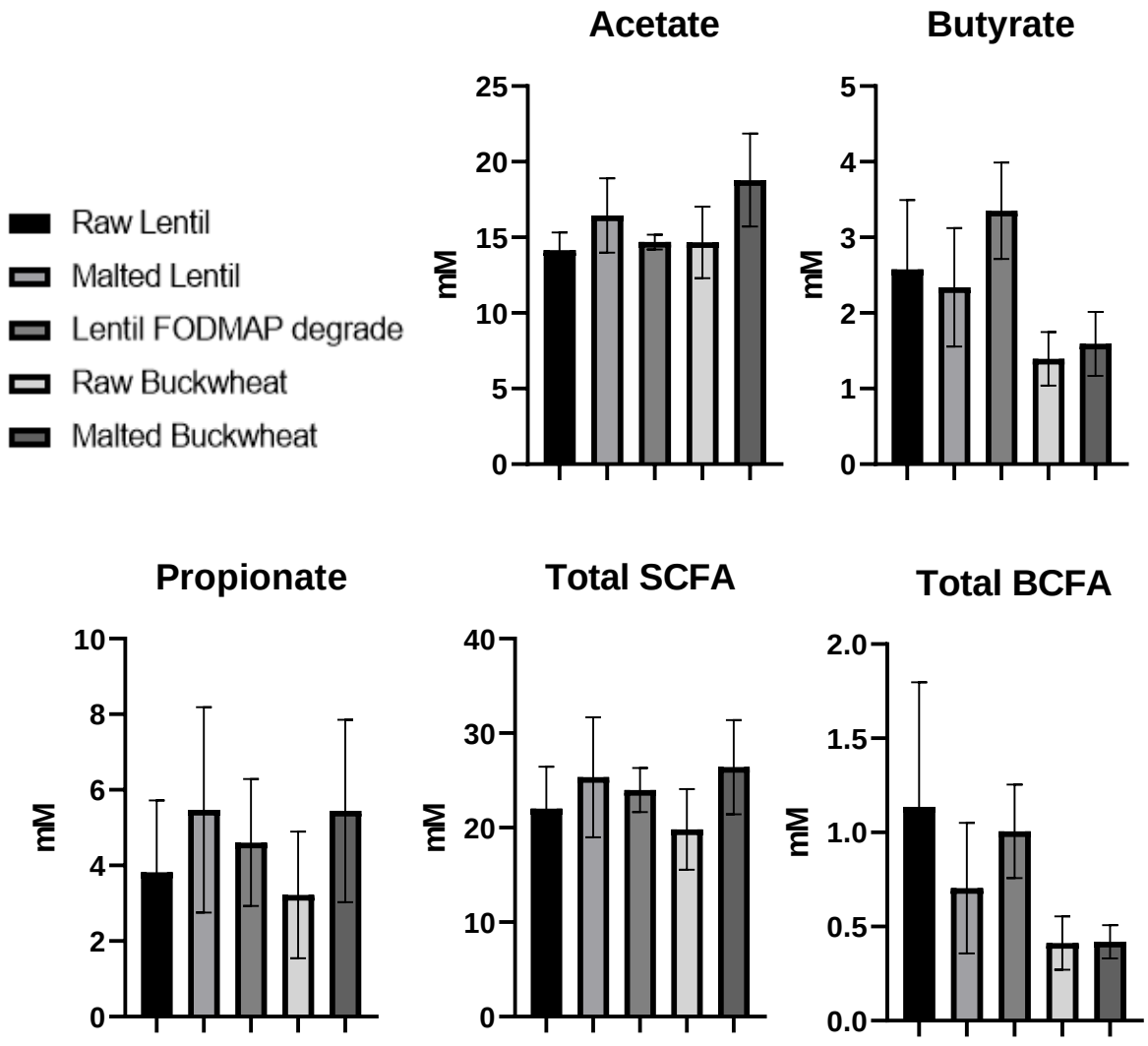

Figure 6. SCFA concentration post fermentation of lentil and buckwheat varieties. Total SCFA = acetate + butyrate + propionate + valerate +isobutyrate + isovalerate. Total BCFA = isobutyrate + isovalerate. No significant changes were reported between fermentations seeded with lentil and buckwheat substrates. Values plotted as mean ± SEM (p-value <0.05).*

5.4.2 | Fermentation in the presence of a high FODMAP bread substrate, a high FODMAP pasta substrate and their low FODMAP alternatives resulted in modest changes to the diversity and composition of the microbiome

High FODMAP bread (1.56 g / 100 g DM), low FODMAP bread (<0.005 g / 100 g DM), psyllium added bread (0.056 g / 100 g DM), inulinase treated bread (0.19 g / 100 g DM), high FODMAP pasta (undetermined FODMAP content) and a low FODMAP pasta (undetermined FODMAP content) were fermented to examine their influence on the human microbiome. The microbiome after the fermentation of three breads of lower FODMAP content, low FODMAP bread, inulinase treated bread and psyllium added bread, were compared to the microbiome after fermentation of high FODMAP bread. Similarly, the microbiome after fermentation of high FODMAP pasta was compared to the microbiome after fermentation of low FODMAP pasta. Comparing these microbial profiles revealed any changes in the alpha diversity, beta diversity, microbial composition and SCFA production caused by the lower FODMAP foods.

Microbiome alpha diversity after fermentation in the presence of bread and pasta prototypes.

The diversity of OTUs in each reaction after fermentation was measured using four alpha diversity metrics: Simpson index, Shannon index, Chao1 and Richness. The alpha diversity of the microbiome following fermentation in the presence of high FODMAP bread, low FODMAP bread, psyllium added bread, inulinase treated bread, high FODMAP pasta and low FODMAP pasta was assessed. No significant differences in microbiome alpha diversity were observed after fermentation in the presence of high FODMAP bread compared to fermentation in the presence of three lower FODMAP breads. Furthermore, fermentation in the presence of high FODMAP pasta did not result in altered microbiome alpha diversity compared to low FODMAP pasta (Figure 7). These results suggest that reducing FODMAPs in bread and pasta does not negatively affect microbiota alpha diversity.

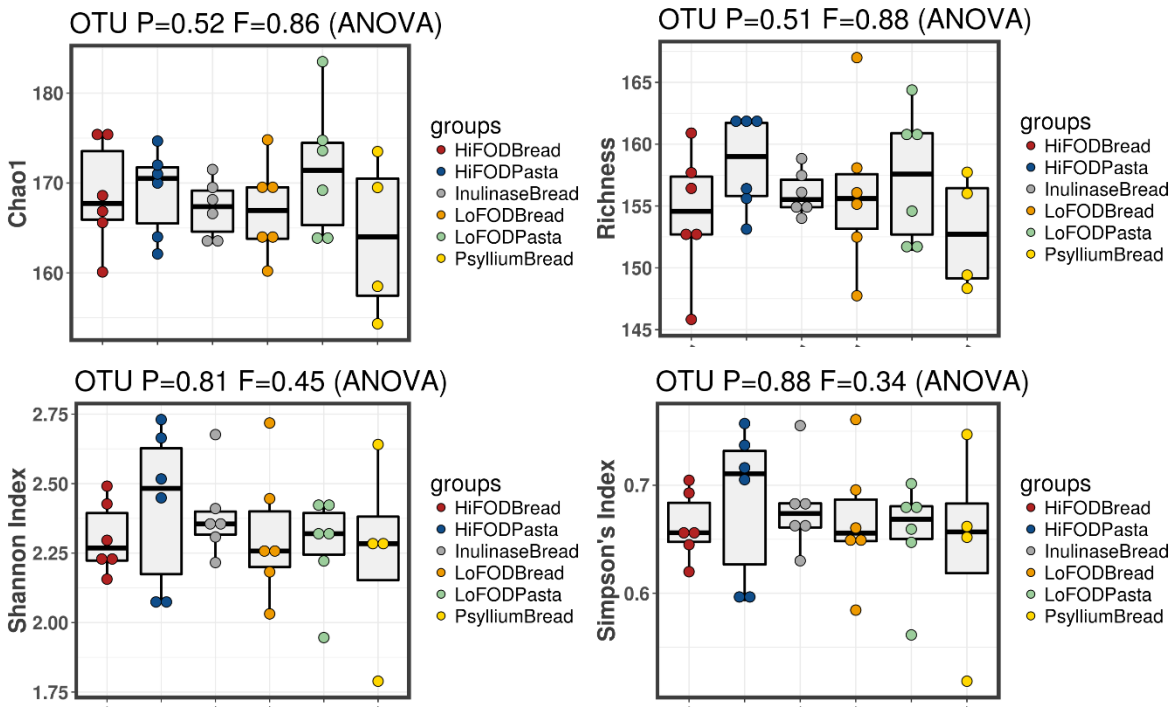

Figure 7 Alpha diversity of the microbiome following fermentation of four bread and two pasta varieties as measured by each metric. No significant differences were revealed between these substrate fermentations. Alpha diversity measured by four metrics: Simpson's index, Shannon index, Richness and Chao1.

Microbiome beta diversity remained stable after fermentation in the presence of bread and pasta prototypes.

RDA and CCA was used to explain to beta diversity of the raw compositional data, whereas the variability of this diversity was analysed using PCoA. No significant differences in microbial beta diversity were observed following fermentation in the presence of high FODMAP bread and all three low FODMAP breads, as determined by RDA (Figure 8). Comparing the microbiome after fermentation in the presence of low FODMAP pasta and high FODMAP pasta revealed no change in beta diversity.

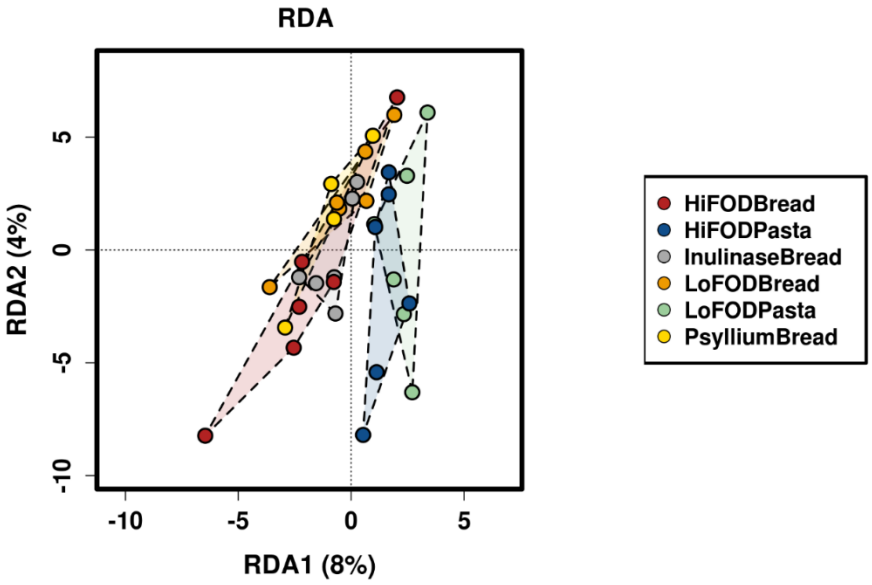

Figure 8 RDA measured beta diversity of the microbiome after fermentation of bread and pasta varieties. No significant differences were observed between groups.

Similarly, beta diversity as measured by CCA revealed no significant changes in microbiome composition after fermentation in the presence of each bread and pasta type (Figure 9). These results indicate that the FODMAP content of bread and pasta does not affect microbial beta diversity after a 12-hour fermentation.

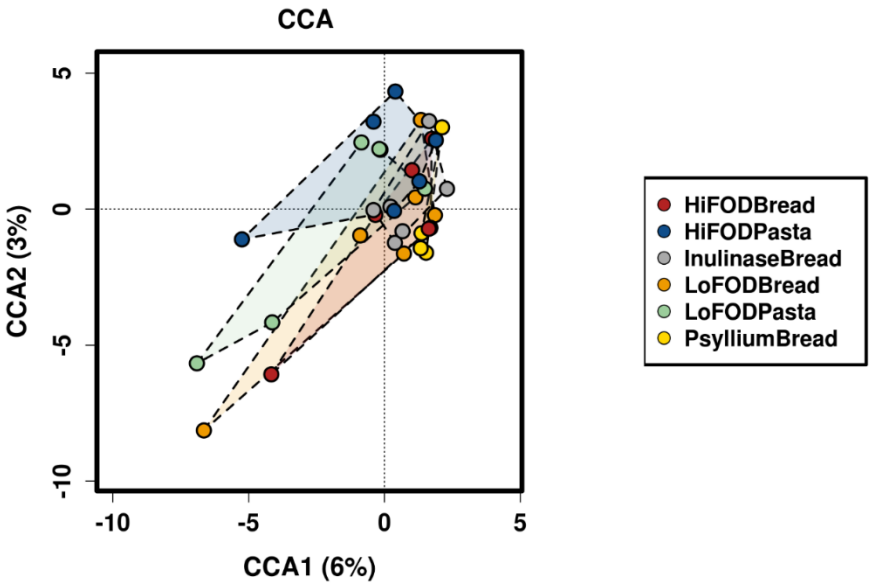

Figure 9 CCA measured microbiome beta diversity of bread and pasta varieties after fermentation. No significant differences were observed after 12-hour fermentation in the presence of each substrate.

The variation in microbiome beta diversity after each fermentation was analysed using PCoA. Fermentation in the presence of bread and pasta substrates with different FODMAPs content did not

cause a significant shift in beta diversity in the post fermentation microbiome. PCoA analysis did not result in groups of each substrate separating together, indicating that the PCoA1 and PCoA2 axis are not explaining differences in FODMAP content (Figure 10). No significant changes in microbiome beta diversity were measured between these six substrates analysed post fermentation.

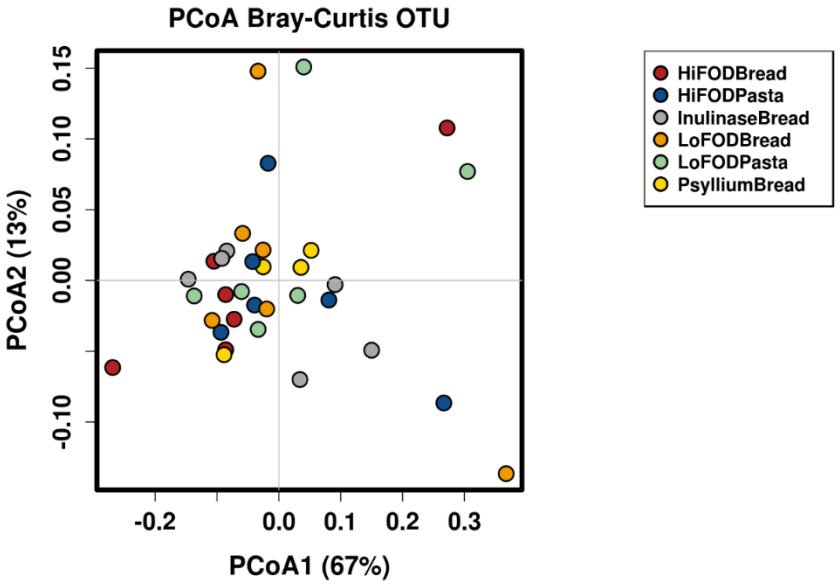

Figure 10 Beta diversity variability was measured using PCoA. Post fermentation samples analysed do not group according to the substrate that was present within the reaction. This indicates that low FODMAP bread and pasta variants have a similar impact on the microbiome compared to the high FODMAP variants.

Analysis of microbial taxa reveal nuanced shifts in response to fermentation of bread and pasta substrates

ANCOM compositional analysis was used to determine the different compositional changes that each substrate elicited, if any were present. Fermentation in the presence of both pasta substrates resulted in significantly increased abundance of *Butyricicoccus* and signifcantly reduced abundance of *Enterococcus* (Figure 11). These changes in abundance appear to be independent of FODMAP content and are potentially caused by fermentation of other components in the bread and pasta foods.

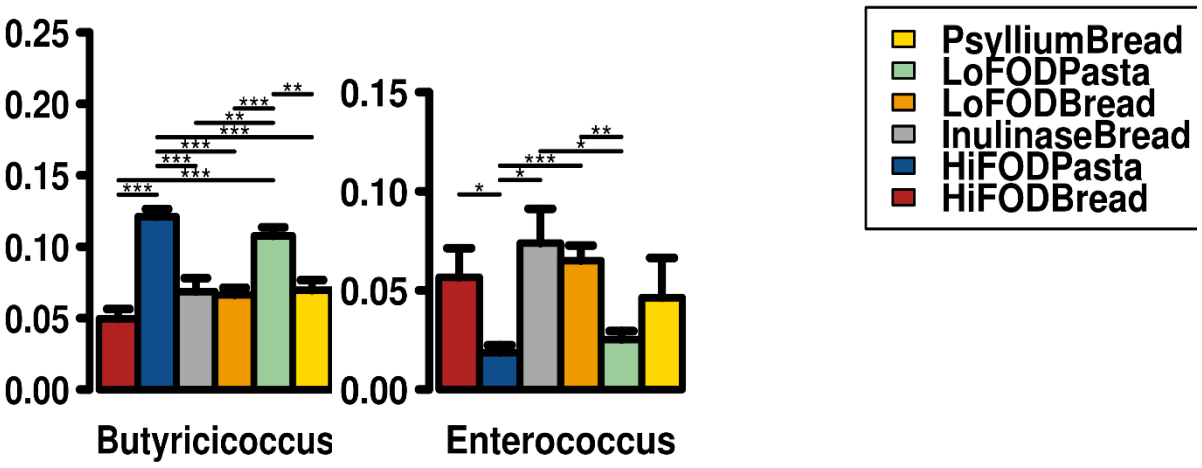

Figure 11 Alteration in Butyricoccus and Enterococcus abundance after fermentation of bread and pasta food varieties as determined by ANCOM. Fermentation of the FODMAP content does not appear to elicit these changes.

LEfSe genus level analysis reveals a number of differentially abundant microbial taxa after fermentation in the presence of each bread and pasta substrate. Fermentation in the presence of high FODMAP bread was associated with increased *Dorea* abundance and fermentation of low FODMAP bread was associated with increased *Libanicoccus* abundance. *Lachnospira* and *Butyricicoccus* were increased in abundance following fermentation in the presence of high FODMAP pasta whereas *Tyzzerella* 3 was associated with fermentation of low FODMAP pasta. Fermentation of psyllium bread elicited an increased abundance of *Anaerostipes* and *Lachnospriraceae* UCG004. Finally, fermentation reactions containing inulinase added bread were associated with increased *Enterococcus* abundance.

SCFAs do not differ significantly after fermentation in the presence of bread and pasta variants.

Similar to the lentil and buckwheat fermentation results reported above, SCFA concentrations did not differ significantly after fermentation of the six distinct bread and pasta variants. Fermentation of high FODMAP bread and high FODMAP pasta did not result in increased SCFA production compared to their lower FODMAP counterparts. A significant difference in total branched chain fatty acids (BCFA) was found after fermentation in the presence of high FODMAP bread compared to fermentation of inulinase added bread (p value < 0.01), and fermentation of low FODMAP bread compared to fermentation of inulinase added bread (p value < 0.05) (Figure 12).

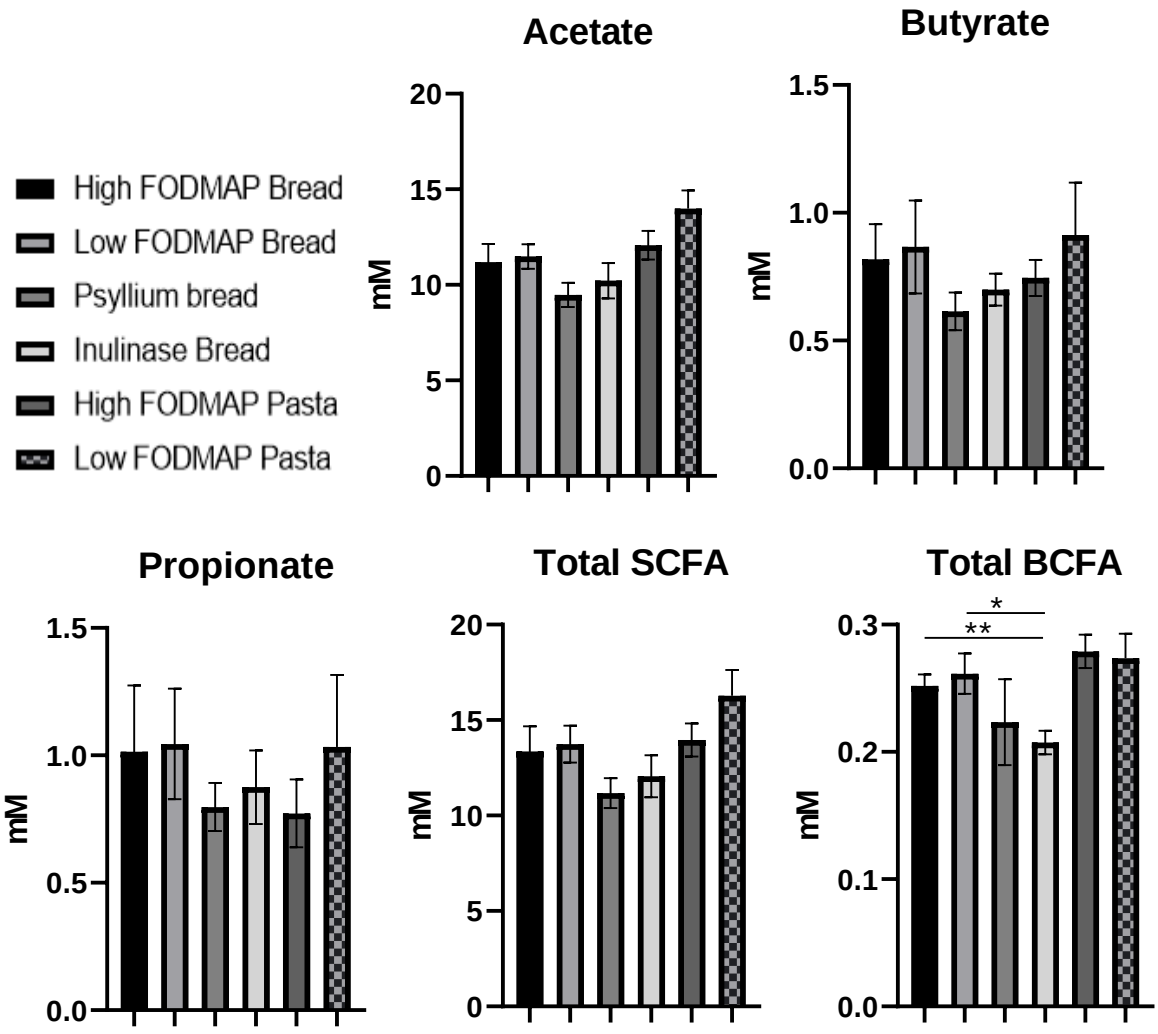

Figure 12 SCFA concentration post fermentation of bread and pasta varieties. Total BCFAs were reduced after fermentation with inulinase added bread when compared to fermentation with high FODMAP and low FODMAP bread. Total SCFA = acetate + butyrate + propionate + valerate +isobutyrate + isovalerate. Total BCFA = isobutyrate + isovalerate. T-test corrected for multiple testing was used to compare groups. Values plotted as mean ± SEM (p-value <0.05, ** p-value <0.01).*

5.4.3 | Substrate fermentation in the presence of *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 modifies the microbiome composition and metabolic output in *ex vivo* reactions

Lactobacillus rhamnosus DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 were added prior to fermentation in reactions with the following substrates: raw lentil, malted lentil, malted buckwheat, high FODMAP bread and high FODMAP pasta. The microbiome after fermentation of each of these substrates was compared to the microbiome after fermentation of the corresponding *Lactobacillus* seeded reaction. The study aims to understand the impact that these three *Lactobacillus* strains have on the microbiome and SCFA production during fermentation in the presence of these five substrates.

Fermentation of raw lentils, malted lentils and malted buckwheat in the presence of added *Lactobacillus* strains resulted in modest changes in the microbiome compared to non-*Lactobacillus* fermentations.

Alpha diversity, beta diversity and compositional changes were examined after fermentation in the presence and absence of the three *Lactobacillus* strains. Alpha diversity was not significantly different between the microbiome after fermentation of raw lentils and the microbiome after fermentation of raw lentil and added *Lactobacillus* strains. *Ex vivo* fermentation of raw lentils and *Lactobacillus* showed a shift in beta diversity compared to fermentation in the presence of only raw lentils (Chi square 0.08, F value 3.77, p value < 0.05) (Figure 13).

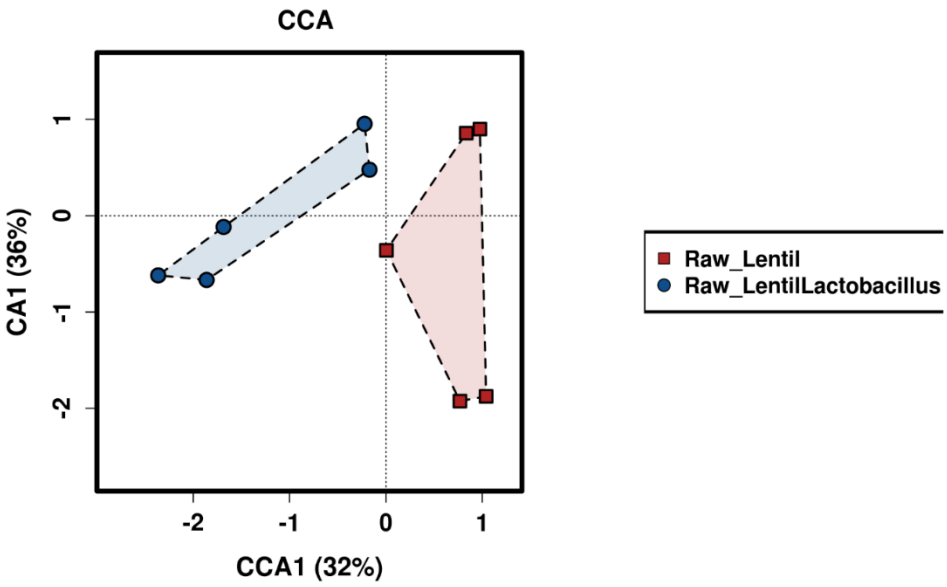

Figure 13 CCA measured beta diversity of bread and pasta varieties. A 12-hour fermentation of three Lactobacillus *strains and raw lentils result in an altered beta diversity compared to fermentation with raw lentils only.*

No significant difference in the microbiome after fermentation of raw lentils and *Lactobacillus* compared to raw lentils alone was observed when beta diversity was analysed using RDA (p values 0.053). Moreover**,** analysis with LEfSe revealed that *Clostridiaceae 1* was reduced while *Lactobacillaceae was* increased after fermentation of raw lentil and *Lactobacillus* compared to raw lentils alone (LDA score of 4.698 and 4.742, respectively).

Fermentation of malted lentils in the presence and absence of the *Lactobacillus* strains revealed no significant changes in alpha diversity, beta diversity and composition, with the exception of increased *Lactobacillus* abundance. Similarly, fermentation with malted buckwheat compared to fermentation of malted buckwheat and *Lactobacillus* revealed no changes to these same metrics, except for an increase in abundance of *Lactobacillus*. These results suggest that fermentation of malted substrates do not provide an environment in which *Lactobacillus* strains can alter microbiome diversity and composition.

Addition of *Lactobacillus* strains prior to the fermentation of high FODMAP bread and of high FODMAP pasta result in significant changes in the microbiome compared to these substrates alone.

Microbiome alpha diversity, beta diversity, microbial composition and SCFA production were analysed after the fermentation of high FODMAP bread, high FODMAP bread with *Lactobacillus*, high FODMAP pasta and high FODMAP pasta with *Lactobacillus* was performed. To understand the influence that these three strains had on substrate fermentation, both high FODMAP bread and high FODMAP pasta fermentations were compared to their *Lactobacillus* seeded reactions.

Fermentation of high FODMAP bread and *Lactobacillus* significantly decreased alpha diversity in the Simpson index (F value 16, p value 0.0025) and Shannon index (F value 20. P value 0.0012) compared to high FODMAP bread with no *Lactobacillus* strains added (Figure 14). Fermentation of high FODMAP pasta and *Lactobacillus* resulted in no change in alpha diversity compared to fermentation of high FODMAP pasta alone.

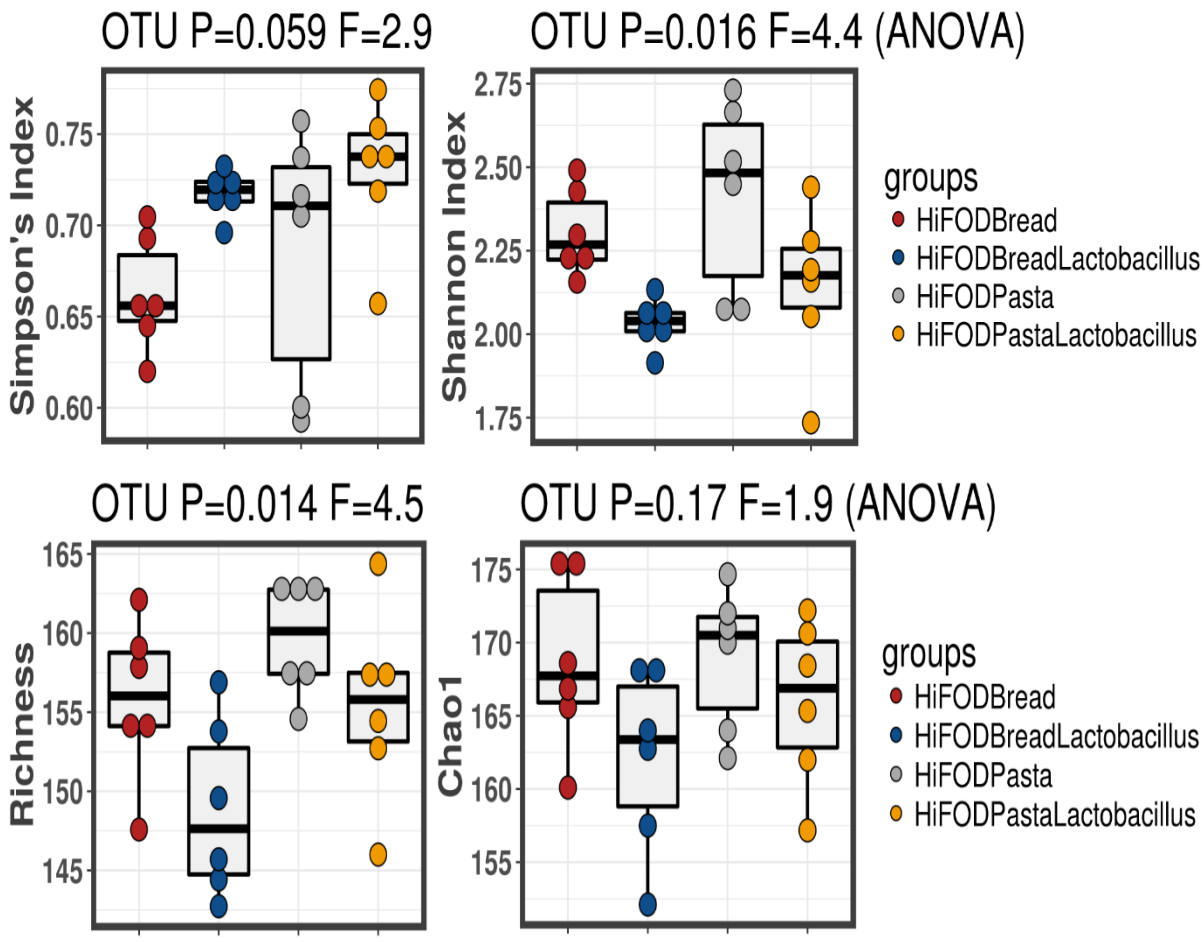

Figure 14 Microbiome alpha diversity after the fermentation of high FODMAP bread and pasta with Lactobacillus *and without* Lactobacillus*. Alpha diversity measured by four metrics: Simpson's index, Shannon index, Richness and Chao1.*

Microbiome beta diversity after each 12-hour fermentation was measured by RDA, CCA and PCoA. These measures indicate that *Lactobacillus* seeded fermentation of high FODMAP bread and high FODMAP pasta show significantly different microbiome diversity than reactions with those substrates alone. Microbiome beta diversity as measured by RDA reveals a significant difference between *Lactobacillus* seeded high FODMAP bread fermentations and high FODMAP bread fermentations alone (variance 56.88, F value 4.74, p value < 0.05). This difference was not observed in the

microbiome after fermentation of high FODMAP pasta compared to high FODMAP pasta with *Lactobacillus* added (variance 36.5, F value 2.62, p value 0.062) (Figure 15).

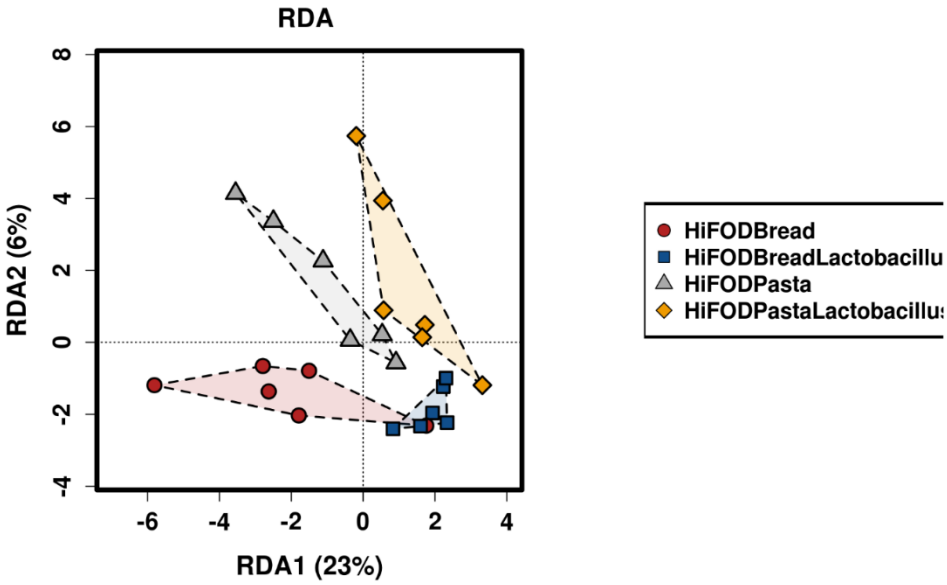

Figure 15 RDA was used to measure beta diversity after 12-hour fermentation in the microMatrix. The microbiome after fermentation of high FODMAP bread and pasta seeded with Lactobacillus *and those not seeded with* Lactobacillus *were compared. Significant differences in the microbiome between high FODMAP bread and high FODMAP bread seeded with* Lactobacillus *were observed.*

Microbiome beta-diversity, as measured by CCA, showed a significant difference after fermentation of both *Lactobacillus* and non-*Lactobacillus* seeded reactions. Beta diversity was significantly different after the fermentation of high FODMAP bread and high FODMAP bread seeded with *Lactobacillus* (Chi square 0.26, F value 35.72, p value < 0.01) and after fermentation of high FODMAP pasta and high FODMAP pasta seeded with *Lactobacillus* (Chi square 0.23, F value 26.13, p value < 0.01) (Figure 16). This result highlights that *Lactobacillus* addition prior to fermentation of these substrates causes a shift in beta diversity.

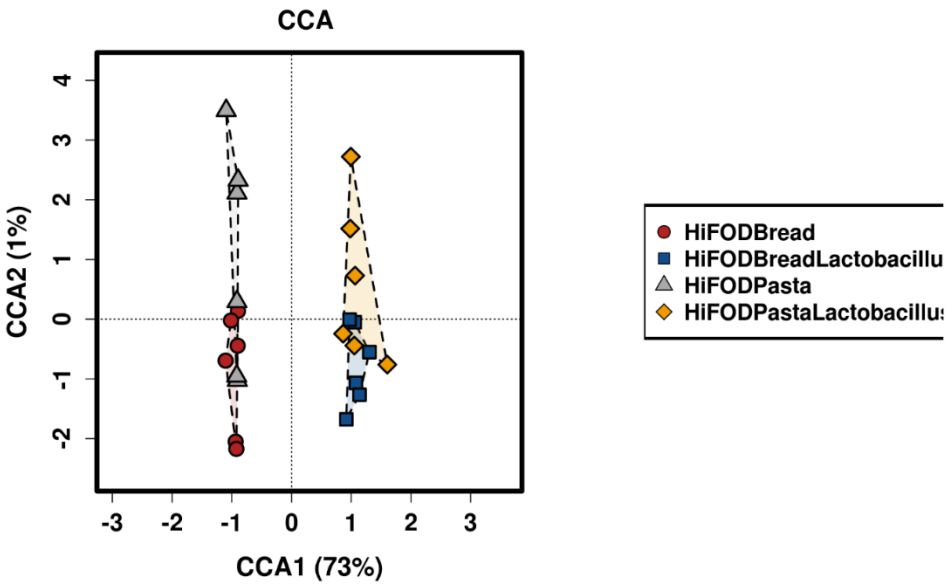

Figure 16. CCA was used to measure the microbiome beta diversity after fermentation in the presence of high FODMAP bread and pasta with and without Lactobacillus *seeded prior to the fermentation. High FODMAP bread and high FODMAP pasta seeded with* Lactobacillus *prior to fermentation elicited a unique shift in microbiome beta diversity compared to those reactions not seeded with* Lactobacillus*.*

The PCoA plot establishes a clear separation based on the presence or absence of *Lactobacillus* strains prior to fermentation with bread and pasta substrates (Figure 17). The microbiome of high FODMAP bread and high FODMAP pasta post fermentation were grouped together, whereas their counterparts that were seeded with *Lactobacillus* are grouped separately. These two groups are clearly divergent along the PCoA1 axis, which explains 72% of the total diversity present between all samples. This indicates that a substantial amount of the microbiome variation between the samples is explained by the addition of *Lactobacillus*.

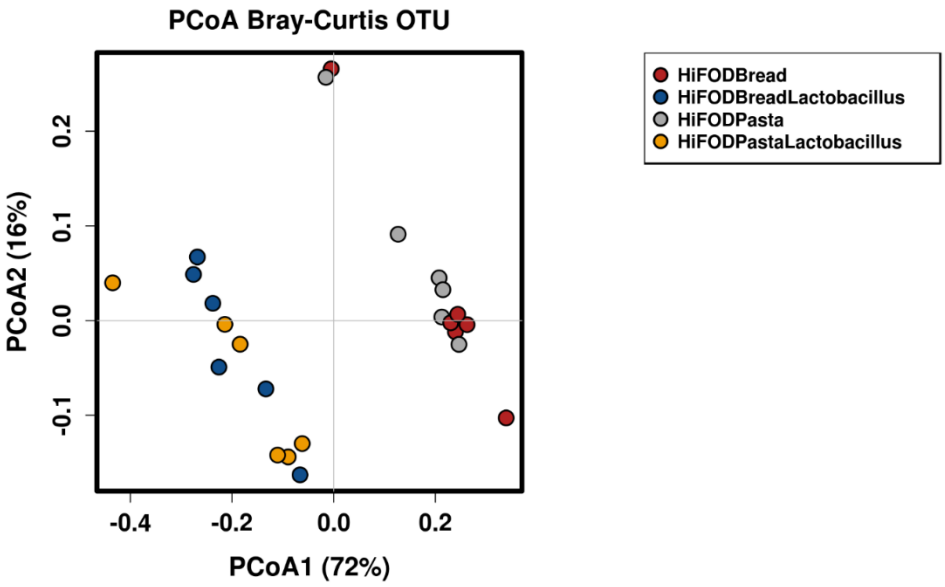

Figure 17. PCoA measures the variability of beta diversity within the microbiome after fermentation in the presence of bread and pasta varieties. The presence of added Lactobacillus *strains prior to fermentation results in a significantly altered beta diversity. The magnitude of this difference is captured on the PCoA1 axis that explains 72% of the total variation in beta diversity between all reactions.*

Compositional analysis of the microbiome after fermentation of *Lactobacillus* seeded reactions reveal significant changes in the abundance of microbial taxa in comparison to reactions with no *Lactobacillus* added. Analysis of the microbiome using ANCOM reveals that fermentations in the presence of high FODMAP bread and *Lactobacillus* have significantly increased *Lactobacillus* (438 fold change, p value <0.001) and a decreased *Anaerostipes* (17 fold change, p value < 0.001) and Clostridium sensu stricto 13 (5500 fold change, p value < 0.05) compared to high FODMAP bread alone. Fermentation in the presence of high FODMAP pasta and *Lactobacillus* reveal an increased abundance of *Lactobacillus* (418 fold change, p value < 0.001) and decreased abundance of *Clostridium sensu stricto 13* (993 fold change, p value < 0.05) compared to fermentation of high FODMAP pasta alone.

Microbiome compositional analysis using LEfSe revealed changes to a broader number of taxonomic groups as a result of adding *Lactobacillus* to the reaction prior to fermentation. These changes are best represented at phylum level. Firmicutes were increased (LDA score 5.09) after fermentation in the presence of high FODMAP bread, whereas Actinobacteria (LDA score 4.52), Bacteroidetes (LDA score 4.36), Verrucomicrobia (LDA score 4.53) and Proteobacteria (LDA score 5.02) were significantly decreased compared to fermentation of high FODMAP bread alone. Similar changes were observed in fermentation reactions in the presence of high FODMAP pasta and *Lactobacillus*. Reactions seeded with *Lactobacillus* prior to fermentation resulted in an increase in abundance of Firmicutes (LDA score 5.10) and decreased abundance of Actinobacteria (LDA score 4.31), Verrucomicrobia (LDA score 4.18)

and Proteobacteria (LDA score 4.99) after fermentation compared to high FODMAP pasta alone. These changes illustrate a substantial rearrangement in microbiome composition at the Phylum level after fermentation in the presence of the three *Lactobacillus* strains. Fermentation in the presence of both high FODMAP bread with *Lactobacillus* and high FODMAP pasta with *Lactobacillus* revealed a common reduction in the abundance of *Bifidobacterium*, *Clostridium sensu stricto 1*, *Corprococcus 3*, *Blautia*, *Lachnospiraceae UCG008*, *Lachnospiraceae UCG004*, *Ruminococcus torques* and *Dorea* compared to fermentation reactions without the addition of *Lactobacillus*. The reduction of these carbohydrate fermenting genera indicate that the *Lactobacillus* strains may be performing carbohydrate degradation during the fermentation instead of them, thus removing their niche and reducing their abundance.

Seeding *Lactobacillus* strains prior to the fermentation of raw lentils, malted lentils and malted buckwheat caused no significant changes to SCFA production compared to their non-*Lactobacillus* counterparts.

Raw lentils, malted lentils and malted buckwheat were fermented in the presence and absence of three *Lactobacillus* strains. SCFA production did not differ after fermentation of lentils and buckwheat in the presence or absence of *Lactobacillus* strains (Figure 18). These data are similar to the diversity and compositional data reported previously, as no significant differences were observed in the microbiome between *Lactobacillus* seeded and non-*Lactobacillus* seeded lentil and buckwheat fermentations.

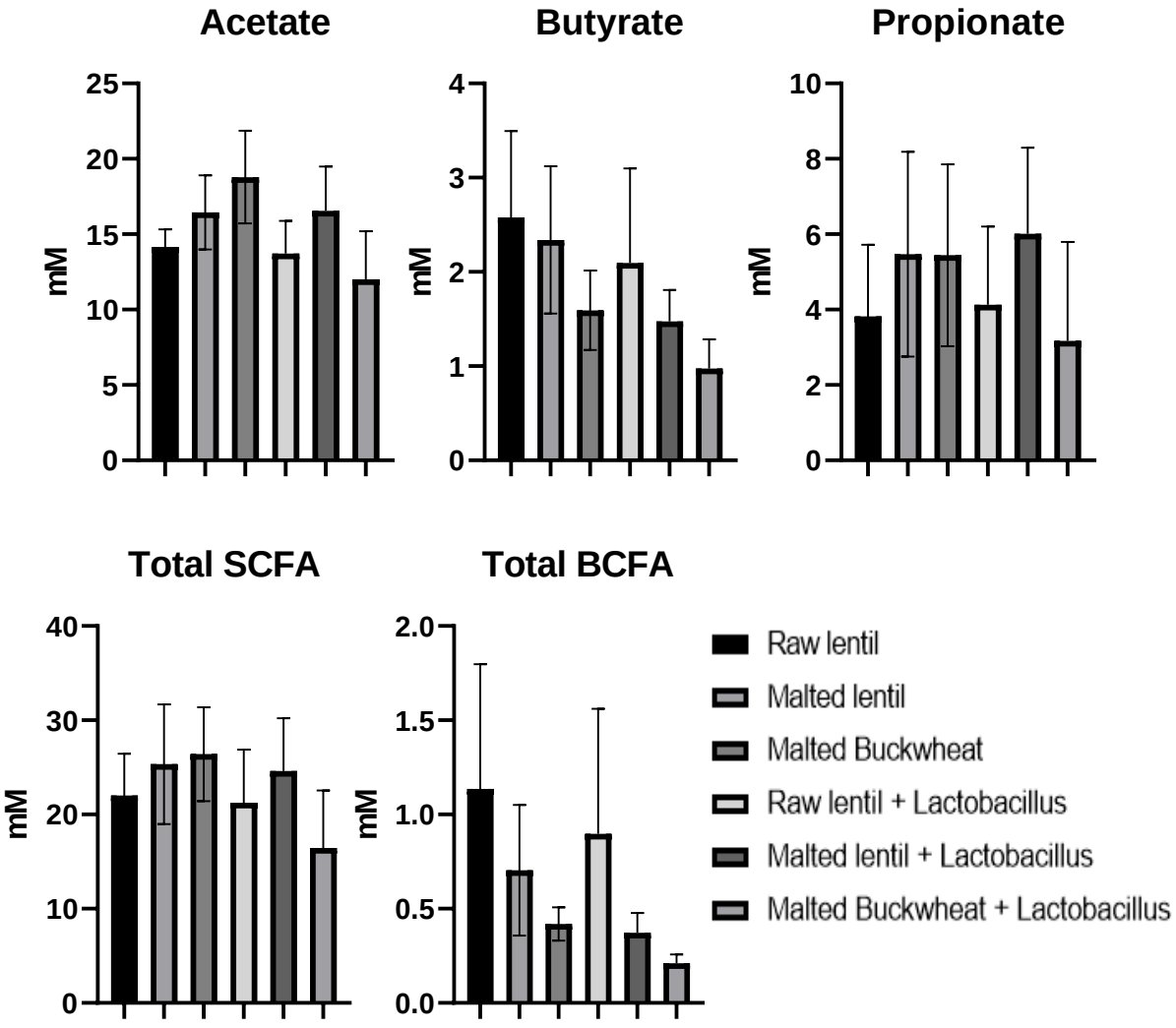

Figure 18 SCFA concentration after fermentation of lentil and buckwheat varieties in the presence and absence of Lactobacillus *strains. Total SCFA = acetate + butyrate + propionate + valerate + isobutyrate + isovalerate. Total BCFA = isobutyrate + isovalerate. T-test corrected for multiple testing was used to compare groups. Values plotted as mean ± SEM (* p-value <0.05, ** p-value <0.01).*

Fermentation in the presence of high FODMAP bread and high FODMAP pasta with *Lactobacillus* strains result altered SCFA profile compared to non-*Lactobacillus* seeded reactions.

Interestingly, fermentation of high FODMAP bread and high FODMAP pasta substrates in the presence or absence of *Lactobacillus* strains resulted in changes in the SCFA profile (Figure 19). Fermentation of high FODMAP bread with *Lactobacillus* strains resulted in decreased acetate (df 10, F value 6.275, p value < 0.01), total SCFA (df 10, F value 9.242, p value < 0.01) and total BCFA (df 10, F value 1.328, p value < 0.05) compared to fermentations of high FODMAP bread without *Lactobacilli* added. Similarly, fermentation of high FODMAP pasta with *Lactobacillus* strains revealed a reduction in acetate (df 10, F value 2.332, p value < 0.001) and total SCFA (df 10, F value 2.329, p value < 0.001) compared to fermentation of high FODMAP pasta alone.

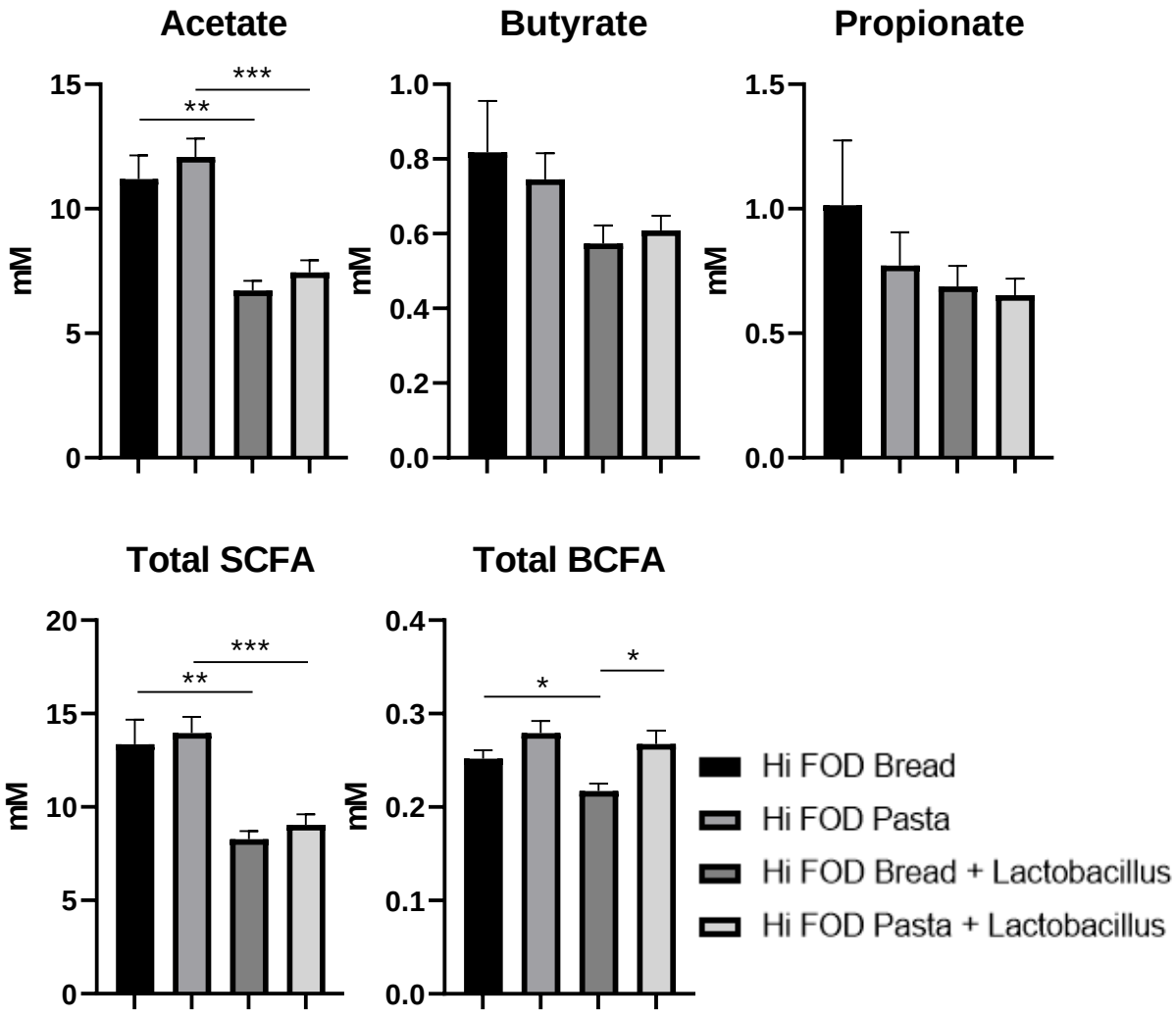

Figure 19 SCFA concentration after fermentation of high FODMAP bread and pasta varieties seeded with Lactobacillus*. Total SCFA = acetate + butyrate + propionate + valerate + isobutyrate + isovalerate. Total BCFA = isobutyrate + isovalerate. T-test corrected for multiple testing was used to compare groups. Values plotted as mean ± SEM (* p-value <0.05, ** p-value <0.01, *** p-value < 0.001).*

These results reveal a correlation between increased *Lactobacillus*, decreased diversity and decreased acetate production.

5.5 | **Discussion**

The data from this study shows that malting lentils and buckwheat resulted in limited differences in the microbiome and no significant differences in microbial metabolism after fermentation, compared to their high FODMAP counterparts. Fermentation of enzymatically degraded lentils resulted in a similar microbiome to malted lentils and raw lentils. Notably, fermentation of malted buckwheat increased the prevalence of two butyrate producers in the microbiome, indicating potentially increased hydrogen production. However, no changes in SCFA production were observed between these groups. The fermentation of four bread and two pasta varieties revealed that low FODMAP bread and low FODMAP pasta varieties result in a similar microbiome to their high FODMAP counterparts. Finally, adding three *Lactobacillus* strains prior to fermentation of high FODMAP bread and high FODMAP pasta resulted in a reduced abundance of other carbohydrate fermenting groups, hydrogen producing groups and decreased acetate levels.

5.5.1 | Fermentation of lentils and buckwheat does not cause substantial changes to microbiome diversity, despite subtle changes to composition.

Understanding the interaction between lentil and buckwheat substrates and the microbiome facilitates choosing the most appropriate food for a low FODMAP diet. Malting legumes has been previously shown to reduce FODMAPs significantly in lentils [27] and the low FODMAP grain buckwheat was found to contain potentially fermentable carbohydrates called fagopyritols [27]. Due to the prevalence of buckwheat in current low FODMAP diets, it is imperative to ensure that fagopyritols are not part of the FODMAP family. Interestingly, the data presented above illustrates no significant changes after comparing the microbiome diversity after the fermentation of three lentil varieties and both buckwheat varieties. A diet high in pulses has previously caused no change in microbiota diversity *in vivo* [40-42], whereas the impact of buckwheat on microbiome diversity has not been directly assessed. This study corroborates this data, revealing no significant differences in microbiome diversity after fermentation in the presence of raw lentils and also after fermentation of the lower FODMAP lentil variants. Furthermore, fermentation of raw buckwheat revealed no significant difference in microbiome diversity compared to malted buckwheat.

Compositional analysis by ANCOM and LEfSe was used to understand the individual taxonomic groups that were altered after fermentation in the presence of these lentil and buckwheat substrates. Interestingly, analysis using LEfSe revealed that fermentation in the presence of malted buckwheat was associated with an increase in two beneficial butyrate producing microbial groups, *Eubacterium ventriosum* and *Ruminiclostridium* 9 [43-46]. Increased abundance of these two genera may indicate that the malting process is increasing availability of fermentable carbohydrates, however malted

buckwheat does not contain detectable levels of fagopyritols [27]. For this reason, no evidence exists that suggests fagopyritols are fermented similarly to other FODMAPs by the microbiome. Despite this, malted buckwheat increases the abundance of the two butyrate producing groups, a potentially undesirable effect due to the increased potential for hydrogen production [44, 47, 48]. Fermentation reactions in the presence of FODMAP degraded lentils were associated with increased *Megasphaera* and *Enterococcus*. *Megasphaera elsdenii* is known for its ability to convert lactate to other SCFAs [49], while *Enterococcus* spp. are known prominent lactate producers [50]. *Enterococcus* are robust microorganisms often used in industrial processes due to their resilience and adaptability to many hostile environments [50-52]. The prevalence of *Enterococcus* in FODMAP degraded lentils may be due to the lack of fermentable carbohydrates proving a difficult environment for other microbes to proliferate, whereas *Enterococcus* is capable of growing in more hostile situations. The subsequent *Enterococcus* driven production of lactate may provide sustenance for *Megasphaera* to increase in abundance within the reaction. *Oscillibacter*, increased in abundance after fermentation of raw buckwheat, is known to produce valerate [53] and was recently reported to exert influence on lactate dehydrogenase levels in chronic kidney disease [54].

Despite the altered abundance of microbial groups responsible for SCFA production, no significant changes were observed in SCFA concentrations after the 12-hour fermentation of these substrates. These data reveal that malted lentils and enzymatically degraded lentils do not have a clear negative impact on gut microbial metabolism. The increase in butyrate producing microbes after the fermentation of malted buckwheat is notable and requires more research to understand. Malted lentils and enzymatically degraded lentils provide suitable alternatives to their higher FODMAP counterparts without negatively impacting the microbiome. Finally, the presence of raw buckwheat does not appear to result in microbial fermentation of fagopyritols.

5.5.2 | Fermentation of four bread and two pasta substrates result in minor changes to their microbiome profiles.

Bread and pasta are commonly reduced or removed in a low FODMAP diet due to their high FODMAP content [55]. Achieving adequate nutritional balance is increasingly difficult without these types of staple foods, negatively impacting the health of these individuals and their gut microbiome [56, 57]. Low FODMAP bread and low FODMAP pasta were produced (Atzler et al, data unpublished) to potentially replace their high FODMAP counterparts. The data presented in this manuscript indicate that fermentation of these new low FODMAP prototypes of bread and pasta do not negatively impact alpha and beta diversity compared to their high FODMAP counterparts. A recent systematic review on FODMAP content and the microbiome suggests that reduced microbiome diversity is not commonly

seen after following a low FODMAP diet [58]. The volume of FODMAP carbohydrates present in a low FODMAP diet may still be sufficient to maintain microbiome diversity. Dietary intake high in fiber is, however, linked to greater bacterial densities and/or higher faecal volumes [59-62]. Indeed, this may highlight a limitation associated with sequencing data and its reliance of % abundance instead of absolute figures. However, our data suggest that microbiome alpha and beta diversity was not significantly different after fermentation in the presence of all four bread substrates or both pasta substrates.

Compositional changes to the microbiome were analysed by ANCOM and LEfSe. ANCOM analysis revealed altered abundance of *Butyricicoccus* and *Enterococcus* between pasta and bread and not as a result of altered FODMAP content. However, LEfSe analysis revealed a significant association between several microbial taxa and individual bread varieties. High FODMAP bread promoted the growth of *Dorea*, a member of the Clostridium cluster XIVa. *Dorea* is a prominent carbohydrate fermenter, producing acetate and subsequently H_2 as its primary metabolites [63, 64]. *Butyricicoccus* and *Lachnospira* were more abundant after fermentation in the presence of a high FODMAP pasta substrate. These two genera are known for beneficial effects through butyrate production [65, 66], however, they are also known to produce hydrogen within the gut environment [67-69]. The increased prevalence of these three genera among the high FODMAP controls highlight the duality of many beneficial butyrate producers in the context of potential IBS symptoms. Low FODMAP bread and pasta varieties may avoid this trade off by stimulating beneficial microbes without inducing IBS symptoms.

Interestingly, psyllium supplemented low FODMAP bread also facilitates the growth of butyrate and H_2 producer Anaerostipes [70-72] and Lachnospiraceae UCG004. An increase in these carbohydrate fermenting microbes after fermentation of psyllium supplemented bread may be explained by the heavier texture of this bread. During post digestion dialysis it was noted that psyllium added bread retained more water and had a heavier, more solid like consistency than other substrates. Psyllium supplementation is known to reduce the glycemic index of bread, thanks in part to this heavy consistency [73, 74]. Indeed, this consistency may have reduced the passage of carbohydrates through the pores of the dialysis tubing, resulting in more carbohydrates reaching the faecal fermentation stage [73, 74]. *Enterococcus* was significantly increased in the microbiome after fermentation of inulinase supplemented bread, potentially indicating that less available carbohydrates preferentially enrich for more resilient genera. Fermentation of low FODMAP bread was associated with a poorly understood and recently characterised genus, *Libanicoccus* [75]. Finally, Tyzzerella 3 and fermentation of low FODMAP pasta were significantly associated, a member of the Lachnospiraceae family previously associated with increased cardiovascular risk [76].

SCFA production was similar between all bread and pasta fermentation reactions with the exception of a reduction in total branched chain fatty acids (BCFAs) (isobutyrate + isovalerate) after fermentation in the presence of inulinase supplemented bread. Significant differences between the total BCFA concentration after fermentation of high FODMAP bread and inulinase bread and fermentation of low FODMAP bread and inulinase bread were revealed. Interestingly, higher inulin concentration appears to negatively affect BCFA production in murine models [77] or have no effect in *ex vivo* experiments [78]. This suggests that inulin is not the factor determining BCFA production. BCFAs have been proposed as markers of protein fermentation and are products of branched chain amino acid fermentation [79]. The lack of inulin-type fructans in the bread structure may facilitate more complete *ex vivo* digestion of this food prototype, resulting in greater amino acid degradation resulting in less amino acids for BCFA production compared to high FODMAP bread and low FODMAP bread.Despite the nuanced changes exhibited after the fermentation of these lower FODMAP bread and pastas varieties, no significant deleterious effects were observed in comparison to high FODMAP varieties. Microbiome alpha and beta diversity remained similar across all fermentations, whereas compositional changes were modest and only affected a limited number of microbial groups. Psyllium added bread may cause unwanted microbial fermentation due to its reduced digestion, however, this may not be a problem *in vivo*. Indeed, fermentation of high FODMAP bread and pasta were associated with increased abundance of hydrogen producers that were not linked to fermentation of lower FODMAP varieties, with the exception of psyllium added bread. These results indicate that the low FODMAP bread, inulinase treated bread and low FODMAP pasta varieties examined in this study are suitable alternatives for higher FODMAP breads and pasta without negatively impacting the gut microbiota.

5.5.3 | *Lactobacillus* supplemented fermentation reactions resulted in substantially altered diversity and composition

Lactobacillus rhamnosus DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 were added prior to a variety of fermentation reactions to understand their potential role in changing microbiome composition and metabolism. Fermentation of raw lentils, malted lentils and malted buckwheat, seeded with *Lactobacillus* prior to fermentation, showed limited changes to the microbiome compared to their non-*Lactobacillus* seeded counterparts. Fermentations of raw lentils and *Lactobacillus* resulted in minor compositional changes and a modest shift in beta diversity compared to fermentation of raw lentils without *Lactobacillus*. However, fermentation of malted lentils and *Lactobacillus* yielded different results. Interestingly, reducing the GOS availability in lentils by malting [27] may prevent *Lactobacillus* from inducing changes during the reaction. Fermentation of malted lentils in the presence of *Lactobacillus* did not alter diversity or composition of the

microbiome compared to fermentation of malted lentils alone. This indicates that the three *Lactobacillus* strains are fermenting GOS in raw lentil reactions and competing with other GOS fermenters, such as *Clostridiaceae 1*, resulting in a reduction in its relative abundance. Due to the known hydrogen production capabilities of *Clostridiaceae 1*, it is promising to see these *Lactobacillus* strains outcompeting them in *ex vivo* reactions [80, 81]. Despite the microbial changes, SCFA production was not impacted and high metabolite variability within groups was observed.

Fermentation of high FODMAP bread and high FODMAP pasta seeded with *Lactobacillus* demonstrated substantial changes compared to non-*Lactobacillus* seeded reactions. Alpha diversity, as measured by Simpson and Shannon index, were significantly reduced in high FODMAP bread and *Lactobacillus* seeded reactions compared to fermentations of high FODMAP bread alone. Previously selected for their FODMAP degrading ability, it is likely that these *Lactobacillus* strains are dominating the fermentation reaction due to this advantage as they take over this FODMAP fermenting niche, reducing carbohydrate fermenting competitors [82, 83]. Research on *Lactobacillus* in general [84] and in *ex vivo* models in particular [85], indicate that *Lactobacillus* are known to ferment these longer chain carbohydrates that are present in bread and pasta [84]. Indeed, fermentation of high FODMAP substrates in this *ex vivo* system provided an environment in which *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 proliferated and reduced competing strains [12, 86]. The presence of *Lactobacillus* within the fermentation reaction caused a reduction in alpha diversity. Despite the negative association with lower diversity, Coyte et. al. (2015) has postulated that a microbiome with highly varied microbial taxa relying on cooperating networks within the gut are inherently more susceptible to collapse than ecosystems with a smaller number of prominent groups [87, 88]. Prominent *Lactobacillus* strains may provide a more stable microbiome due to reduced reliance on other microbial groups [88]. Furthermore, fermentation of high FODMAP bread in the presence of added *Lactobacillus* strains yielded a reduction in the prominent hydrogen producing group Anaerostipes, after analysis by both ANCOM and LEfSe [70]. LEfSe analysis also showed a broad reduction in carbohydrate fermenting and hydrogen producing genera after fermentation of high FODMAP bread and fermentation of high FODMAP pasta in the presence of the *Lactobacillus* strains compared to non-*Lactobacillus* seeded counterparts. This may highlight a potential limitation of abundance-based measurements, as a substantial increase in the genus *Lactobacillus* may result in an apparent reduction in a large swath of other groups with limited knowledge about the absolute increase or decrease that occurred. Despite this, it is tempting to suggest that the three *Lactobacillus* strains added prior to fermentation are competitively reducing many other microbial groups within the reaction, causing a reduction in hydrogen production potential in the microbiome. Notably, *Dorea* and *Clostridium sensu stricto* are reduced in abundance

after fermentation of high FODMAP bread and fermentation of high FODMAP pasta in the presence of *Lactobacillus*. These two genera have been implicated in hydrogen production and IBS symptoms specifically and reducing their abundance may reduce hydrogen genesis [89, 90]. Indeed, the SCFA results appear to corroborate this suggestion. Fermentation of high FODMAP bread and high FODMAP pasta seeded with *Lactobacillus* strains resulted in significantly reduced acetate production compared to their non-*Lactobacillus* counterparts. The production of acetate generates the most hydrogen of all SCFAs measured, indicating that increased abundance of *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 may result in reduced hydrogen production [25, 91]. Total BCFAs are also reduced after fermentation of high FODMAP bread in the presence of *Lactobacillus* strains compared to non-*Lactobacillus* seeded reactions. The reduction in acetate did not result in a concurrent increase in other SCFAs, indicating a reduction in the amount of fermented substrates within the gut. This result may in part explain the reduced diversity due to fermentation in the presence of *Lactobacillus* strains. The presence of *Lactobacillus* strains during this fermentation coincided with reduced alpha diversity in the microbiome. *Ex vivo* gut models [92], animal studies and human intervention studies [93, 94] have provided evidence that increased complex carbohydrates result in reduced BCFA production. This may indicate that there are competing mechanisms between carbohydrate SCFA production and BCFA production in the MicroMatrix *ex vivo* system. In such an environment, the presence of dominant *Lactobacillus* strains may suppress BCFA yield in favour of SCFA production.

5.6 | **Conclusion**

Ex vivo fermentation of lentils, buckwheat, bread and pasta prototypes provided information on the impact these foods may have on the healthy microbiome. A 12-hour fermentation in the presence of raw lentils (3.89 g FODMAP / 100 g), malted lentils (0.84 g FODMAP / 100 g) and FODMAP degraded lentils (<0.005 g FODMAP / 100 g) resulted in limited changes in the microbiome with no detectable shift in alpha or beta diversity. Similarly, fermentation of raw buckwheat (0.33 g FODMAP / 100 g) and malted buckwheat (<0.005 g FODMAP / 100 g) revealed no changes in microbiome alpha and beta diversity. Noteably, malting the buckwheat grain may increase availability of fermentable carbohydrates resulting in increased abundance of two butyrate producing groups, *Eubacterium ventriosum* and *Ruminiclostridium* 9. These groups were not enriched by fermentation of raw buckwheat, highlighting a potentially undesirable effect as this fermentation may generate hydrogen during the process of butyrate production. Fermentation in the presence of a high FODMAP bread and three low FODMAP breads resulted in no changes to both alpha and beta diversity metrics. Fermentation of high FODMAP pasta also revealed no change in diversity compared to fermentation of low FODMAP pasta. Despite this, fermentation of higher FODMAP bread and pasta increased the abundance of a number of carbohydrate fermenting, hydrogen producing microbes in the microbiome compared to the lower FODMAP content alternatives. Psyllium supplemented bread may hinder *in vitro* digestion due to its heavy consistency, harbouring undigested carbohydrates through the simulated *in vitro* digestion. Fermentation of low FODMAP bread and pasta did not reduce diversity, nor did it increase abundance of hydrogen producing microbes. These data highlight that low FODMAP bread, low FODMAP pasta and inulinase treated bread may be suitable foods for a low FODMAP diet. A larger scale fermentation unit targeting these specific substrates may increase the reliability of these results. At this scale, a more robust picture of SCFA fermentation products may be gathered which may reveal significant differences after substrate fermentation. Furthermore, seeding the reaction with three *Lactobacillus* strains, *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083*,* prior to fermentation of raw lentils resulted in a shift in diversity and reduction in *Clostridiaceae* 1 compared to fermentation without *Lactobacillus*. Fermentation of high FODMAP bread and high FODMAP pasta in the presence of the three *Lactobacillus* strains resulted in substantial changes in microbiome diversity and composition, in conjunction with reduced acetate production. These results indicate that *Lactobacillus* is dominating the carbohydrate fermenting niche within these reactions and potentially reducing the hydrogen production simultaneously. Low FODMAP bread, low FODMAP pasta and inulinase treated bread may act as suitable replacements for high FODMAP bread and high FODMAP pasta as part of a low FODMAP diet. *Lactobacillus rhamnosus* DPC 6071, *Lactobacillus casei* DPC 6072 and *Lactobacillus casei* DPC 6083 may act *in vivo* to reduce

hydrogen production after FODMAP fermentation. Further in vivo studies in animal and human trials are required to corroborate these results.

5.6 | **References**

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Chapter VI

General Discussion

6.1 | **Overview and Summary**

The research presented in this thesis demonstrates considerable advances in therapeutic approaches to FODMAP-induced IBS. The results discussed in previous chapters highlight the potential for both novel bacterial strains and new food prototypes to ameliorate FODMAP-induced IBS. The broad reach of this thesis, encompassing *in vitro*, *in vivo* and *ex vivo* work, presents a unique and balanced view of the impact that these potential therapeutic measures have on host physiology, the immune system and the microbiome. This chapter will focus on final considerations of each distinct theme in this thesis, discussing the collated data presented in several chapters. The implications of this research in the context of current research will be explored and used to discuss where it is positioned among previously published data. Finally, the avenues for future research will be discussed focusing on how to increase the impact of the research completed in this thesis.

6.1.1 | The impact of *Lactobacillus* strains both *in vivo* and *ex vivo*.

L. rhamnosus DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 were selected based on the presence of several potentially probiotic traits and their ability to ferment FODMAP carbohydrates, as outlined in Chapter 2. These attributes confirmed their suitability for further research *in vivo* and *ex vivo*. The animal models used in Chapter 3 and Chapter 4 presented distinct challenges for the survival and efficacy of the three strains. Despite this, the microbiome sequencing data clearly indicates that the *Lactobacillus* strains increased in abundance among the gut microbiota of *Lactobacillus* fed mice. The impact that these strains had on the broader microbiome and host response was significant. Diversity within the microbiome was markedly reduced in *Lactobacillus* fed animals in both animal studies. A reduction in diversity has been previously observed after probiotic supplementation [1-3]. In both murine trials performed it is likely that the *Lactobacillus* supplementation caused reduced diversity due to the concurrent consumption of a high FODMAP diet. *Lactobacillus* performance during *ex vivo* fermentation, described in Chapter 5, revealed the difference between reactions seeded with *Lactobacillus* and those seeded without *Lactobacillus* strains. *Lactobacillus* were seeded directly into the reaction prior to the 12-hour fermentation, accounting for 5% of the total bacteria. The fermentation resulted in an increase in *Lactobacillus* abundance to approximately 25%, indicating a significant surge compared to other microbial groups present. Furthermore, *Lactobacillus* seeded fermentations were associated with significantly decreased diversity compared to those not seeded with *Lactobacillus* strains, indicating that *Lactobacillus* dominated the reaction and filled suitable niches within the reaction. However, adding the *Lactobacillus* strains directly before the reaction takes place without treating them similarly to the other microbes present within the FSI, i.e. faecal sample preparation and freezing at -80°C (Section 5.3.5), may cause a confounding factor. Despite this, the addition of *Lactobacillus* strains both *in vivo* and *ex vivo* resulted in reduced microbial diversity. Reduced diversity in the microbiome may in part be explained by the competitive inhibition of carbohydrate degrading microbes by the three *Lactobacillus* strains. Moreover, reduced diversity may be caused by a destabilized microbiome [4] or indeed a microbiome with increased robustness [5]. These conflicting results indicate that diversity may no necessarily be the most appropriate measure of resilience and functionality. Indeed, recent interventional studies have indicated that substantial increases in dietary fibre can temporarily reduce diversity as they selectively enrich particular microbial groups [6]. Interestingly, our research presented both a highly fermentable diet and a microbial group that is specifically enriched by that diet. This combination resulted in colonization of the murine gut in two separate murine trials. Lasting colonization was demonstrated in Chapter 4, as the *Lactobacillus* strains appeared to survive in the murine gut for seven days in DSS treated mice and during seven days of recovery without further *Lactobacillus* supplementation.

L. rhamnosus DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 were consumed by mice (Chapter 3 & 4) and fermented *ex vivo* in the presence of high and low FODMAP food substrates and a human microbiome (Chapter 5). The impact of ingesting *Lactobacillus* strains on microbial metabolic output (SCFA production) was altered depending on the FODMAP content and other microbial constituents. SCFA concentration was measured after *ex vivo* faecal fermentations and *in vivo* murine trials to understand the primary metabolic output of the microbiome. SCFAs are known for their beneficial effects on the host, implicated in improved outcome of a variety of disorders through mediation of tight junctions, immune response and cell signalling [7-9]. However, FODMAP carbohydrates often act as precursors to SCFA production in microbial metabolic pathways [10]. Analysis of the SCFAs produced after FODMAP fermentation revealed information regarding the increased and decreased production of hydrogen gas, a compound responsible in part for IBS symptoms of pain and bloating [11-14]. The aims of the murine trial described in Chapter 3 include determining if *Lactobacillus* consumption may persist in the murine microbiome and exert an effect on the microbial metabolism. Results from this study indicate a significant increase in propionate in the gut of animals consuming the *Lactobacillus* strains, whereas *ex vivo* fermentations seeded with *Lactobacillus* in Chapter 5 revealed reduced acetate. Interestingly, both these changes are linked by their effect on hydrogen production, as acetate is a net producer and propionate a net consumer during fermentation [15, 16]. These results indicate that during both *in vivo* and *ex vivo* experiments the three *Lactobacillus* strains were capable of potentially reducing hydrogen output.

Finally, *Lactobacillus* strains evoked important immune changes in the host, as reported in Chapter 3 and Chapter 4. CD19+ immune cells were increased in concentration in the mesenteric lymph nodes in Fat1 mice in Chapter 3 (Figure 3.6). A strong inverse association between IBS severity and CD19+ concentration has been previously reported [17], suggesting that these strains may reduce IBS severity via modulation of the immune response within the murine gut. Chapter 4 detailed a substantial reduction in pro-inflammatory immune cells five days after DSS treatment began. IFN-γ and MCP-1 were significantly reduced, whereas TNF-α trended towards a reduction. Reducing these prominent pro-inflammatory agents has been associated with marked improvements in IBD symptoms and severity [18-21]. Probiotics have previously demonstrated their ability to reduce the severity of murine DSS induced colitis through the suppression of pro-inflammatory cytokines [22, 23] Moreover, this probiotic effect on pro-inflammatory cytokines has been observed in human ulcerative colitis patients [24]. Indeed, oral supplementation of two *Lactobacillus* strains over an 8-week period was sufficient to decrease the concentration of IL-6, expression of TNF- α and NF-κB p65 proteins. The efficacy of *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 in reducing pro-inflammatory cytokines indicates their potential therapeutic role in IBD management. Furthermore, colonising the gut

ecosystem with *Lactobacillus* strains capable of exerting an anti-inflammatory influence may provide long lasting protection against the recurrence of IBD symptoms. Due to their ability to exert an anti-inflammatory response and robustly colonise the murine gut microbiome, *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 present potential in ameliorating IBD symptoms.

6.1.2 | The influence of genotype in both murine trials

Two separate mouse genotypes were used in the studies described in Chapter 3 and Chapter 4. Fat1 mice are capable of maintaining an optimum N-6 PUFA to N-3 PUFA ratio by converting N-6 to N-3 endogenously. These animals provide a unique platform for N-3 PUFA research, removing many confounding factors. A plethora of evidence exists detailing the health benefits of N-3 PUFAs [25-27]. N-3 PUFAs are capable of ameliorating low grade inflammation present in the gut [28], a mechanism that is understood to occur in IBS patients [29]. Research on N-3 PUFA has also demonstrated a potentially beneficial influence on the maintenance of remission in IBD [30, 31]. These studies indicate a potential effect, however, do not provide conclusive evidence, often citing the need for further research.

The data presented in this thesis indicate that an optimum N-6 to N-3 ratio proves instrumental in providing a protective effect to mice with DSS induced colitis. Indeed, the *Lactobacillus* strains acted synergistically with the Fat1 N-3 profile to provide protection against weight loss and modulate the immune system (Figure 4.5). Prevention of weight loss was not observed in WT mice, indicating that the *Lactobacillus* strains worked in an N-3 PUFA dependent manner. Previous research has suggested that high concentration of N-3 aids in the colonization of probiotic strains, potentially indicating a mechanism for the increased protection observed in the study described in Chapter 4 [32-34]. The anti-inflammatory effect of N-3 PUFA may reduce the mammalian immune response to probiotics that are introduced, increasing their adherence and colonization. Indeed, it is tempting to suggest that the optimum N-3 ratio aided the colonization of *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 within the murine gut, resulting in a protective phenotype due to a greater abundance of these strains within the gut microbiome.

6.1.3 | The influence of varied FODMAP content on the microbiome

The impact of FODMAP content on the microbiome was explored both *in vivo* and *ex vivo*. High and low FODMAP diets were fed to mice, detailed in Chapter 3, whereas prototype substrates with varied FODMAP content were seeded within fermentation reactions as described in Chapter 5. FODMAP carbohydrates have a substantial impact on the gut microbiota, enriching saccharolytic microbial groups capable of fermenting them. Interestingly, it is hypothesized that individuals with gut microbes adept at fermenting these carbohydrates may benefit the most from a low FODMAP diet [35]. This presents an issue in individuals that consume a healthy diet high in fermentable carbohydrates. These individuals may be enriching their microbiota for carbohydrate fermentation and potentially creating an environment for FODMAP-induced IBS symptoms to arise [35]. This phenomenon highlights the need for longer term solutions to FODMAP-induced IBS. Conversely, underlying conditions such as fructose malabsorption, sucrose malabsorption and lactose intolerance are also considered under the term FODMAP-induced IBS [36-38]. These disorders manifest as FODMAP-induced IBS symptoms due to microbial fermentation of fructose, sucrose and lactose within the gut. Although low FODMAP food variants will not directly resolve these issues, providing more dietary options with lower FODMAP content results in a greater likelihood that IBS symptoms can be avoided. Interestingly, these disorders would be treated by probiotic supplementation, demonstrating further use cases for *Lactobacillus* supplementation. Low FODMAP prototype foods that do not negatively affect diversity or enrich potentially problematic microbial groups are also an exciting solution to more conventional FODMAP-induced IBS.

The tools available for examining the impact of FODMAPs on the microbiota are varied. *In vivo* human trials are the most appropriate method for understanding the impact of specific FODMAP combinations on the gut microbiota. Despite this, human studies have inherent limitations due to the lack of control over confounding factors [39]. Animal trials present more control over the diet and environment, however, introduce an issue in translating research to humans [40]. *Ex vivo* fermentation systems provide more control over the input parameters and are conducted without living subjects. However, these fermentation systems do not possess many important factors that must be considered before interpreting results [41, 42]. *Ex vivo* systems, by their nature, are devoid of host-microbe interactions. Host factors include bactericidal activity, availability of host-derived glycans, absorption of metabolic products and facilitating gaseous accumulation. Indeed, the loss of these *in vivo* traits provides a system unsuitable for directly studying hydrogen production, a parameter of interest in understanding the degradation of FODMAP carbohydrates [43]. It is appropriate to note that neither human nor animal trials have a suitable method for calculating hydrogen production within the gut, indicating a limitation in understanding microbial FODMAP

fermentation [35, 44]. Furthermore, a lack of host absorption of metabolic products in *ex vivo* systems limit the accuracy of measured SCFA concentrations. These compounds accumulate during the fermentation run instead of interacting with the host through absorption and signalling [45, 46]. Despite this, SCFA accumulation can be compared between groups and microbe-microbe cross feeding will occur within the *ex vivo* reactions.

The interaction between a high FODMAP diet and the *in vivo* murine microbiome resulted in a familiar response. The high FODMAP diet resulted in significantly increased diversity within the murine microbiome and enrichment of microbial groups capable of carbohydrate fermentation and SCFA production (Figure 3.13) [47-49]. High FODMAP substrates in the *ex vivo* fermentation system did not result in a significant increase in diversity compared to low FODMAP substrates (Figure 5.17). This result suggests a considerable difference between the *in vivo* and *ex vivo* experiments. This may be due to the limited resources available within the *ex* vivo fermentation units. An *ex vivo* system acts similarly to a batch system, potentially running low on easily fermentable carbohydrates by the end of the fermentation run. The finite amount of each substrate seeded within the reaction may not be sufficient to cause substantial shifts in species diversity and richness. Conversely, the ready supply of highly fermentable carbohydrates in the murine trial consistently enriches the host gut microbiome, increasing microbial diversity and richness. This demonstrates that not only were the FODMAP carbohydrates enriching the groups directly fermenting them, but they were also providing fuel for the complex cross feeding structure throughout the microbiome. Given the limitations outlined regarding the MicroMatrix *ex vivo* fermentation system, the use of a larger bioreactor may reveal further information regarding the most suitable substrates for human trials. Two directly modified substrats, inulinase treated bread and psyllium added bread, demonstrated significant potential for further research. Microbial fermentation in the presence of each of these resulted in non-significant reductions in SCFA and acetate that bordered on significant. Larger scale fermentation units may grant greater clarity on this trend and also may facilitate the direct measurement of hydrogen gas. Combining both these metrics may reveal a significant benefit by consuming these two substrates instead of their higher FODMAP counterparts. Ultimately, based on *in vivo* and *ex vivo* studies, high FODMAP carbohydrates appear to increase carbohydrate fermenting groups and diversity within the murine microbiome and, as mentioned above, potentially facilitate the colonization of *Lactobacillus* strains *in vivo*.

6.1.4 | Assessing the murine response to models of maternal separation and DSS induced colitis

The efficacy of two murine disease models used in this body of research was varied. Maternal separation is a model used to induce a stress-like phenotype in mice and rats [50, 51]. This stress-like phenotype is used to study visceral pain, depression, anxiety-like behaviour and IBS [52-54]. The research, detailed in Chapter 3, was aimed at inducing an IBS phenotype. It was hypothesized that this IBS phenotype could then be exacerbated by the consumption of a high FODMAP diet, as is the case in human subjects. However, the maternal separation model did not appear to have any deleterious impact on the mice after analysing their body weight, MLN immune profile and performance in behavioural tests (Figure 3.5, Figure 3.7, Figure 3.8). Higher body weight was observed at weaning in mice that were subjected to maternal separation, contrary to previous successful models of maternal separation [55]. CD4+CD8+ and CD25+ were significantly reduced in maternally separated mice indicating reduced immune stimulation. Finally, no significant differences were reported after behavioural examinations were performed. We concluded that the maternal separation effect did not induce the desired IBS-like phenotype and may have elicited greater care from the dams prior to the weaning period. Despite the research describing successful maternal separation, many studies also indicate that maternal separation is ineffective [56-58]. Own et al. (2013) reported increased maternal care as a function of maternal separation in C57/Bl6 offspring [57]. This observation appears similar to our results reported in Chapter 3, suggesting that C57/Bl6 mice were resilient to maternal separation and may even benefit from it.

C57/Bl6 mice were subjected to DSS treatment, detailed in Chapter 4, to induce a colitis phenotype. DSS induced colitis is a frequently used model in studying IBD, resulting in a reliable and consistent phenotype [59, 60]. Our research examined the impact that *Lactobacillus* strains, selected previously based on their ability to degrade FODMAPs, and an optimum N-3 PUFA concentration may have on experimental colitis. Indeed, mice consuming 2.5% DSS for seven days developed a robust colitis like phenotype characterized by reduced body weight, increased pro-inflammatory immune cells and reduced colon length [59]. The severe reaction described facilitated the analysis of *Lactobacillus* and N-3 status in a colitis environment. These two distinct animal models provided differing results regarding their efficacy. The data presented in this thesis and other previously published studies describe the ineffectiveness of this maternal separation model. As the maternal separation model is considered the most efficacious IBS model in mice, it is apparent that a more appropriate model for IBS research is required [50]. An effective IBS mouse model may be a significant step towards revealing more regarding this complex disorder.

6.2 | **Implications for related research**

Research on the gut microbiome is gathering pace, reaching many distinct diseases and disorders. Therapeutic opportunities in modulating the microbiome are now presented for disorders such as Type II diabetes, cardiovascular disease and autoimmune disease [61]. However, diseases located within the gut may yet see the greatest benefit from microbiome modulation. IBS and IBD are intimately linked to the gut microbiome and the host-microbe interactions that it entails [62, 63]. Due to the proximity of gut microbial constituents to the diseased areas, it is possible that modulating the microbiome may directly ameliorate these gut-based disorders. However, introducing microbial strains to exert a beneficial outcome in these disorders has yielded mixed results thus far [64-68]. Many of these probiotics target bile salt deconjugation, bactericidal activity against enteropathogens or immune modulation [69]. However, these mechanistic targets may not have robust ties to the underlying disorder. FODMAP-induced IBS, however, presents a distinct mechanism of action that is directly targetable by the introduction of microbial strains. The results reported in this thesis present *Lactobacillus* strains that are capable of degrading FODMAP carbohydrates, colonizing the gut and exerting an impact on the metabolic output. By targeting this specific mechanism, *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 may reduce symptoms by shifting the microbiome metabolism away from hydrogen production, without a requirement for reducing FODMAP consumption. IBS research targeting this mechanism may yield exciting results.

The potentially protective effect probiotic strains exert against colitis has been reported previously [69-73]. However, the persistence of at least one of these three strains through DSS challenge and recovery is notable and indicates robust colonization of the murine gut. This colonization occurs in the presence of a diet high in fermentable carbohydrates by *Lactobacillus* strains adept at fermenting these carbohydrates. Research in this area may benefit from matching strains with prebiotics to aid colonization of the host microbiome. Furthermore, low FODMAP food prototypes are presented as a novel approach in supplementing a low FODMAP diet. In selecting the most appropriate low FODMAP prototype, one must attempt to limit the potential negative implications on the gut microbiome. *Ex vivo* fermentation models may not be exact replicas of *in vivo* systems, however, they present a suitable screening method for choosing the most efficacious prototypes for further analysis [74]. Indeed, *ex vivo* trials demonstrating the impact of low FODMAP prototype foods on the microbiome produced valuable information supporting their potential in a low FODMAP diet. These results lend further evidence for the use of *ex vivo* faecal fermentations in determining the interaction between food prototypes and the microbiome.

6.3 | **Avenues for future research**

The information presented in this thesis opens several avenues for future research. The primary objective of future studies in this area is the development of novel approaches to reduce the severity of FODMAP-induced IBS. To that end, further research on microbes that target the FODMAP-hydrogen production paradigm may result in more appropriate strains capable of reducing symptoms *in vivo*. Targeting this paradigm may not be exclusive to current probiotic genera such as *Lactobacillus* and *Bifidobacterium*. Next generation probiotics may be more suitable for this task, none more so than a prevalent saccharolytic genera such as *Bacteroides* [75]. This genus harbours many species capable of considerable FODMAP degradation and predominant propionate production [76]. With these combined attributes, specific *Bacteroides* spp. may be best positioned to steer the FODMAP fermentation within the human microbiome away from hydrogen production. Given the role of *Bacteroides* in the production of hydrogen, seeding a *Bacteroides* sp. within the microbiome may result in the direct competition with other *Bacteroides* spp. This direct competition may be aided by *Bacteroides* Type VI secretion system, a known anti-microbial system that often preferentially targets other *Bacteroides* spp [77]. *Bacteroides* are, however, opportunistic pathogens and may be more difficult to grow to high concentrations than current generation probiotics. Despite these limitations, their attributes present them as more than suitable candidates as microbial therapeutic agents in ameliorating FODMAP-induced IBS.

Our data demonstrate a successful colonization of *L. rhamnosus* DPC 6071, *L. casei* DPC 6072 and *L. casei* DPC 6083 within the murine gut and modulation of host microbiome metabolic output. These results present an opportunity to examine these *Lactobacillus* strains in human trials. Further research assessing the efficacy of these strains in human trials should focus solely on individuals with FODMAP-induced IBS. Translating these results from murine trials to humans may prove difficult, however, targeting FODMAP-induced IBS patients will increase the likelihood of successful results. In particular, patients that have previously achieved reduced IBS symptoms after undergoing a low FODMAP diet would be the ideal candidates for a trial of this nature. Furthermore, tailoring an improved delivery system to increase the viable cell counts that reach the microbiota may increase the likelihood of a successful outcome [78].

Finally, analysing the impact of novel low FODMAP food prototypes on the human gut microbiota *in vivo* may provide a more accurate understanding of their influence on microbiota diversity and richness. Future research on these food prototypes may take the form of human trials assessing their impact on the gut microbiome. Due to the low risk associated with consumption of these foods, human trials may be best suited to deliver robust results. A consistent feeding regimen including

regular consumption of the food prototype may provide a more balanced indication of how the microbiome is affected by the components of each prototype in the longer term.

The avenues of research outlined above would propel the current research forward, providing potential therapies that directly aid in FODMAP-induced IBS. Notably, the treatment approaches mentioned may also act synergistically, potentially amplifying their effects. These three separate approaches require more successful research to lead to these results, however, progress in these areas would result in real improvements in treatments for FODMAP-induced IBS.

6.4 | **Conclusion**

The data collated as part of this thesis present therapeutic approaches to managing FODMAP-induced IBS. The *in vitro* performance of three *Lactobacillus* strains demonstrated probiotic potential and indicated their suitability for *in vivo* trials. During murine trials, the impact of the selected *Lactobacillus* strains on the murine microbiome composition, diversity and metabolic output were consistent with the reduction of hydrogen production *in vivo*. Furthermore, these strains are capable of persisting in the gut throughout DSS challenge while exerting an anti-inflammatory immune response. Finally, low FODMAP food prototypes analysed using an *ex vivo* fermentation system did not result in reduced diversity. These results highlight a number of potential therapeutic approaches capable of targeting FODMAP fermentation for reducing IBS symptoms.

6.5 | **References**

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