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# Novel insights into the influence of fibre and lipid substrates on the microbiome

Thesis presented by

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for the degree of

**Doctor of Philosophy**

**University College Cork**

**School of Microbiology**

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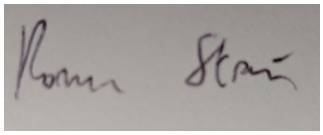
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## Declaration

I hereby declare that the contents of this thesis, except where otherwise acknowledged, is the result of my own work and has not been submitted for a higher degree in another university.

Ronan Strain, BSc, MSc



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## Abstract

Since the turn of the millennium, advances in sequencing technology have uncovered a plethora of microorganisms that live with and inside us. There are some indications that a considerable part of the environmental influence on human health and disease is mediated by this microbial community and it is recognised that diet massively dictates the structure and function of these symbionts. Identifying dietary components, therefore, that impact this internal community can reveal novel insights into how these symbionts affect and promote human health and well-being.

This thesis investigated the effects of dietary fibre and lipid components on the human gut microbiota using *in vitro, in vivo* and *in silico* methods. Attempts were made to characterise the effects of these dietary components on gut microbiota composition from an alternative perspective in relation to human health; how they impact colonisation resistance against pathogens, influence bacterial taxa associated with metabolic disease and how they affect an overlooked proportion of the microbiota, the human gut virome. An extensive literature review was undertaken in Chapter 1 to examine how diet may impact colonisation resistance against invading pathogens, exploring how different food components and metabolites either protect or promote gastrointestinal pathogen colonisation.

In Chapter 2 and Chapter 3, it was found that dietary fibres have differential effects on colonisation resistance against pathogens by employing an *ex vivo* model of the distal colon and assessing pathogen growth following 24-hour faecal fermentation. Glucose was used as a positive control to compare the success of pathogen growth in the presence of various pre-digested dietary fibres. Pathogen abundance was evaluated by 16S rDNA sequencing, qPCR and the effects on metabolic activity by short chain fatty acid (SCFA) analysis. We found that SCFA concentrations were increased in the presence of pathogens.

The same *ex vivo* model was implemented, as described in Chapter 4 to study the effects of various dietary fibres and lipids on gut microbiota, using faecal samples collected from subjects with various stages of metabolic syndrome. Effects of microbial composition and diversity were assessed by 16S rDNA sequencing and SCFA analysis was performed for some of the treatments. Dietary fibre supplementation increased bacterial taxa negatively associated with metabolic disease including *Bacteroides* and *Faecalibacterium*. Fish oil treatment exhibited a decrease in pro-inflammatory *Enterobacteriaceae* and an increase in *Bifidobacterium* and *Veillionella.*

A pilot study, described in Chapter 5, using faecal samples taken from a randomised controlled trial (RCT) on a dietary oily fish intervention was used to study dietary relationships with the human gut virome. The purpose was to evaluate how diet might influence this overlooked proportion of the microbiota and if oily fish intake selects for viral taxa negatively associated with autoimmune disorders. Metagenomic shotgun sequencing was performed on faecal samples taken from the RCT at baseline and at 8 weeks for 2 different fish types (sardine & tuna) eaten at either one portion or two portions of fish per week; these data were compared with a no fish intake group. The analysis was limited to diversity and viral load assessments, owing to the fact that a large fraction of our sequences had no matches in the viral database. There was a trend towards oily fish intake having a transient association with the human gut virome, which may be an artefactual signal from the high degree of inter-individual variation observed in humans.

In conclusion, these results highlight the potential of dietary fibre and lipid food components in modulating microbiota composition. Further research is required to assess whether these changes in microbiota composition have a beneficial effect on human health.

# General Introduction

There are hundreds of trillions of microorganisms that populate the mammalian gastrointestinal system and they have been shown to impact host physiology and host health. These microorganisms, collectively referred to as the microbiota, include a myriad of interconnected bacteria, bacteriophage, archaea, fungi and eukaryotic viruses (Fan & Pedersen, 2021). This assemblage of cross-kingdom entities is intrinsically tied to many aspects of host health, including immune function, digestive capabilities and gut-brain interactions. Many factors influence this community, with diet among the most potent modulators of microbiota composition and function. It is important to deduce the specific food substrates which influence this community of microbes, which in turn, impact the metabolism, absorption and storage of ingested nutrients and ultimately, host health. While much of the focus of microbiota research is concentrated on the bacterial component of the microbiota, little attention has been made on the viral component, which is primarily comprised of bacteriophage. Bacteriophages are viruses that infect bacteria and are the most abundant biological entity on the planet (Weinbauer, 2004). This thesis will attempt to uncover novel aspects of diet-microbe interactions, with a particular focus on the bacteriome and later, the virome, by utilising the latest *in vitro* and *in silico* techniques to provide an innovative approach to the intersect of diet and human health.

Colonisation resistance is the ability of the microbiota to block infections by invading pathogenic microbes, and is a fundamental aspect of microbiota-host association which is highly conserved among the animal kingdom (Ducarmon et al., 2019). This protection is not fully understood; however, it is believed to be primarily mediated by bacteria, through antimicrobial secretion, nutrient competition, maintenance of the gut barrier integrity and distribution of bacteriophage. The antibiotic associated susceptibility of the microbiota can lead to a more vulnerable state of the host through the rapid loss of protective commensal microorganisms, provoking an increased susceptibility to infection from enteric pathogens. Owing to the fact that diet is a dominant force that shapes bacterial communities, similar to the perturbation effect of antibiotics, one can speculate that diet has the

potential to mediate colonisation resistance. It was realised that little research has been carried out on how food substrates could potentially provide a means of protection against gastrointestinal pathogen colonisation through dietary-mediated encouragement of growth of protective commensal bacteria. Chapter 1 provides a detailed literature review and perspective on diet and colonisation resistance of gut pathogens.

Chapter 2 attempts to address the paradigm of diet and colonisation resistance through application of a state-of-the-art *ex vivo* model of the distal human gut. Probiotics were historically considered to provide a health benefit if possessing the ability to inhibit pathogen growth; however, the same definition is not included for prebiotics (Hill et al., 2014). Prebiotics are non-digestible substrates that beneficially influence the host by invigorating growth and the associated function of bacteria in the colon, and thus improving host health (Davani-Davari et al., 2019). The idea of a prebiotic inducing a beneficial nutrient-niche for commensal bacteria has been explored previously (Shepherd et al., 2018), but little research has been performed in terms of those producing an anti-pathogenic effect. An *ex vivo* model of the distal colon, the MicroMatrix fermentation unit, was used to emulate the conditions in the mammalian gastro-intestinal tract. This *ex vivo* system allows for multiple, miniature faecal fermentations (x24) to be performed in parallel, allowing for a much more controlled environment as opposed to traditional large-scale bioreactors (O'Donnell et al., 2018). A novel prebiotic fibre, yeast β-glucan, was chosen and compared to a control carbohydrate, glucose. A cocktail of spiked *Enterobacteriaceae* pathogens was introduced to the faecal fermentation unit and the total microbiome analysis by 16S and SCFA production by GC-FID was assessed after 24-hours in the presence of each of the test carbohydrates. There were issues regarding the specificity of 16S rRNA amplicon sequencing for quantifying relative abundances of the spiked pathogens in the complex background of the human gut microbiota. Nevertheless, we observed how pathogens behave in an ex vivo system in the presence of different carbohydrate sources.

Following on from the problems encountered in the faecal fermentation experiment, it was decided to repeat the procedure but using a different approach. In chapter 3, a range of prebiotic

fibres were selected and subjected individually to the faecal fermentation procedure and spiked with a variety of pathogens. We chose four clinically relevant gastrointestinal pathogens: *Escherichia coli, Salmonella enterica, Enterococcus faecium* and *Listeria monocytogenes.* The spiked pathogens were quantified after 24-hours fermentation using quantitative real-time PCR (qPCR) targeting a unique region of the genome of the specific pathogen being tested. This approach allowed the assessment of any impact of prebiotic fibre on pathogen growth in the *ex vivo* distal model of the human gut. To our knowledge, this was the first experiment of this type employing this approach. While the *ex vivo* conditions are far from those that would be encountered in the human gastro-intestinal tract, they open up a new avenue of research to support the use of various prebiotic compounds to provide colonisation resistance in animal models and the human gut.

The next two chapters focus more on the influence of dietary substrates on the microbiota, as opposed to promoting colonisation resistance. In chapter 4, the *ex vivo* model was employed to examine the impact of prebiotic fibres and dietary lipids on faecal samples collected from patients with metabolic disorders: obesity and type 2 diabetes. There are some indications from animal models (Khan et al., 2014) that there might be differences in microbiota composition between healthy and metabolic syndrome patients (Dabke et al., 2019); the latter, in turn, may have abnormal metabolic function such as aberrant digestion of dietary substrates (Turnbaugh et al., 2006). Given that these conditions are characterised by a microbiome imbalance, loss of genomic diversity and enrichment of certain microbes, we sought to test if a range of prebiotic and lipid substrates can promote gut diversity and encourage the growth of beneficial bacteria associated with a healthy gut microbiome. Considering the host variability, which has profound effects on reproducibility and robustness in human microbiota studies (Vujkovic-Cvijin et al., 2020), the high-throughput *ex vivo* model and extensive 16S analysis were employed to enable multiple dietary conditions to be tested in tandem, and for each human subject, in order to attempt to elucidate microbiota members that are truly enriched in response to dietary treatment.

The final chapter in this thesis attempts to explore an aspect of diet-microbiome interactions which is barely covered in the literature. Oily fish is considered to be an important component of the Mediterranean diet which has been found to have measurable benefits on the microbiome (De Filippis et al., 2016). While there are some studies on oily fish consumption on the bacteriome (Parolini, 2019), to our knowledge, there are no studies to date on effects of oily fish intake and the human gut virome. The gut virome is primarily composed of bacteriophage, viruses that infect bacteria, and they have been shown to be inter-individually unique, relatively stable in human subjects over time, and potentially implicated in certain gastrointestinal disorders such as inflammatory bowel disease (IBD) (Norman et al., 2015). However, very little is known about the influence of diet on this component of the microbiome. Furthermore, the potential anti-inflammatory effect of polyunsaturated fatty acids (PUFAs) in fish have been linked with reduction of ischaemic pathologies such as atherosclerosis (Yamagata, 2017); however the link between PUFAs and gastrointestinal inflammatory disorders is limited. The pilot study described in Chapter 5 uses faecal samples from a previous fish consumption intervention study in human volunteers and seeks to answer two questions: whether the stability of the virome can be impacted by diet and whether oily fish consumption selects for bacteriophage that are associated with a healthy virome state i.e., those negatively associated with gastrointestinal disorders. As little research has been performed on the human virome, the majority of sequencing data from virome studies have no homology in the genomic databases and are termed “viral dark matter”. The combination of these unknowns and inter-individual variation, makes it difficult to pinpoint precisely the link between the gut virome and human health and disease. It was hoped that by demonstrating that diet can influence certain viral taxa between subjects, it can help shed light on the darkness of this unknown fraction of the microbiome.

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# Chapter 1: Literature review

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## Effect of diet on pathogen performance in the microbiome

## (Submitted for publication in Microbiome Research Reports)


## Abstract

Intricate interactions among commensal bacteria, dietary substrates and immune responses are central to defining microbiome community composition, which play a key role in preventing enteric pathogen infection; a dynamic phenomenon referred to as colonisation resistance. However, the impact of diet on sculpting microbiota membership, and ultimately colonisation resistance has been overlooked. Furthermore, pathogens have evolved strategies to evade colonisation resistance and out-compete commensal microbiota by using unique nutrient utilisation pathways, by exploiting microbial metabolites as nutrient sources or by environmental cues to induce virulence gene expression. In this review, we will discuss the interplay between diet, microbiota and their associated metabolites, and how these can contribute to or preclude pathogen survival.


## Introduction

The gut microbiota has coevolved with its host over millions of years and augments the coding potential of the human genome (~22,000 genes) by upwards of 500-fold (J. Li & Jia, 2014). Indeed, the human genome itself, encodes at most only 17 enzymes involved in food digestion, mainly the digestion of starch, sucrose and lactose (Kaoutari et al., 2013). On the other hand, our gut microbiota encodes upwards of 60,000 CAZymes (Carbohydrate-Active Enzymes), with diverse specificities, facilitating the depolymerisation and fermentation of complex dietary polysaccharides into host utilisable short chain fatty acids (SCFAs) (E. D. Sonnenburg & Sonnenburg, 2014). This gut microbiota is heterogeneous and highly personalised and while bacterial enterotypes cluster independently of nationality, ethnicity, sex, age or BMI (G. D. Wu et al., 2011), diet is the dominant selective force that

defines microbiota membership and functionality (David et al., 2014). Diet is also the simplest to customise and therefore presents the most straightforward route for therapeutic intervention.

Distribution of bacteria throughout the gastrointestinal tract varies, from $10^3$–$10^4$ cells/ml in the stomach and upper small intestine, to $10^{11}$ cells/ml in the colon (Sender & Fuchs, 2016). Furthermore, taxonomic composition of these communities is niche-specific, and largely defined by the nutritional requirements of the residing bacteria. These contentions are supported by observations in gnotobiotic mouse models whereby concentrations of individual dietary components correlate with the relative abundance of specific microbiota members (Faith et al., 2011). For example, *Bacteroides cellulosilyticus* is controlled by administration of different concentrations of the prebiotic fibre arabinoxylan (M. Wu et al., 2015). This phenomenon opens up potential for therapeutic probiotic colonisation, which has been demonstrated by Kearney and colleagues; administration of the seaweed polysaccharide polyphyran and a polyphyran-degrading commensal *Bacteroides plebius* enabled successful engraftment of this species (Kearney et al., 2018).

Bacterial pathogens must overcome an array of obstacles such as oesophageal peristalsis, stomach pH and locating a permissible niche in the intestine in order to access nutrients to begin replication and to achieve successful colonisation in the GI tract. The final hurdle is to overcome resident commensal bacteria of the large intestine. However, continuous competition for nutrients, and compartments, the production of antimicrobial substances by commensals, and the barrage of immune responses evoked by the commensals themselves collectively give way to the phenomenon of colonisation resistance. For example, commensal symbionts and their related pathogens often compete with each other for metabolic resources compared with distant unrelated species. These metabolites include diverse carbon sources, bile acids, trace metals and vitamins. Those bacteria which possess high-affinity transporters for available nutrients will ultimately define the microbial community structure, but also serve as a barrier for gastrointestinal pathogens. However, many pathogens confer the ability to subvert competition with commensal bacteria, often by generating

their own specific niche to suit their metabolic needs. As an example, invasion of epithelial cells provides an environment suited to intracellular pathogens. Additionally, there is accumulating evidence to suggest that intestinal pathogens/pathobionts may subvert and exploit the host immune response to induce microbial dysbiosis and improve conditions for their subsequent colonisation (Darfeuille-Michaud et al., 2004; Lupp et al., 2007; Stecher et al., 2007). Furthermore, intestinal pathogens have evolved unique nutrient utilisation pathways in relation to their symbiotic counterparts; *Escherichia* can utilise alternative sugar sources to that of their commensal rivals (Bertin et al., 2011; Fabich et al., 2008), and gain a competitive advantage. In this regard, what we consume may have the potential to alter bacterial networks and shift the balance in favour of or against pathogen survival.

Indeed, accumulating evidence over the past decade has linked the high-fat/high-sugar/low-fibre 'Western diet' with a myriad of ever-increasing GI and metabolic disorders. It is widely acknowledged that the microbial dysbiosis resulting from this lifestyle is a major contributing factor to the epidemic of GI and metabolic disorders (Turnbaugh et al., 2009). One could speculate whether long-term dietary regimes could increase or decrease the host's colonisation resistance to enteric pathogens. In this review, the interactions among diet, the microbiota, colonisation resistance and pathogen performance will be examined by focusing on the keystone taxa and metabolites involved.

## How Neonatal and Infant Diet Protect against Enteric Infection by Modulating the Gut Microbiota

Neonates and the elderly face the greatest susceptibility to gastrointestinal infections. This susceptibility in new-borns has been generally attributed to the immaturity of the adaptive and innate immune systems; premature new-borns often display a heightened risk of suffering from excessive inflammation, which decreases as they age (Kollmann et al., 2012). Given that the gut microbiota is involved in the development of the immune system (Kamada et al., 2013) and the neonatal microbiota

is less diverse than the adult microbiota, research is shifting toward the potential role that specific gut taxa play in maturation of immune function and colonisation resistance.

Naturally delivered infants are generally dominated by bacterial groups associated with maternal vaginal microbiota (e.g., *Atopobium, Bacteroides, Clostridium, Escherichia coli, Streptococcus* spp. and *Prevotella)*, whereas C-section-born infants are dominated by the taxa associated with the skin microbiota such as *Staphylococcus* spp. (Dominguez-Bello et al., 2010). Breastfeeding aids in the initial colonisation of key taxa: *Bifidobacterium* and *Lactobacillus* (Solis et al., 2010), with the former involved in the digestion of human milk oligosaccharides (HMOs) which are resistant to human enzymatic digestion (Sela & Mills, 2010). The resulting fermentation of these compounds produces lactate (Z. T. Yu et al., 2013) and SCFAs, specifically acetate, which accounts for 80% of the total SCFA production in the infant gut (Bridgman et al., 2017) compared with 50% in the adult gut (Louis, Scott, Duncan, & Flint, 2007). The interactions between pathogens and SCFAs will be discussed later.

The distinction between microbiota community composition of human breast milk-fed and formulae-fed infants is clear; with the general consensus that human breast milk directs the propagation of beneficial bacteria and their related metabolites (Lara-Villoslada et al., 2007; Zivkovic et al., 2011). Lactation drives the colonisation of *Bifidobacterium* in infants, and has been shown to play a key role in maturation of the immune system (for a review, see (Ruiz et al., 2017)). Moreover, in mice, IgG in breast milk derived from mothers previously infected with *Citrobacter rodentium*, is passed to the offspring, enhancing colonisation resistance when challenged by this pathogen (Caballero-Flores et al., 2019). Probiotics derived from breast milk have shown great promise in mitigating the risk of necrotising enterocolitis (NEC), the most common GI disease in preterm infants and the leading cause of death in extremely preterm infants from 2 weeks to 2 months of age (Patel et al., 2015). NEC is a proinflammatory disease, in which an accumulation of inflammatory cells infiltrates the intestinal mucosa leading to subsequent sepsis (Egan et al., 2016), while functional regulatory T cells (Treg) are significantly decreased. Perhaps the most promising “probiotic” is *B.*

*longum* subsp. *infantis,* an extremely proficient coloniser of the infant gut, exhibiting decreasing NEC incidence in neonates (Underwood et al., 2015). More recently, a human breast milk-derived commensal *Propionibacterium* strain UF1, belonging to the same phylum as *Bifidobacteria*, the *Actinobacteria*, has been shown to mitigate NEC-like injury in mice (Colliou et al., 2017) and conferred protection against *Listeria monocytogenes* infection (Colliou et al., 2018) via Treg17 cell upregulation.

Along with *Bifidobacterium*, the other key taxa involved in the fermentation of HMOs are *Bacteroides* (Marcobal et al., 2011). Vaginal birth and breastfeeding significantly improve *Bacteroides* colonisation (Jakobsson et al., 2014); suggesting a long co-evolved symbiotic relationship between the host and taxa, and this symbiosis is reflected in studies demonstrating their role in immune system development (Round & Mazmanian, 2010; Telesford et al., 2015). The immunosuppressive effect of polysaccharide A from *B. fragilis* is achieved by promoting differentiation of $FoxP3^+$ regulatory T cells (Telesford et al., 2015), which may be beneficial to the host given that many pathogens favour a gut-inflamed environment. However, the supposed reliance on *Bacteroides* spp. for the development of the infant immune system is not clear, as an observational study in Northern Europe observed reduced prevalence of *Bacteroides* and Type I diabetes (T1D) in Russian children, relative to their counterparts from Finland and Estonia, where *Bacteroides* and T1D were more common (Vatanen et al., 2016). The Bacteroidetes phylum primarily produces acetate and propionate (Macfarlane & Macfarlane, 2003). Acetate has been observed to decrease the frequency of autoreactive T cells, and a diet designed to release large amounts of acetate protected against the development of T1D in mice (Marino et al., 2017). However, *Bacteroides* spp. that may be a risk factor for T1D, might be unrelated to the production of acetate and its associated metabolites, but more likely related to the overexposure to *Bacteroides* – associated LPS derived from HMO-utilising *Bacteroides* (Vatanen et al., 2016).

As the diet changes from maternal milk to fibre-rich foods as the infant matures, the infant microbiome acquires members of *Bacteroidales* and the butyrate-producing *Clostridiales*. *Clostridium* species have been associated with the increasing abundance and activity of $T_{reg}$ cells in the colon

(Atarashi et al., 2013) by providing bacterial antigens. Administration of members of the Clostridiales, but not Bacteroidales, provided protection to germ-free adult mice, colonised with neonatal microbiota, against infection from *Salmonella* and *Citrobacter* (Y. G. Kim & Sakamoto, 2017). These researchers hypothesise that in the first days of life, oxygen consumption by aerobic or facultative anaerobes enhances the ability of the strict anaerobes, *Clostridiales*, to colonise the gut, which, in turn, provides protection against pathogen infection. Furthermore, when succinate was administered in the drinking water to mice, it reduced oxygen intestinal content, which in turn, enhanced *Clostridales* colonisation. Interestingly, the authors demonstrated that it was these bacterial groups that abrogated infection and that host immunity did not contribute to the *Clostridia*-mediated effect. Similarly, a longitudinal study examining faecal samples from an asymptomatic infant carrier of *Clostridioides difficile* (an infant female born by C-section), from pre-weaning to weaning, revealed a dramatic change in microbiota composition within the first five days of transition from breast milk to cow's milk and solid foods (Davis et al., 2016). A rapid decline and eventual disappearance of *C. difficle,* accompanied with an increase in relative abundance of Bacteriodales/Clostridiales observed during weaning, were likely responsible for the expulsion of *C. difficile*.

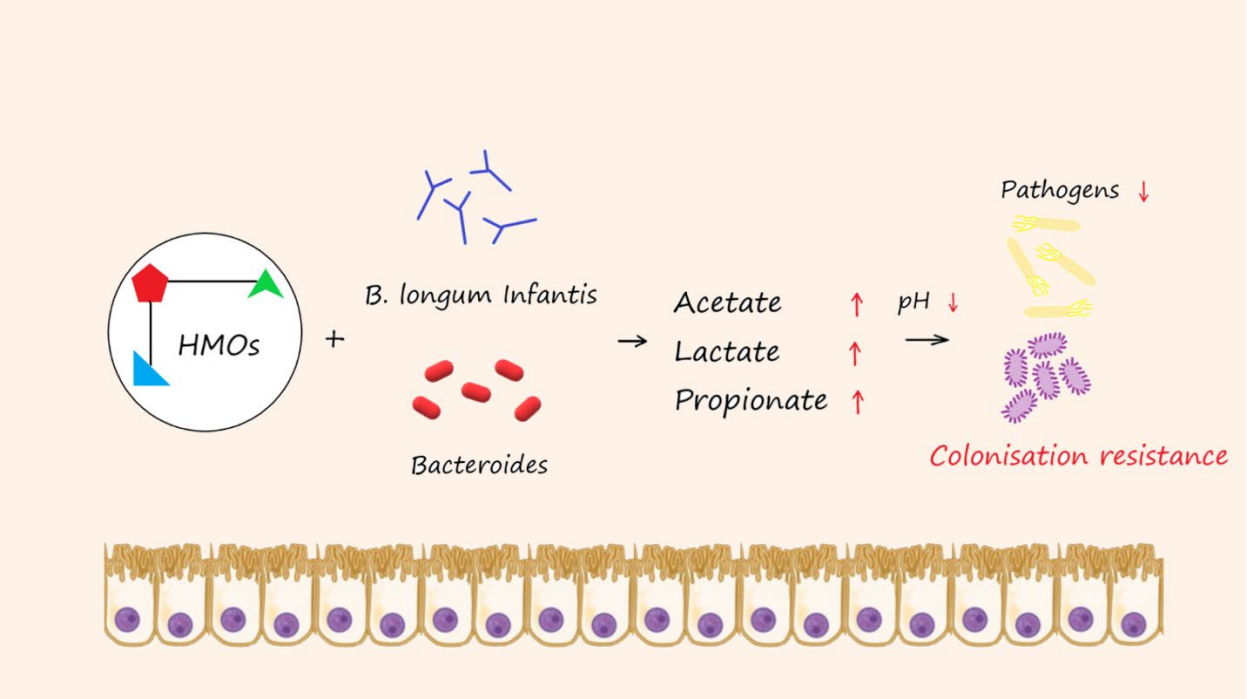



*Figure 1.1 – Metabolism of HMOs lowers gut pH boosting colonisation resistance*

*Human Milk Oligosaccharides (HMOs) are metabolised by initial colonisers (Bifidobacterium longum ssp. infantis & Bacteroides) in the infant gut, producing SCFAs and subsequently lowering pH & increasing colonisation resistance against gastrointestinal pathogens.*

## The Role of Dietary Fibre in Promoting Microbial Diversity, Maintaining the Mucus Barrier and the Link with Colonisation Resistance

There is an appreciation that the diet of westernised nations has declined in the quantity of fermentable fibre intake, which has been associated with the coincidental rise in diseases such as heart disease, diabetes and colorectal cancer (Burkitt et al., 1972). Human populations with a diet rich in dietary fibre exhibit increased diversity of the microbiota, such as experienced by the Hadza tribal people from Tanzania (Schnorr et al., 2014). Conversely, a 'westernised' low fibre diet can result in a progressive loss of microbial diversity (E. D. Sonnenburg et al., 2016). While there is greater prevalence of gastrointestinal pathogen infections in developing countries compared with their western counterparts (S. M. Fletcher et al., 2013), this may be attributed to poverty related risk factors and sanitation practices. Interestingly, in a study comparing the microbiota of children from Burkina Faso and their European counterparts, it was observed that *Enterobacteriaceae* (*Shigella/Escherichia*) were underrepresented in the Burkina Faso cohort relative to European children (De Filippo et al., 2010). The authors hypothesized that the high fibre diet consumed by the Burkina Faso children selects for a bacterial community capable of maximising energy intake from the fibres, while at the same time protecting them from potential enteropathogens. However, whether diet-induced increases in microbial diversity or specific taxa and their related metabolites can improve colonisation resistance in humans is an intriguing but an essential question to answer. The majority of studies to date supporting the inverse link between diversity and infection stem from murine models. Both germ-free and antibiotic-treated mice display increased susceptibility to enteric infections, which are associated with reduced microbiota diversity (Antonopoulos et al., 2009; Kamada et al., 2012). While the bulk of studies support microbial-induced benefits derived from fermentable fibre, recent evidence suggests that cellulose, an insoluble fibre, also exerts enrichment in protective microbial species and provides colonisation resistance (Morowitz et al., 2017). A comparison of the impact of soluble dietary fibre (oat β-glucan) versus insoluble dietary fibre (microcellulose) in mice gut microbiota reported reduced alpha-diversity and higher relative abundance of fibre-degrading *Bacteroides* and pathogenic

*Proteobacteria* in the former (Luo et al., 2017), indicating potential cross feeding between commensals and pathogens. Higher alpha-diversity was observed when the two fibres were mixed. Such mixtures would reflect better a human diet, which contains a mixture of soluble and insoluble fibres.

SCFAs are the end products of the fermentation of dietary fibres and have a broad range of effects on mammalian host physiology, can attenuate inflammation, and alter microbial composition and pathogen virulence. SCFAs are saturated aliphatic organic acids comprised of one to six carbons of which acetate (C2), propionate (C3) and butyrate (C4) are the most abundant (>95%) (Cook & Sellin, 1998). The success of the invading pathogen depends on the biotic interactions within the community, including exchanging and competing for metabolites (Kinnunen et al., 2016). The effect of SCFAs can be a double-edged sword for invading pathogens, with beneficial or inhibitory effects depending on concentration and environmental pH.

The glycoprotein-rich layer that covers the gut epithelium provides the first line of defence against both commensal and pathogenic bacteria. Evidence has suggested that reduced dietary fibre intake is associated with a thinner colonic mucus (Hedemann et al., 2009). Indeed, a recent study demonstrated that during chronic or intermittent fibre deficiency in mice, the gut microbiota degraded the host-secreted glycoproteins as an energy source, and in turn, resulted in an unstable mucus barrier function, increasing susceptibility to infection by *Citrobacter rodentium* (Desai et al., 2016). One of the key players in the mucosal-microbiota environment is *Akkermansia muciniphila,* comprising 1-4% of colonic microbes, which preferentially degrades mucin as its primary nutrient source (Ottman & Davids, 2017) and is inversely correlated with a myriad of GI diseases. The distribution of *Akkermansia* in the GI tract of vertebrates is vast (Belzer & de Vos, 2012), suggesting a long-term co-evolutionary relationship with their hosts and underlining their symbiotic importance. Mucus consists primarily of the heavily O-glycosylated protein, mucin 2, which *Akkermansia* can degrade with an arsenal of enzymes (Ottman et al., 2016). Continuous production of mucin by the goblet cells contributes to both mucin presence in the mucus layer and in the colonic lumen

(Johansson, 2012). *Akkermansia* degradation of mucin releases fewer complex carbohydrates and acetate, providing a food source for butyrate-producing bacteria. Thus, *Akkermansia* turnover of mucin contributes to maintenance of intestinal integrity and microbial community homeostasis. Other species, including *Bacteroides thetaiotaomicron* and *Bifidobacterium bifidum,* that possess the capacity to break down mucus O-glycans have been identified in some studies (Martens et al., 2008; Turroni et al., 2010). Similarly, *Akkermansia*, acetate producers like *B. thetaiotaomicron* may require a commensal adjuvant e.g., *Faecalibacterium prausnitzii,* an acetate consumer and butyrate producer, in order to maintain colonic epithelial homeostasis (Wrzosek et al., 2013). *F. prausnitzii* is capable of immunosuppression through blocking of NF- κB activation and anti-inflammatory cytokine production, and reduced abundance of *F. prausnitzii* has been observed in inflammatory bowel disease subjects (Lopez-Siles et al., 2018), indicating a harmonious relationship between mucosal commensals and the host in a healthy gut environment. Conversely, the glycans released from the mucin may actually provide a food source to GI pathogens. Ng et al. demonstrated that *Salmonella* and *C. difficile* thrived on *B. thetaiotaomicron*-liberated glycans following antibiotic induced disruption of mono-colonised mice compared to germ-free mice (Ng et al., 2013). Furthermore, *B. thetaiotaomicron* is capable of altering the metabolic landscape by secretion of metabolites such as succinate that enhance Enterohemorrhagic *Escherichia coli* (EHEC) (Curtis et al., 2014) and *C. difficile* expansion (Ferreyra et al., 2014). It must be noted, however, that increases in luminal oxygen, such as those observed as a result of inflammation or antibiotic treatment contribute to a reduction of obligate anaerobes including members of the *Clostridia* e.g., *F. prausnitzii*, and enabling the expanse of facultative anaerobes of the *Proteobacteria* phylum e.g., *Salmonella.* Interestingly, a recent study demonstrated that commensal *Enterobactericeae* in combination with *Clostridia* conferred colonisation resistance against *Salmonella* through competition for luminal oxygen in neonatal chicks (Litvak et al., 2019).

The western-style diet (high-fat/low-fibre) has been associated with a decrease in *Bacteroides, Bifidobacterium* and *Akkermansia* (Everard et al., 2011; Everard et al., 2014), subsequently affecting intestinal mucosal homeostasis and permeability; the effects of which can be

ameliorated by addition of dietary fibres (Everard et al., 2011; Everard et al., 2014; Schroeder et al., 2018), and thereby potentially protecting against infection (Desai et al., 2016). Moreover, the Western diet is characterised by an increase in the Firmcutes/Bacteroidetes ratio and weight gain in humans. A link between obesity and risk of infection of the enteric pathogen *C. difficile* has been identified (Bishara et al., 2013), and *C. difficile* colonisation has been attenuated in mouse models by addition of dietary fibre (Hryckowian et al., 2018a), suggesting a fibre deficient diet may be a risk factor for *C. difficile* infection and persistence. Collectively, these data suggest that individual fibres select for bacteria that are best at metabolising the specific fibre, leading to reduced diversity and hence a higher risk for colonisation of pathogens. Mixtures of dietary fibres that better represent a human diet promote higher gut microbiota diversity, and thus improve colonisation resistance and mucosal integrity.

## The Double-Edged Sword of Short Chain Fatty Acids

Of the three major SCFAs, acetate is the most abundant, constituting approximately 60% in the colon and stool (Cummings et al., 1987). It can be produced from pyruvate via acetyl-CoA by most of the enteric bacteria (*Akkermansia muciniphila*, *Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp., *Ruminococcus* spp.) or from pyruvate via the Wood-Ljungdahl pathway (*Blautia hydrogenotrophica, Clostridium* spp., *Streptococcus* spp.) (Louis et al., 2014; Rey et al., 2010). Acetate may have anti-inflammatory effects *in vivo*, by decreasing the LPS-stimulated TNFα response from neutrophils (Tedelind et al., 2007), albeit to a lesser extent than butyrate and propionate. Moreover, SCFAs derived from a high-fibre diet have been shown to protect against food allergy (Tan et al., 2016); *Bifidobacterium* alleviated food allergy in mice (J. H. Kim et al., 2016) and *Bifidobacterium* supplementation to pregnant women significantly reduced allergy in offspring (Enomoto et al., 2014). Whether these effects are directly attributed to changes in microbiota composition, or indeed SCFA concentrations, have yet to be determined.

Many pathogens use SCFAs as environmental cues to determine their biogeographical location within the gut and switch on genes accordingly e.g., virulence factors to colonise the preferential location. For example, *Salmonella typhimurium* preferably colonises the ileum (Carter & Collins, 1974), where the typical concentration of acetate is 30mM. This concentration enhances the expression of SPI-1 (*Salmonella* Pathogenicity Island 1)-encoded T3SS (Type three secretion system), which is involved in invasion of the host. Similarly, SPI-1 gene expression is promoted in the presence of minute concentrations of formate (~8mM), like those encountered in the ileum (Huang et al., 2008), suggesting *S. typhimurium* has multiple mechanisms to determine its biogeographical location. Furthermore, streptomycin-treated mice were more susceptible to *S. typhimurium* infection in the ileum compared with untreated mice, where the SCFA concentrations remained unchanged, suggesting that ileal commensal bacteria can also affect *S. thyphimurium* virulence, likely by physically blocking colonisation or contributing to the immune response (Garner et al., 2009). On the contrary, higher concentrations of propionate and butyrate, or the absence of formate i.e., similar to conditions found in the colon, suppress the expression of T3SS (Lawhon et al., 2002) and invasion is inhibited. Colonic environmental cues likely initiate adaptation of *S. typhimurium* gene expression to endure environmental insults and/or preparation for transmission to a new host. Interestingly, a recent study observed reduced ileal colonisation of *Salmonella* in mice which were pre-treated with a consortium of *Bacteroides spp.* with a high capacity for production of propionate (Jacobson et al., 2018), through disruption of intracellular pH homeostasis.

Similarly, Enterohaemorrhagic *E. coli* (EHEC) utilises SCFAs for virulence gene regulation; its preferred site of colonisation and infection is the colon (Croxen et al., 2013) where the ratio of acetate:butyrate tends to be lower. However, some studies have demonstrated that acetate can be refractory to the virulence of EHEC (Shin et al., 2002), by lowering intestinal pH (Cherrington et al., 1991). Mixtures of SCFAs that represent the small intestine significantly upregulate EHEC flagellar genes and motility whereas colonic SCFA concentrations have a down-regulatory effect (Lackraj et al., 2016). Expression of the *iha* gene that encodes an adherence-conferring outer membrane protein,

however, is upregulated by EHEC in the small intestine (Herold et al., 2009), and is crucial for colonisation and infection. Consequently, ileal SCFA concentrations activate EHEC flagellar production and motility, followed by expression of genes involved in type III secretion and adherence when approaching colonic SCFA concentrations (Tobe et al., 2011), and thereby permitting efficient adherence in EHEC's preferred niche. Production of acetate from *Bifidobacterium* has been demonstrated to inhibit the translocation of Shiga toxin of the EHEC 0157:H7 from the gut lumen (Fukuda et al., 2011) and prevent 0157:H7-induced colonic epithelial cell death via *Bifidobacterium* acetate-upregulated carbohydrate transporters (Fukuda et al., 2012). *Campylobacter jejuni* has similar mechanisms to sense metabolites and hence spatial distribution. In avian hosts, where *Campylobacter jejuni* behave as symbionts in the lower GI tract, concentrations of acetate are high and allow for expression of genes that permit commensal colonisation (Luethy et al., 2017). Conversely, high concentrations of lactate, similar to those observed in the upper GI tract, where *C. jejuni* colonises less efficiently, repress the genes involved in colonisation. The authors speculate whether *C. jejuni* utilises similar environmental cues in order to colonise humans and, thereby, causing diarrhoeal disease.

SCFAs have also been associated with concentration-dependent negative effects on *C. difficile* growth (Rolfe, 1984) and as SCFA concentrations are reduced following antibiotic treatment, this could be a contributing factor to its subsequent colonisation. Other studies have demonstrated that SCFAs increase the expression of Toxin B (TcdB) an essential virulence factor (Hryckowian et al., 2018a; Lyras et al., 2009). SCFAs may serve as a signal to *C. difficile* of an inhospitable and competitive environment; therefore, upregulation of TcdB may provide a survival mechanism. The success of faecal microbiota transplants (FMT) in treatment of *C. difficile* infection (CDI) reinforces the role of commensal microbiota (*Bacteroides, Clostridium* clusters IX and XIVa) in treatment of CDI (van Nood et al., 2013). Early *in vitro* studies have demonstrated that dietary fibre polysaccharides induce a bifidogenic effect and hence increase SCFA production, which may result in enhanced colonisation resistance against *C. difficile* (Hopkins & Macfarlane, 2003). Likewise, an *in vivo* study found that *C. difficile*-infected mice fed a diet rich in dietary fibre had stimulated growth of fibre-utilising taxa (*Bacteroides* spp.) and their

associated metabolites, i.e. SCFAs, (Hryckowian et al., 2018a), with decreased *C. difficile* fitness and numbers, while toxin expression was increased (Hryckowian et al., 2018a).

Among the major SCFAs, butyrate is the most extensively studied, in large part, owing to the beneficial effects on both colonocyte energy metabolism and intestinal homeostasis (Guilloteau et al., 2010). Butyrate is the least abundant of the three SCFAs produced, comprising 15% of the total SCFA in humans (Spiller et al., 1980). Butyrate can upregulate mucin 2, reinforcing the mucus layer of the intestinal mucosa, and leading to enhanced protection against luminal pathogens (Willemsen et al., 2003). Butyrate is formed in the so-called 'classical pathway', by the condensation of two molecules of acetyl-CoA, and the subsequent reduction to butyryl-CoA, which can be converted to butyrate by members of the *Clostridia* family (*Anaerostipes* Spp., *Coprococcus catus*, *Eubacterium rectale, Eubacterium hallii, Faecalibacterium prausnitzii, Roseburia* spp.) (Duncan et al., 2002; Louis et al., 2014). *Anaerostipes* spp. and *Eubacterium hallii* are also capable of utilising lactate as the substrate for production of butyrate. Alternatively, butyrate can be synthesised from butyryl-CoA by the phosphotransbutyrylase/butyrate kinase route (*Coprococcus comes, Coprococcus eutactus*) (Duncan et al., 2002).

Butyrate is at its highest concentration in the colon, and specific pathogens use the high concentrations of butyrate as an environmental cue in order to express virulence factors. For example, EHEC exhibited high adherence to Caco-2 cells in the presence of butyrate, whereas acetate and propionate had little effect (Nakanishi et al., 2009). Similarly, shiga toxin-producing *E. coli* (STEC) exhibit increased adherence in the presence of SCFA concentrations that reflect those that are found in the colon (Herold et al., 2009). In a study by Zumbrun et al, the authors demonstrated that high fibre diet contributed to elevated butyrate and reduced commensal *Escherichia* compared to a low fibre diet; these resulting changes led to higher STEC colonisation, more weight loss and higher mortality in high fiber diet-fed mice (Zumbrun et al., 2013). In IBD patients, faecal butyrate concentrations are higher than healthy controls, despite lower abundance of butyrate-producing taxa

(Ferrer-Picon et al., 2019), and this may be explained by its impaired uptake and oxidation by inflamed colonocytes. While colonic SCFA concentrations seem to exacerbate certain *Escherichia* pathologies, colonic concentrations of butyrate (Gantois et al., 2006) and propionate (Hung et al., 2013) have an antagonistic effect towards invasion gene expression in *Salmonella*, by down-regulating expression of SPI-1.

The butyrate-producing *Clostridia* are obligate anaerobes capable of maintaining a healthy gut homeostasis. Under eubiosis, the *Clostridia*-derived butyrate is the major energy source of the colonocytes and activates epithelial signalling through the intracellular butyrate sensor PPAR-y, driving mitochondrial β-oxidation of this substrate. *Salmonella* virulence factors induce inflammation during the early stages of infection and these virulence factors have been shown to deplete the butyrate-producing *Clostridia* from the gut-associated community, leading to an epithelial aerobic environment which ultimately favours the aerobic expansion of the pathogen (Rivera-Chavez et al., 2016). Moreover, antibiotic treatment resulting in a reduction of PPAR-y signalling i.e. increased bioavailability of oxygen, has been shown to exacerbate this effect (Byndloss & Olsan, 2017). The reduced butyrate concentrations observed during *Salmonella* infection stimulates the colonocytes to switch from β-oxidation of butyrate to lactate fermentation and increasing luminal lactate. *Salmonella* exploit this increase in lactate and utilise this carbon source for subsequent expansion (Gillis et al., 2018). Recently, genes involved in the direct β-oxidation of butyrate have been identified in *Salmonella*, and excision of the operon drove the transition from a gastrointestinal to an extraintestinal pathogen i.e., non-typhiodal to typhoidal (Bronner et al., 2018), suggesting utilisation of butyrate plays a crucial role in *Salmonella* GI disease. Moreover, specific members of *Clostridia* are some of the few bacterial species capable of utilising fructose-asparagine, a known food source of *Salmonella* which improves its fitness (J. Wu et al., 2018), and could explain an evolutionary competition between these species.

In silico analysis of butyrate production pathways in GI pathogens has identified members of the *Fusobacterium* genus and a few pathogenic strains of *Clostridium* (*C. tetani* and *C. tetanophorum*) with the ability to produce butyrate (Anand et al., 2016). However, their capacity to synthesise butyrate involves amino acids, primarily 4Ab, glutarate and lysine, as substrates, and are different to those observed in commensals. The end product of this fermentation yields ammonia, higher concentrations of which are associated with colorectal cancer (Corpet et al., 1995). On the whole, the effects of SCFAs on pathogen colonisation are concentration dependent, with higher or lower concentrations having the capacity to be either antagonistic or hospitable, respectively, depending on the species and its preferred niche.

**Prebiotics**

Prebiotics are selectively fermented ingredients that beneficially affect the host by stimulating the growth and/or functional activity of one or a limited number of bacteria in the colon, and thus improve host health (Gibson et al., 2017). The premise that these bacteria can potentially inhibit or obstruct the growth or virulence of pathogens is nothing new, and has been suggested as an alternative to antibiotic growth promoters in animals. The best documented benefits stem from the use of indigestible oligosaccharides, such as fructans and galactans (Rastall & Gibson, 2015); however, consideration into the ability of pathogens to ferment or utilise the prebiotics or the associated metabolites should be taken into account (Schouler et al., 2009). Cereals, fruits and legumes are natural sources of prebiotics, whilst the active components are often synthesised using industrial chemical and enzymatic methods. The majority of prebiotic studies on pathogen inhibition to date involve livestock and animal models, which vary in terms of physiology and microbiota composition; therefore, care should be taken in translating these results to humans.

Prebiotics such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are preferentially fermented into SCFAs by *Bifidobacterium* and *Lactobacillus* which have historically been viewed as beneficial bacteria, resulting in lowered luminal pH. Addition of oligosaccharides to poultry

feed have been shown to increase *Bifidobacterium* and *Lactobacillus* populations, while also being refractory to *E. coli* (Baurhoo et al., 2007) and *Salmonella* colonisation (Agunos et al., 2007; Azcarate-Peril et al., 2018). This outgrowth of prebiotic-utilising taxa has also been demonstrated to eliminate *C. difficile* in mice (Hryckowian et al., 2018a). The prebiotic inulin is primarily composed of FOS and has been shown to ameliorate low-grade inflammation through microbiota-dependent induction of IL-22 expression (Zou et al., 2018) and was able to prevent increased bacterial mucus penetration in high fat diet-fed mice (Schroeder et al., 2018). Furthermore, supplementation of high fat diet-fed mice with *Bifidobacterium longum* restored mucus growth (Schroeder et al., 2018), suggesting that prebiotic and probiotic treatments have the potential to prevent intestinal mucus abnormalities, which is a consequence of a high fat diet. However, a study by Miles et al. demonstrated that inulin may actually have the potential to exacerbate disease severity in response to inducers of colitis such as dextran sodium sulphate (DSS) in both low-fat and high-fat diets (Miles et al., 2017). Moreover, inflammation is crucial for the successful colonisation of *Salmonella*, through inflammatory-mediated expression of virulence factors; studies in rats have shown increased translocation of *Salmonella* when FOS is added to the diet (Petersen, Heegaard, Pedersen, Andersen, Sorensen, et al., 2009; Ten Bruggencate et al., 2005). Although the overwhelming data suggest the beneficial effects of inulin supplementation, more care is needed to define the specific mechanisms by which inulin impact the gut microbiota to protect against the effects of inflammation and improve mucosal integrity.

The other extensively studied prebiotic is GOS, which is commercially produced from lactose using glycoside hydrolases that catalyse transgalactosylation reactions (Gänzle, 2012). *In vitro* studies have demonstrated the protective effect of GOS against EHEC and *Cronobacter sakazakii*, through an anti-adherence mechanism (Quintero et al., 2011; Shoaf et al., 2006). Interestingly, a recent study on the *in vivo* protective effects of GOS on the murine EHEC model pathogen, *Citrobacter rodentium*, showed that GOS treatment prevented pathogen-induced intestinal tissue damage, independent of anti-adherence activity and *C. rodentium* numbers (Kittana et al., 2018). The authors speculate whether this effect may be a result of 'disease tolerance', whereby the host protects from direct tissue

damage through indirect immunopathological damage, a potentially overlooked mechanism in many animal studies (Medzhitov et al., 2012). Indeed, commensal bacteria that promote disease tolerance have recently been identified; such as an *E. coli* 021:H strain that protected against the gut pathogen *S. typhimurium* and Pneumonic pathogen *Burkholderia thailandensis*, through activation of the inflammasome and IGF-1 signalling (Schieber et al., 2015). In terms of the effect of GOS on microbiota composition, studies have identified a clear bifidogenic effect of GOS, while simultaneously lowering *E. coli, Helicobacter* and *Clostridium* spp. concentrations (Monteagudo-Mera et al., 2016). The combined effects of the prebiotic on SCFA production, microbiota composition, pathogen virulence and fitness make it difficult to pinpoint the exact mechanism involved in providing a protective effect. This lack of a known mechanism underscores the importance of thorough analysis in animal studies before extrapolating results to humans.

### Dietary lipids and bile acids

Bile acids are amphipathic biological detergents produced by the liver with the primary function of metabolising lipids in the GI tract (Chiang, 2009), and their production is linked to the ingestion of fatty foods. Those bile acids, which are not reabsorbed into the liver (~5%), can serve as substrates for colonic microbial metabolism i.e., hydrolysis of conjugated bile acids by bile salt hydrolases (BSH) or biotransformed into secondary bile acids by 7α-dehydroxylation, where they are either excreted in faeces or recirculated back into the liver through the enterohepatic circulation (Ridlon et al., 2006). Thus, changes in microbiota composition culminate in changes in the bile acid pool and this homeostatic imbalance is associated with a range of disease states including colorectal cancer (CRC), IBD and recurrent *C. difficile* infection (Staley et al., 2017). Recent advances have identified specific OTUs involved in bile acid biotransformations and correlated loss of specific taxa with development of disease. Conversely, previous infection of mice with *Yersinia pseudotuberculosis* remodels the microbiota to enrich for Deltaproteobacteria, a taurine metabolising class of bacteria which provide colonisation resistance to the pathogen *Klebsiella pneumoniae* (Stacy et al., 2021). Supplementing

mice with taurine alone, remodels the microbiota to enrich for bacteria that possess a sulfite reductase (dsr) gene, which catalyses taurine metabolism and releases the by-product hydrogen sulphide, an inhibitor of cellular respiration and consequently limits pathogen's ability to access oxygen and non-fermentable substrates. Thus, commensal bile acid interactions are intrinsically linked in both mitigation and amplification of colonisation resistance. Identifying the biochemical mechanisms which underpin the effect on colonisation resistance will improve our knowledge going forward and open up new avenues for therapeutic manipulation of the microbiota.

Antibiotic-induced destruction of the microbiota is associated with recurrent *C. difficile* infection (CDI). The protective role of the microbiota against *C. difficile* can be consolidated by the success of FMT (van Nood et al., 2013). *C. difficile* spores must germinate *in vivo* to develop into actively growing bacteria to produce enough toxins to initiate infection. *In vitro*, primary bile acids stimulate germination and secondary bile acids inhibit this process (Francis et al., 2013). Indeed, these interactions have been shown *in vivo* whereby depletion of secondary bile acids in the ileum resulted in *C. difficile* germination and growth (Theriot et al., 2014). Moreover, inflammation induced by *C. difficile* toxins subsequently changes this pathogen's nutrient metabolism pathways and enables it to thrive in the inflamed gut, particularly on products of collagen degradation, outcompeting commensal bacteria aside from members of *Bacteroides* genus, which can also utilise collagen degradation products (J. R. Fletcher et al., 2021). In a study by Buffie et al, the authors identified a single commensal that conferred resistance to *C. difficile; Clostridium scindens*, a bile acid 7α-dehydroxylating intestinal bacterium, which enhanced resistance in a secondary bile acid dependent fashion (Buffie et al., 2015). *C. scindens*-mediated restoration of secondary bile acids from host-derived bile salts were sufficient in inhibiting *C. difficile* germination, underpinning the pivotal role commensal bile acid-metabolising bacteria play in preventing recurrent *C. difficile* infection. Patients successfully treated for recurrent CDI have an enrichment of bile salt hydrolase-producing bacteria, the abundance of which negatively correlates with faecal concentrations of taurocholic acid, a primary bile acid (Mullish & McDonald, 2019). While obesity has been identified as a risk factor for *C. difficile*

infection (Bishara et al., 2013), and the microbiota composition of obese subjects is characterised by a decrease in the Bacteroidetes:Firmicutes ratio, there is no evidence to suggest that bile acid synthesis or enterohepatic circulation is altered by obesity. However, an obesity-driven altered and less diverse microbiota coupled with antibiotic elimination of bile-acid metabolising bacteria may very well contribute to increased risk of *C. difficile* infection.

High-fat diets promote the biosynthesis of bile, which can impact commensal microbiota that are sensitive to bile acid concentrations. Additionally, pathogens such as *S. typhimurium* are quite resilient to high bile acid concentrations and colonisation resistance to this pathogen is alleviated upon oleic acid or high fat diet supplementation in mice (Wotzka et al., 2019) and colonisation resistance is improved when switched back to a plant-based diet. Commensal *E. coli* that can compete with *S. typhimurium* through bile acid (Wotzka et al., 2019) or oxygen (Litvak et al., 2019) competition could provide a means of protection against this pathogen.

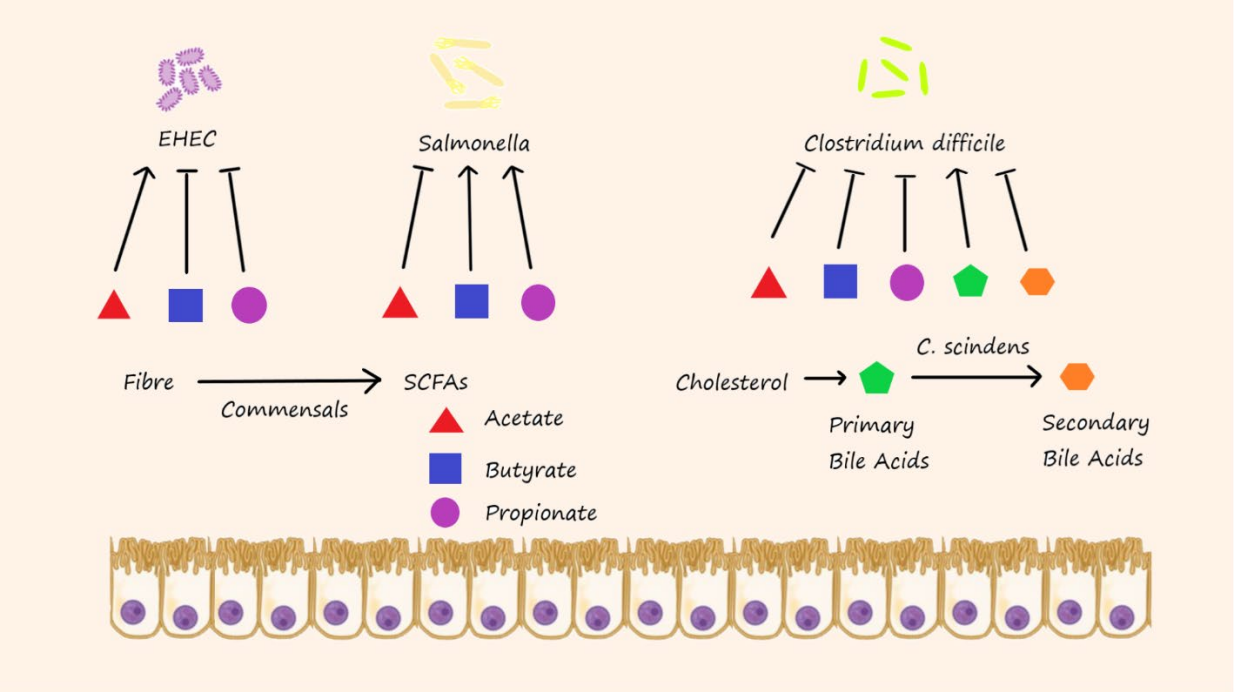



*Figure 1.2 – Differential effects of diet metabolites on pathogens*

*Metabolites from fibre degradation by commensals have differential effects on the success of an invading pathogen. Acetate promotes the growth of Enterohemorrhagic E. coli (EHEC), whereas butyrate and propionate repress growth. Conversely, acetate represses Salmonella growth, whereas butyrate and propionate promote growth. Colonisation of Clostridium difficile is increased by the cholesterol metabolites, primary bile acids. The commensal Clostridium scindens can limit the availability of primary bile acids by converting these to secondary bile acids, thus increasing colonisation resistance to C. difficile.*

## Protein and amino acids

Aberrations in microbiota community structure driven by antibiotics, infection and/or diet will affect protein homeostasis and can increase free amino acids in the gut, providing a nutritional niche upon which some pathogens can capitalise. Indeed, many gut pathogens such as EHEC (Warr et al., 2019), *V. cholerae* (Fu et al., 2013), *C. jejuni* (Gao et al., 2017), and *C. rodentium* (Caballero-Flores et al., 2020) have genes involved in amino acid biosynthesis upregulated upon gut colonisation. Moreover, the host relies on amino acid metabolism to support its immune responses against invading pathogens, with diets deficient in protein having a counterproductive effect on immune function, independent of the microbiota (Ochi et al., 2016). On the other hand, dietary administration of high protein and amino acid by-products stimulates the growth of pathogens (Caballero-Flores et al., 2020) and protein fermenting bacteria contributing to disease susceptibility (N. Ma et al., 2017). Therefore, identifying commensal competitors and pathogen metabolic pathways involved in protein fermentation and amino acid biosynthesis may help in developing new strategies to encourage colonisation resistance through diet.

D-amino acids are biosynthesised by gut bacteria as opposed to L-amino acids which humans biosynthesise or obtain from the diet. Tryptophan is an essential amino acid from diet in mammals and is primarily catabolised by commensal bacteria into various indole-containing metabolites. While tryptophan is required for optimal immune responses, such as T-cell proliferation, the commensal-mediated tryptophan metabolites can have disparating effects on gut pathogens. In *S. typhimurium,* indole induced expression of genes related to efflux-mediated multidrug resistance (Nikaido et al., 2012), while concomitantly decreasing the expression of genes involved in invasion located on the SPI-1 pathogenicity island (Kohli et al., 2018). Similarly, indole upregulates EHEC secretion of EspA and EspB via the type III secretion system enhancing this pathogen's ability to form attaching and effacing (A/E) lesions (Hirakawa et al., 2009), while other indole-derivatives can inhibit biofilm formation, motility and formation of A/E lesions (Bommarius et al., 2013). The enzyme indoleamine 2,3-

dioxygenase (IDO) catalyses the conversion of tryptophan into kynurenine, reducing the tryptophan pool in the gut, therby directly impacting various immune responses. *C. difficile* infection upregulates the expression of IDO, increasing the production of kynurenine subsequently depleting the tryptophan pool, and thereby diminishing the immune responses of the host toward this pathogen (El-Zaatari et al., 2014). All-in-all, tryptophan and its associated metabolites have direct effects on immune function and pleiotropic effects on gut pathogens, suggesting that this molecule will be of interest in colonisation resistance studies moving forward.

Many gut pathogens switch metabolic pathways depending on the environment e.g., in inflammation. The pathobiont Adherent Invasive *E. coli* (AIEC) shifts its metabolism to catabolise L-serine in the inflamed gut to maximise growth potential (Kitamoto et al., 2020), with L-serine having little effect on AIEC fitness in a healthy gut environment. Interestingly, AIEC bloom in the inflamed gut, and are significantly reduced when amino acids are reduced from the diet. *C. difficile* exploits the niche created following particular antibiotic treatments and this dysbiotic environment has increased availability of amino acids. Specifically, a recent study demonstrated that *C. difficile* is dependent on L-proline metabolism, as L-proline knockout *C. difficile* strains were unable to colonise the gut of germ-free mice transplanted with either a dysbiotic or healthy microbiota (Battaglioli et al., 2018). Furthermore, low-protein or low-proline diets given to mice substantially decreased wild-type *C. difficile* expansion suggesting that *C. difficile* is dependent on proline for adequate colonisation which can potentially be mediated through dietary intervention. Furthermore, commensals such as members of the *Clostridia* class, decreased the fitness advantage of *C. difficile* ability to ferment proline, through competition for this amino acid (Lopez et al., 2020). Likewise, EHEC was found to be reliant on proline for colonisation, with commensal *E. coli* that compete for proline attenuating the expansion of EHEC (Momose et al., 2008).

As discussed above, the microbiota can limit the colonisation of invading pathogens by depleting the concentration of amino acids in the gut. Some pathogens can overcome this problem by

inducing amino acid biosynthesis to subvert such a deficiency. Transposon sequencing is a powerful tool and allows for generation of a library of random pathogen mutants, for example, those defective in amino acid biosynthesis pathways (van Opijnen et al., 2015). This technique provides a means to estimate fitness contribution or essentiality of each genetic component in a bacterial genome. Caballero et al. applied this to *C. rodentium* and found that specific mutants deficient in the production of arginine, threonine, histidine, tryptophan, or isoleucine lost their competitive advantage in mice, compared to wild-type *C. rodentium* (Caballero-Flores et al., 2020). Moreover, these genes were significantly upregulated in conventional mice as opposed to germ-free mice, suggesting that *C. rodentium* specifically use these pathways to outcompete the microbiota. Feeding of high-protein diet to mice produced markedly better colonisation of *C. rodentium* compared to normal chow. While mouse studies like these inform a mechanistic understanding of pathogen colonisation, the importance of these findings in relation to human disease warrent further investigation.

### Trace elements

The majority of human enteric pathogens are part of a small group of bacterial families that belong to the phylum Proteobacteria; the *Enterobacteriaceae* (*E. coli, Yesinia, Salmonella, Shigella*), the *Vibrionaceae* (*Vibrio cholera*) and the *Camplyobacteriaceae* (*Camplyobacter*) (Stecher et al., 2013). In the normal intestine which is largely inhabited by commensals, mainly *Bacteroides* and *Firmicutes*, Proteobacteria only constitute <1% of microbiota populations. Outgrowth of Proteobacterial 'blooms' are a hallmark of gut 'dysbiosis' resulting from microbial perturbations caused by antibiotic therapy, dietary changes or inflammation.

The availability of micronutrient trace elements is essential to the successful colonisation of pathogens during infection. Nearly 60% of known enzymes contain at least one metal co-factor, with zinc being the most common, followed by iron and manganese (Andreini et al., 2008). In the inflamed gut, these dietary trace elements are heavily sequestered by high affinity binding proteins or kept in organelles that are not accessible to bacteria, in a process known as 'nutritional immunity' (Hood &

Skaar, 2012). Many proteobacterial pathogens are equipped with an array of high affinity siderophores, to help them overcome restriction of available metals and ultimately drive key cellular processes, which in turn sustains and propagates infection. Deficiency or increased supplementation of dietary trace elements may disrupt the commensal microbial populations and predispose individuals to infection.

Zinc deficiency is associated with increased *Enterobacteriaceae* and *Enterococcus,* with concomitant decreases in abundance of *Clostridiales* and *Verrucomicrobia* (*A. muciniphilia)* (Lopez & Skaar, 2018). Moreover, in a mouse model of enteroaggregative *E. coli* (EAEC)*,* a cause of traveller's diarrhoea, zinc deficient mice exhibited altered immune responses and an increase in EAEC virulence factors (Bolick et al., 2014). Furthermore, dietary zinc supplementation abrogated disease progression, reduced EAEC colonisation and expression of virulence factors (Medeiros et al., 2013). In another study, zinc supplementation protected from uropathogenic *E. coli* haemolysin-induced gut barrier dysfunction (Wiegand et al., 2017). These observations indicate a beneficial impact of zinc supplementation on zinc deficient subjects; conversely, excessive zinc supplementation can have a detrimental impact on the microbial homeostasis and host immune responses. In a study by Zackular et al, zinc supplementation stimulated the growth of *Enterococcus* and *Clostridium* XI cluster while concomitant reductions in *Turicibacter* and *Clostridium* (unclassified) were observed. Ultimately, excess zinc selected for a microbiota that was much more prone to destruction by antibiotics, and thus exacerbating *C. difficile* colonisation and associated disease (Zackular et al., 2016).

For many bacterial pathogens, availability of iron is often the limiting factor for colonisation and infection. During inflammation, nutritional immunity limits the bioavailability of iron in the gut, and thus bacterial species equipped with an array of iron acquisition systems are often the most successful and pathogenic. Given this ability of bacterial siderophores to hijack host iron homeostasis, it is not surprising that the innate immune system has evolved mechanisms to counteract bacterial iron acquisition, such as the production of Lipocalin-2 (LCN2). In the acute phase response to infection,

LCN2 is expressed to bind bacterial siderophores and neutralises bacterial capacity to sequester iron. However, some bacteria have evolved resistance mechanisms to counteract this immune response, such as the stealth siderophore salmochelin produced by *Salmonella*, and thereby gaining a competitive advantage in the inflammatory milieu (Raffatellu et al., 2009). Interestingly, the probiotic *E. coli* strain Nissle shares many fitness properties to uropathogenic *E. coli*, including iron uptake systems. In the presence of LCN2, Nissle is capable of outcompeting *Salmonella* in a mouse model (Deriu et al., 2013), underscoring the evolved synergy between commensal and host immune response in thwarting pathogen colonisation. Some pathogens such as *Vibrio cholerae* have the ability to obtain iron from haem only when Cholera toxin (CTX) is produced (Rivera-Chávez & Mekalanos, 2019). The production of CTX induces inflammation and thus decreases gut iron concentrations but enables the bioavailability of host haem, while concurrently changing the transcriptomic gene signature of *V. cholerae* to one that is capable of utilising iron from haem. This change allows expansion of *V. cholerae* by providing an iron limited metabolic niche and a competitive advantage over commensals by this pathogen's unique ability to acquire iron from haem.

In both developing and developed nations, iron deficiency remains the most common form of nutritional deficiency, in many cases prompting iron supplementation to alleviate symptoms of malnutrition. Given the importance of iron to GI pathogens, the effect of iron in bolstering colonisation resistance should perhaps be considered as a detrimental effect by inducing microbial dysbiosis. Indeed, an outgrowth of *Enterobacteriaceae* and increased risk of infection has been observed in both mice (Constante et al., 2017) and humans (Jaeggi et al., 2015), following iron supplementation. Intriguingly, the adverse effects can be mitigated simply by addition of prebiotics to the diet (Lin et al., 2018; Paganini et al., 2017), inducing growth of beneficial *Bifidobacterium* and *Lactobacillus*. *Bifidobacterium* have also been demonstrated to efficiently sequester iron (Vazquez-Gutierrez et al., 2015). The reliance of the host immune system on sequestration of iron, coupled with commensal sequestration capacity and subsequent SCFA production, plays a multifactorial role in reducing pathogen colonisation.

In addition to the production of LCN2, the host can produce another antimicrobial molecule, Calprotectin, whose primary function is to bind to free zinc and manganese in the gut lumen. Bacteria utilise manganese as a cofactor for a number of proteins; perhaps the best studied is the role of manganese as a detoxifier of reactive oxygen species (ROS), of which numerous are encountered following an immune response. *Salmonella* have evolved high affinity cation transporters to bypass the action of calprotectin and therefore promoting growth in an inflamed intestine (Diaz-Ochoa et al., 2016; Kehres et al., 2002). It is unclear how excess or deficient dietary manganese influences gut microbiota populations. It is possible many pathogens behave similarly to *Salmonella* in subverting calprotectin when manganese is in excess, or perhaps behave similarly to *Staphylococcus aureus*, which can switch to iron in manganese-deplete conditions; thereby bypassing nutritional immunity and causing infection (Garcia & Barwinska-Sendra, 2017).

Pinpointing the delicate balance between trace element toxicity and deficiency while simultaneously understanding the mechanisms involved in both nutritional immunity and colonisation resistance remains complicated. Understanding the complex pathways dietary trace elements play in microbial respiration in infection and inflammation will undoubtedly uncover novel treatments. For example, recently, dysbiotic *Enterobacteriaceae* blooms were ameliorated by tungstate treatment, which inhibited molybdenum-cofactor-dependent respiratory pathways and reduced the severity of inflammation in mouse models (Zhu et al., 2018).

## Conclusions and Recommendations

Disentangling the direct and indirect impact of dietary ingredients and nutrients on commensal bacteria in the gut, their associated metabolites, immune function and pathogen virulence will no doubt be challenging. This knowledge will require multidisciplinary collaborations between experts in nutrition, immunology and microbiology to name a few. Given that gnotobiotic and antibiotic-treated mice are more susceptible to infection, and this phenotype can be reversed upon supplementation with even a simplified consortium of commensal bacteria (Brugiroux et al., 2016) strongly supports

the paradigm of colonisation resistance. Reductionist or modular approaches like these can help identify potential probiotic or synbiotic candidates and generate insights into diet-microbe/host-microbe/microbe-microbe interactions.

Studies in neonates and infants support the importance of breast milk-feeding, in providing antibodies and colonisation of human breast milk-metabolising taxa, both of which have been shown to enhance colonisation resistance in the offspring. Moreover, C-section delivery disrupts the mother to infant transmission of specific commensals, resulting in a reduced immunostimulatory potential passed to the offspring (Wampach & Heintz-Buschart, 2018). Perhaps a critical, yet, unexplored area of research would be the impact of infant formula-metabolising taxa in reducing/providing protection against pathogens. Identifying specific species and their associated functions, which improve neonatal colonisation resistance, has the potential to optimise production of infant formula through selection of desired carbon sources.

The mucus layer provides the first line of defence against exogenous microorganisms, the integrity of which is greatly determined by microbial composition, which in turn, is influenced by dietary components, in particular fibre. Patients with IBD should be cautious when consuming specific dietary fibre or prebiotics; inulin has been demonstrated to exacerbate DSS-induced colitis in mice, whereas others such as psyllium have been successful in ameliorating gut inflammation (Llewellyn et al., 2018). In the context of human GI disease, it is equally ambiguous; as a study observed avoidance of dietary fibre associated with flares in Crohn's disease patients but not in ulcerative colitis patients (Brotherton et al., 2016). Research focusing on individual fibres or prebiotics must be interpreted cautiously as they may inadvertently select a small subset of taxa, while care must be taken when comparing to mouse chow diet controls, which contain a mixture of fibres, and thus selecting for a larger subset of taxa. More research is required in humans, in terms of the impact of dietary fibre and prebiotics on the function, stability and characterisation of specific taxa and their associated

metabolites. These data could complement in vitro, ex vivo and 'humanised' mouse studies to identify mechanisms when the host is challenged with a GI pathogen.

Recent attention has been shifted towards the role of other components of the microbiome, such as fungal or viral constituents and how they impact human health. However, very little research has been made with regards these lesser studied kingdoms and how they influence colonisation resistance against pathogens. Recently, alterations in gut microbiota composition have been observed in some patients infected with SARS-CoV-2 (S. Li et al., 2022), suggesting an immune cross-reactivity between commensal bacteria and SARS-CoV-2. It has been demonstrated that prophage can influence host fitness in commensal bacteria (Reyes et al., 2013), suggesting that phage can contribute to maintaining and/or having a detrimental effect on a healthy gut microbiome. Interestingly, a recent study found that a fructose-rich diet stimulates *Lactobacillus reuteri* to induce prophage (Oh, Alexander, et al., 2019), providing the first evidence that diet can influence phage populations in the gut. How the gut virome contributes to colonisation resistance either through direct effects or indirect immune stimulation could be an unrevealed aspect of colonisation resistance going forward.

Seasonal variations in the mouse chow diet, industrial variations in the processing of prebiotics and feed supplements, choice of laboratory pathogen/commensal strains and breed of mouse all contribute to disparities among research groups. Thus, tightly controlled models are a necessity, before translating to novel therapeutics and functional foods. Moreover, the fine line between commensal and pathogen in genetically predisposed individuals only adds to the uncertainty and personalised dietary interventions (Zeevi et al., 2015) in these individuals for the prevention of infection is an interesting prospect, but further research is warranted.

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# Chapter 2 - Assessment of pathogen performance in the presence of yeast β-glucan in an *ex vivo* model of the human gut

Notes:

Simulated Digestion, pathogen culturing, micromatrix fermentation, DNA extractions, sequencing library preparation and fatty acid analysis was carried out by Ronan Strain (author).

Analysis of sequencing data was carried out by Dr. Tam Tran.

## Abstract

Dietary fibre plays a pivotal role in shaping the community of microbes in our gut, affecting its composition, function and influencing host-microbe interactions. Commensal bacteria can protect against invasion of pathogens through immune system signalling, production of antimicrobial molecules and competing for nutrients or niches. In this study, we employed an *ex vivo* gut model to study the impact of a dietary fibre, yeast β-glucan, on pathogen performance. Faecal samples from healthy subjects were pooled, spiked with a cocktail of gastrointestinal pathogens (*Citrobacter rodentium, Cronobacter sakazakii, Escherichia coli* and *Salmonella enterica)* and fermented with yeast β-glucan for 24 hours, and comparisons were made with glucose as a control. Relative abundance of taxa was assessed by 16S rRNA sequencing and short chain fatty acid (SCFA) and branched chain fatty acid (BCFA) production was analysed by gas chromatography – Flame ionization detector (GC-FID). Presence of fibre selected for higher proportions of *Bacteroides* (p=0.0005) and *Clostridia* (p=0.005) while species of *Escherichia/Shigella* (p=0.021) and *Lactobacillus* (p=0.007) were higher in the glucose control. In the absence of pathogens, higher production of BCFAs (p=0.002) and SCFAs (p=0.002) was observed for the fibre group compared with the glucose group. There was no significant difference in pathogen abundances between fibre and glucose control following fermentation. Interestingly, in the presence of glucose, SCFA concentrations were much higher when pathogens were present compared with their absence. Yeast β-glucan supplementation selects for fatty acid producing bacteria while concomitantly lowering pathogen abundance in an *ex vivo* model of the human gut.

## Introduction

The gut microbiome is a diverse and metabolically active community, consisting of up to 1000 various bacterial species, with total cell estimates of approximately ~3.9 x $10^{13}$ microbial cells (Sender & Fuchs, 2016). These bacterial symbionts perform a broad range of functions such as the digestion of complex dietary polysaccharides, production of vitamins and metabolites, interaction with and maintenance of the immune system, and defence against pathogens. Factors including age, genetics and lifestyle

influence microbiome composition (Gill et al., 2006). Furthermore, dietary components that are recalcitrant to digestion by host enzymes provide energy sources for bacterial growth and metabolism in the colon (Chung et al., 2019). Thus, diet can also be considered a dominant selective force that drives microbiota community structure and function.

Dietary fibre is defined as non-digestible carbohydrates of ≥3 monomeric units naturally present in foods, but also includes isolated or synthetic counterparts, that have been determined to provide physiological benefits (Jones, 2014). Accordingly, many species of the gut microbiota are well equipped with an enzymatic repertoire that is capable of converting these substrates into monosaccharides and their associated metabolites. The major end products of these reactions are short chain fatty acids (SCFAs), which have generally favourable physiological consequences for the host, ranging from glucose or lipid homeostasis to tumour protection (Russell et al., 2013). Intake of dietary fibre is widely considered to contribute to a more 'homeostatic' diverse gut community (Heiman & Greenway, 2016), through interactions among hundreds of bacterial species, driven primarily by cross-feeding and competition (Rakoff-Nahoum et al., 2016).

The importance of the indigenous gut microbiota in protecting against enteric pathogens (termed 'colonisation resistance') has been realised as far back as the 1950s, when Bohnhoff and colleagues demonstrated that antibiotic treatment significantly increases murine susceptibility to *Salmonella* infection (Bohnhoff et al., 1954). Similar mechanisms of colonisation resistance exist in humans, with antibiotic-mediated destruction of the microbiota leading to an expanse of opportunistic pathogens such as *Clostridioides difficile* (Slimings & Riley, 2014). Interestingly, an effective approach to treatment for these stubborn colonists involves reintroduction of a diverse faecal microbiota from a healthy donor via a procedure known as Faecal Microbiota Transfer (FMT), further supporting the protective role of commensal bacteria against invasive species (van Nood et al., 2013). Applying a systematic approach to identifying individual microbiota members (Caballero et al., 2017; Reeves et al., 2012) and their associated metabolites (Jacobson et al., 2018; J. A. K. McDonald

et al., 2018) in terms of providing colonisation resistance remains a popular area of microbiome research.

While it is apparent that antibiotics have a detrimental effect on the gut microbiota, similar perturbations stemming from a diet absent in dietary fibre have been observed in mice (Erica D. Sonnenburg et al., 2016) (Martinez-Medina et al., 2014) with concomitant increased risk of pathogen infection (Desai et al., 2016) (Hryckowian et al., 2018a). Interestingly, the reduced gut diversity and pathogen sensitivity can be mitigated through dietary interventions containing Microbiota-Accessible Carbohydrates (MACs) (Erica D. Sonnenburg et al., 2016) (Hryckowian et al., 2018a). There is no doubt that murine infection models are indispensable in pathological microbiome research; most involve either antibiotic-treated or germ-free animal models, both of which increase susceptibility to colonisation to human pathobionts or enteric pathogens, which otherwise remain refractory to infection in conventional mice (Mullineaux-Sanders et al., 2018). However, both of these treatments result in anatomical changes to the mouse gastrointestinal tract, and potentially confounding data interpretation. This problem, coupled with differences in microbiota composition, whereby many of the predominant human taxa fail to colonise the mouse gut or bloom to concentrations that are no longer representative of the concentrations found in human faeces, further highlights the difficulties in mechanistic studies (Julie A.K. McDonald, 2017).

To overcome some of these limitations, many researchers employ the use of *ex vivo* human gut fermentation models, often to complement human and animal studies or to provide a rationale for proceeding with the more expensive *in vivo* models. Similar to human gut mouse models, *ex vivo* models cannot maintain all of the species found in human donor faeces; however, they can provide a cheaper, reproducible, ethical and highly controlled environment, ideal for studying the measurable effects of individual dietary substrates on microbiome composition, in the absence of confounding effects of the host (Julie A.K. McDonald, 2017). For studies involving pathogens, dietary intake and associated metabolites, *ex vivo* models are at present a valuable option, bypassing the complications

discussed above and the ethical roadblocks in human studies. Indeed, novel discoveries, such as the inhibitory effect of valerate on *C. difficile* growth (J. A. K. McDonald et al., 2018), have been made using this approach, and thereby, enabling new avenues for therapeutic interventions.

Many enteropathogens possess virulence factors that restructure the environment to establish a niche from which they can flourish. One such strategy is employed by *Salmonella* whereby the virulence-associated type III secretion system elicits host immune responses, which in turn creates inflammatory-mediated reduction in commensal diversity, and ultimately a less competitive environment from which it can expand (Stecher et al., 2007). Additionally, *Salmonella* is equipped with high-affinity transporters to scavenge the newly available terminal electron acceptors resulting from inflammation. For example, during inflammation, a depletion of butyrate-producing Clostridia is observed, and this in turn promotes colonocytes to undergo lactate fermentation, elevating luminal oxygen and lactate (Rivera-Chavez et al., 2016), and promoting aerobic expansion of *Salmonella* utilising this newly acquired energy source (Gillis et al., 2018). While it is impossible to recreate the intricate interactions between pathogens, commensals and human immune systems in an *ex vivo* setting, *ex vivo* culture fermentations provide a framework from which to build.

We employed the use of a mini-bioreactor (O'Donnell et al., 2018) as an *ex vivo* batch colon model to study survival of various pathogens under the influence of individual complex dietary fibres. We hypothesised that increases in microbial diversity would create an environment that is more hostile to selected enteropathogens, and thereby enabling identification of potential probiotics, prebiotics and/or metabolic pathways that are antagonistic to pathogen growth. In order to overcome individual bias, we used pooled faecal samples inoculated with a select panel of enteropathogens, in the presence of the non-digestible carbohydrate yeast β-glucan, and assessed the survival of the pathogens after 24 hours of fermentation. We show here that a prebiotic fibre (yeast β-glucan) influences bacterial composition and diversity but did not appear to limit the growth of specific pathogens *ex vivo.*

## Methods

### Simulated human digestion of fibres

To replicate the digestion process in the upper gastrointestinal tract, 30 grams of yeast β-glucan (Kerry Group, Ireland) was subjected to a simulated gastric digestion process in order to produce a substrate that would be similar to that which enters the colon *in vivo*, and was adapted from a previous study (Minekus et al., 2014). This process represents three enzymatic phases of the digestion process; oral, gastric and intestinal phase. In the oral phase, yeast β-glucan was mixed in a 50:50 (w/v) ratio with simulated salivary fluid (KCl 15.1 mmol $L^{-1}$; $KH_2PO_4$ 3.7 mmol $L^{-1}$; $NaHCO_3$ 13.6 mmol $L^{-1}$; $MgCl_2(H_2O)_6$ 0.15 mmol $L^{-1}$; $(NH_4)_2CO_3$ 0.06 mmol $L^{-1}$; adjusted to pH 7.0) and human salivary α-amylase (Sigma; Ireland) (pre-warmed to 37°C) was added to achieve 75 U $ml^{-1}$, followed by addition of $CaCl_2$ to achieve 0.75 mM, and the enzyme digestion was allowed to proceed for 2 minutes at 37°C. The resulting digested mixture was subjected to a simulated gastric digestion with the mixture added in a 50:50 (v/v) to simulated gastric fluid (KCl 6.9 mmol $L^{-1}$; $KH_2PO_4$ 0.9 mmol $L^{-1}$; $NaHCO_3$ 25 mmol $L^{-1}$; NaCl 47.2 mmol $L^{-1}$; $MgCl_2(H_2O)_6$ 0.1 mmol $L^{-1}$; $(NH_4)_2CO_3$ 0.5 mmol $L^{-1}$; adjusted to pH 3.0). Porcine pepsin (Sigma; Ireland) was added to achieve 2000 U $ml^{-1}$ and $CaCl_2$ added to achieve 0.075mM. pH was adjusted to 3.0 with 1M HCl and the mixture was incubated for 2 hours at 37°C in a shaking incubator. The mixture product from the gastric phase was then subjected to a simulated intestinal digestion using a 50:50 (v/v) simulated intestinal fluid (KCl 6.8 mmol L-1; KH2PO4 0.8 mmol L-1; NaHCO3 85 mmol L-1; NaCl 38.4 mmol L-1; MgCl2(H2O)6 0.33 mmol L-1; adjusted to pH 7.0). A pancreatin (Sigma; Ireland) solution was prepared in advance using the simulated intestinal fluid as a solute and this was added to the mixture at a final concentration of 100 U $ml^{-1}$. Bile salts were added at a final concentration of 10 mM, $CaCl_2$ at a final concentration of 0.3 mM and the pH adjusted to 7.0 prior to a 2-hour digestion at 37°C. The end product was dialysed in a 1KDa dialysis membrane to account for absorption in the small intestine, and subsequently lyophilised and stored at -20°C.

### Faecal sample collection and processing

Stool samples were collected from consenting healthy volunteers (n=6) under the approval of the Clinical Research Ethics Committee (CREC) of the Cork Teaching Hospitals according to the study protocol APC 055. A standardised faecal inoculum was prepared as described previously (O'Donnell et al., 2016a), by pooling together 60g of each (n=6) subject's faecal sample. Faecal samples were pooled to reduce inter-individual donor bias and to obtain a better representation of general population. Briefly, 60g of each donor's faecal sample was placed in a stomacher bag containing a 70μm filter insert (Sparks lab supplies, Ireland). To this, 400 ml of 50mM phosphate buffer and 0.05% w/v L-cystine hydrochloride (Sigma, Ireland) was added and the mixture was homogenised. The filtered faecal homogenate was centrifuged at 4000xg for 25 mins and resuspended in 400ml 50mM phosphate buffer. This suspension was adjusted with sterile glycerol to a final concentration of 25% (v/v). The standardised faecal slurry was immediately frozen in aliquots (100ml) at -80$^{o}$C and on the day of the fermentation experiment, it was thawed at 37$^{o}$C for 1 hour prior to use.

**Gastrointestinal pathogens**

A four-strain pathogen cocktail was prepared on the day of the fermentation experiment. All the test pathogens were obtained from the DPC culture collection (Teagasc Moorepark, Ireland). *Citrobacter rodentium* (DPC 6470; ICC168), *Cronobacter sakazakii* (DPC 6090; ATCC 12868) and *Escherichia coli* (DPC 6054; P1432) were routinely grown aerobically overnight at 37$^{o}$C in LB broth (Merck Millipore, Address) and *Salmonella typhimurium* LT2 (DPC 6048; ATCC 700720) was routinely grown aerobically overnight at 37$^{o}$C in BHI broth (Merck Millipore). On the day of faecal fermentation, overnight cultures of pathogens were subcultured into fresh aliquots of their corresponding media and grown to mid-log phase (OD600 ~0.1), cells were harvested by centrifugation at 4000xg at 4$^{o}$C for 15 min and resuspended in fresh faecal fermentation medium (see below) to give a final concentration of 1 x $10^{5}$ cfu/ml of each pathogen.

***Ex vivo* model of the distal colon**

A no-carbon faecal fermentation medium was prepared as described previously (Fooks & Gibson, 2003) and allowed to reduce overnight in an anaerobic chamber. The Applikon MicroMatrix (Applikon Biotechnology, The Netherlands) *ex vivo* model was employed to model the human distal colon as described previously (O'Donnell et al., 2018). Each vessel contained 4.75ml of faecal fermentation medium combined with 0.25ml faecal slurry and 1% w/v test carbohydrate. Two millilitres of a $T_0$ baseline sample containing 4.75ml of fermentation media and 0.25ml faecal slurry was centrifuged at 16,000xg for 15 mins at 4$^{o}$C and the pellet and supernatant immediately frozen at -80$^{o}$C. The 24 well MicroMatrix cassette was divided into 4 groups, n=6 for each group; consisting of (1) glucose with no pathogen added, (2) yeast β-glucan with no pathogen added, (3) glucose with pathogen cocktail added, (4) yeast β-glucan with pathogen cocktail added. The MicroMatrix fermentation experiment was allowed to run for 24 hours with the temperature kept constant at 37$^{o}$C, pH at 6.8, anaerobiosis maintained by addition of $N_2$ gas and orbiter set at 300rpm. Following 24 hours of fermentation, 2mls of each vessel fermentate were collected and centrifuged at 16,000xg for 15 min at 4$^{o}$C and the pellet and supernatant were immediately frozen at -80$^{o}$C.

**DNA extraction**

Bacterial DNA was extracted using the Repeated Bead Beating (RBB) plus column method adapted from (Z. Yu & Morrison, 2004), in combination with a Qiagen Blood & Tissue Kit (Qiagen, Address). DNA was extracted from the pellets collected from baseline ($T_0$) and $T_{24}$ fermentation vessels.

**Bacterial 16S rRNA sequencing**

The V3-V4 region of the 16S rRNA gene was amplified according to the Illumina 16S metagenomics sequencing protocol, creating a 460bp amplicon using the forward primer 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and reverse primer 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC (Klindworth et al., 2013). Indexed products were cleaned with AMPure XP beads (Beckman Coulter, Address), quantified with Qubit dsDNA HS Assay kit (Life technologies, Address) and subsequently pooled in an equimolar

fashion. Samples were sequenced on the Illumina MiSeq Sequencing platform (Clinical Microbiomics, Denmark) using a 2 x 250 cycle kit (Illumina).

**Short & Branched Chain Fatty Acid analysis**

A 2ml aliquot of fermentation liquid was syringe-filtered (0.2um) to remove bacterial debris and 1ml of this was subsequently subjected to gas chromatography as described previously (Cussotto et al., 2019). Briefly, 1ml of filtered faecal fermentate was mixed with 2-Ethylbutyric acid (Sigma, Ireland) as an internal standard. SCFAs were measured by gas chromatography, using a Varian 3500 GC flame-ionization system fitted with a ZB-FFAP column (Phenomenex, UK). Conditions for chromatography were as follows: GC oven temperature was pre-heated to 50$^{o}$C for 30s, then incremented stepwise, by 10$^{o}$C/min until a temperature of 140$^{o}$C was reached. This was followed by 20$^{o}$C/min increments until a temperature of 240$^{o}$C was reached and this was held for 5 min. Injector temperature was set at 240$^{o}$C and the detector set at 300$^{o}$C. Helium gas was used as the carrier at a flow rate of 1.3 ml/min$^{-1}$. A standard curve was constructed based on increasing concentrations of SCFA and BCFA solutions (Sigma, Ireland). The Varian Star Chromatography Workstation v6.0 software was used to integrate peaks from the test samples and the concentrations of SCFA and BCFA were calculated using the linear regression equations ($R^2 \geq 0.999$) from the inputted standard curve. SCFA and BCFA standards were included on the run to check for calibration. The data was presented as mMol.

**Analysis of sequencing data**

Illumina MiSeq reads were analysed using the Quantitative Insights into Microbial Ecology (QIIME) v.1.9.1 and uSearch v.8.1 software as described previously (Tran, Corsini, et al., 2019). Paired-end reads were merged using FLASH v.1.2.8 and adaptors were removed using cutadapt v.1.8.3. An operational taxonomic unit (OTU) table was obtained using uSearch. Classification of representative sequences for each OTU was carried out using mothur v.1.36.1. against the 16S rRNA reference of Ribosomal Database Project database trainset 14. Scripts for generation of alpha and beta diversity can be found in (Tran, Corsini, et al., 2019).

**Statistical analysis**

All statistical analyses were performed using the software package R v.3.5.1. Kruskal-Wallis *H* test with Dunn's multiple comparison test performed for significant differences in alpha diversity and abundances of each OTU. A Benjamini-Hochberg correction was employed to correct for P values to control false discovery rate. The permutational multivariate ANOVA (PERMANOVA) analysis was used to determine significant differences in beta diversity.

For SCFAs, data normality was assessed using the Shapiro–Wilk test using SPSS Version 22.0. Differences in total SCFAs and BCFAs were analysed using the univariate ANOVA with Bonferroni correction.

**Results**

**Alpha diversity**

High-throughput paired-end sequencing of the V3 and V4 region of the 16S rRNA gene was performed on the faecal fermentation samples using the Illumina MiSeq platform. Alpha diversity (expressed as the number of species or richness present in a group of samples) indices (Chao1; Phylogenetic diversity; Observed species; Shannon; Simpson) were determined and p values were calculated from the Krustal-Wallis test. Alpha diversity matrices can be found in Figures 2.1 and 2.2. All the diversity indices were significant when comparing the baseline ($T_0$) and the fermentation condition groups, indicated by the p1 value; Chao1 p=0.03, Phylogenetic diversity p=0.004, Observed species p=0.01, Shannon p=0.003, Simpson p=0.001. When comparing between the fermentation groups, the alpha diversity indices Shannon p=0.01, Simpson p=0.004 and Phylogenetic diversity p=0.02 were the only indices found to be significant, indicated by the p2 value.

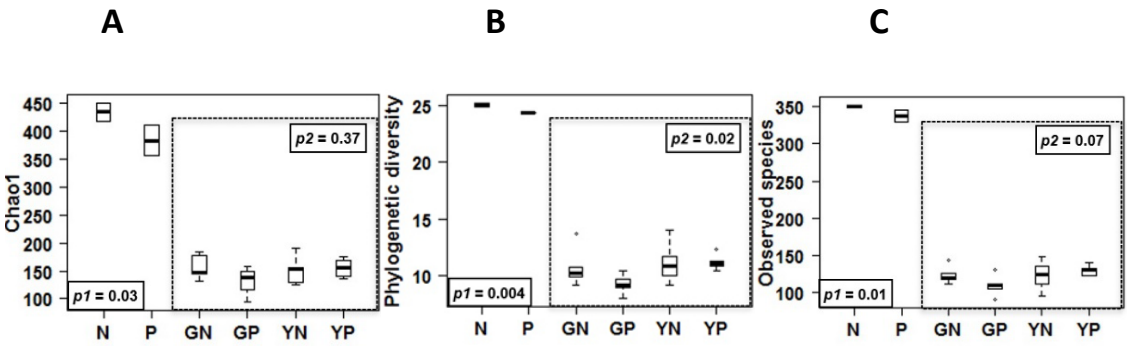



*Figure 2.1 – Chao1, phylogenetic and observed species alpha diversity metrics in treatment groups*

**Alpha diversity indices–** Alpha diversity measured by (A) Chao1, (B) Phylogenetic diversity and (C) Observed species metric is plotted for inocula no-pathogen (N), baseline pathogen (P), 24-hour glucose no-pathogen (GN), 24-hour glucose pathogen (GP), 24-hour yeast β-glucan no-pathogen (YN), 24-hour yeast β-glucan pathogen (YP). The line inside the box represents the median, boxes represent the interquartile range and the whiskers represent the range. P values calculated from Krustal-Wallis test; p1 when comparing inocula and 24-hour fermentation groups; p2 when comparing within fermentation groups.

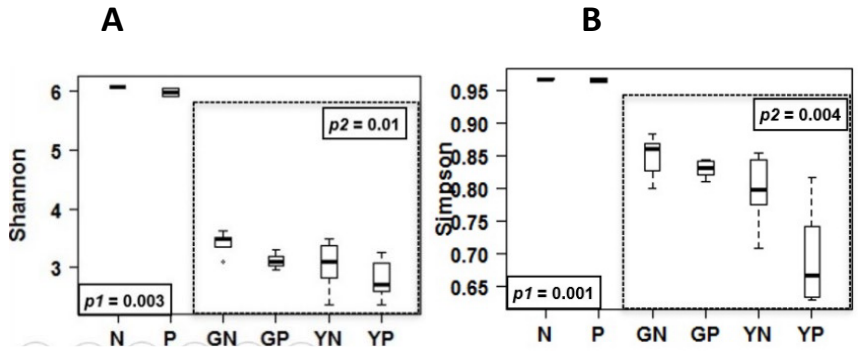



*Figure 2.2 – Shannon and Simpson alpha diversity metrics in treatment groups*

**Shannon and Simpson alpha diversity metrics** - Alpha diversity measured by (A) Shannon and (B) Simpson metric is plotted for inocula no-pathogen (N), inocula pathogen (P), 24-hour glucose no-pathogen (GN), 24-hour glucose pathogen (GP), 24-hour yeast β-glucan no-pathogen (YN), 24-hour yeast β-glucan pathogen (YP). The line inside the box represents the median, boxes represent the interquartile range and the whiskers represent the range. P values calculated from Krustal-Wallis test;

p1 when comparing inocula and 24-hour fermentation groups; p2 when comparing within fermentation groups.

**Beta diversity**

Principal component analysis (PCoA) based on Spearman distances between the fermentation groups using unweighted UNIFRAC, weighted UNIFRAC and Bray-Curtis can be found in Figure 2.3 and account for 31.7%, 90.5% and 68.9% of the variation between samples, respectively. The significant differences between groups were calculated by analysis of similarity (ANOSIM) tests. UNIFRAC measures the phylogenetic differences i.e., evolutionary differences between groups of samples, whereas Bray-Curtis dissimilarity measures the differences in species populations. Weighted UNIFRAC incorporates the relative abundances of species when calculating the differences in phylogenetic branch lengths, whereas unweighted UNIFRAC does not consider relative abundances; therefore, making this measure useful for low abundance species. Figures 2.4, 2.5 and 2.6 show the PCoA plots with the baseline ($T_0$) included illustrating a clear separation or decrease in microbial diversity between the $T_0$ and $T_{24}$ fermentation groups. The larger separation in the weighted UNIFRAC PCoAs compared with unweighted UNIFRAC would explain that there is a wider range of low abundance species that benefit depending on the carbon source.

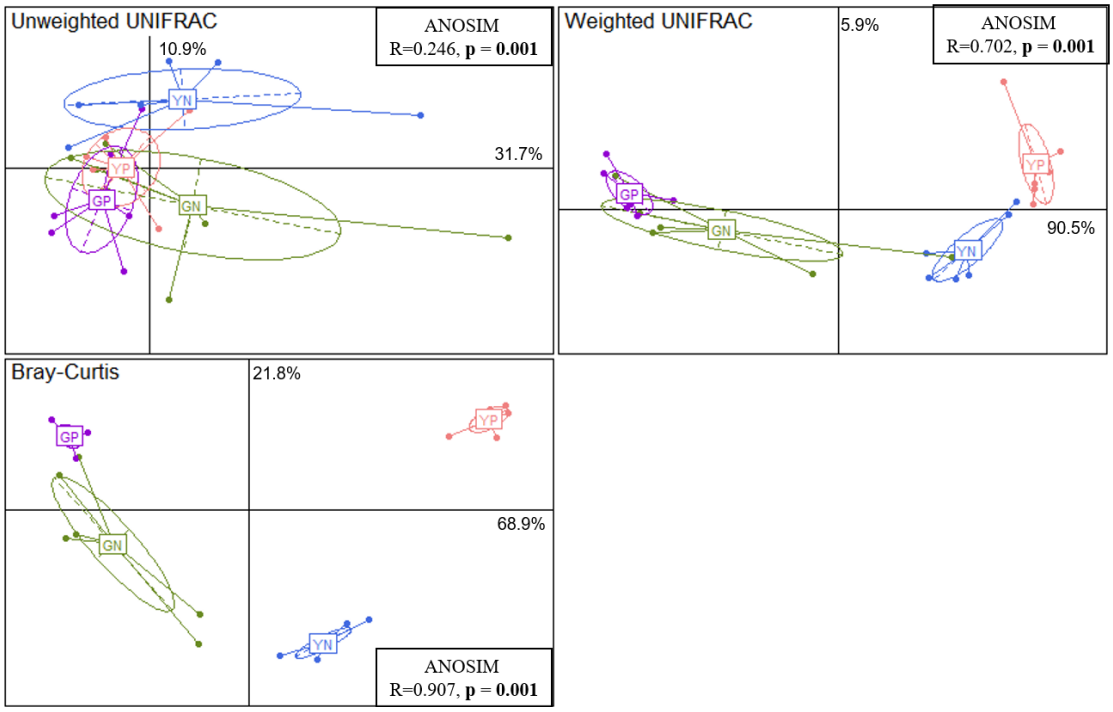



*Figure 2.3 – Unweighted UNIFRAC, weighted UNIFRAC and Bray-Curtis beta diversity metrics in treatment groups*

**Beta diversity matrices –** PCoA plots for unweighted UNIFRAC, weighted UNIFRAC and Bray-Curtis distances for the four fermentation groups; 24 hour glucose no-pathogen (GN), 24 hour glucose pathogen (GP), 24 hour yeast β-glucan no-pathogen (YN), 24 hour yeast β-glucan pathogen (YP). The significant differences between groups were calculated by analysis of similarity (ANOSIM) tests.

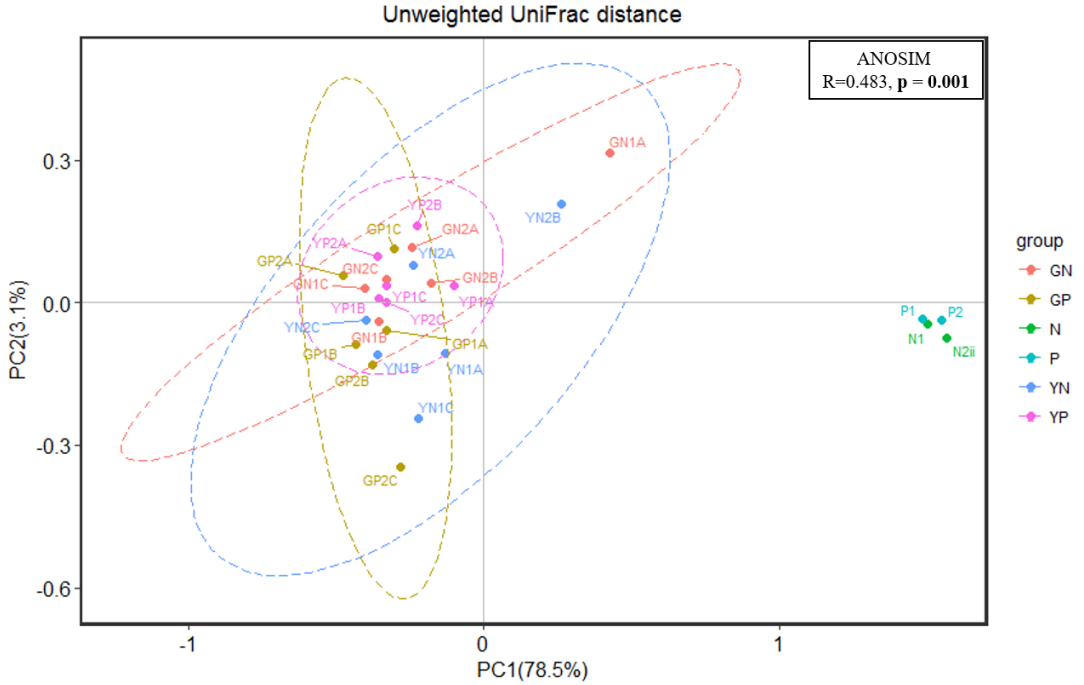



*Figure 2.4 – Unweighted UNIFRAC beta diversity with inocula*

**Figure 2.4 – Unweighted UNIFRAC beta diversity with inocula** - PCoA plot for unweighted UNIFRAC for the four fermentation groups with inocula included; inocula no-pathogen (N), inocula pathogen (P), 24 hour glucose no-pathogen (GN), 24 hour glucose pathogen (GP), 24 hour yeast β-glucan no-pathogen (YN), 24 hour yeast β-glucan pathogen (YP). The significant differences between groups were calculated by analysis of similarity (ANOSIM) tests.

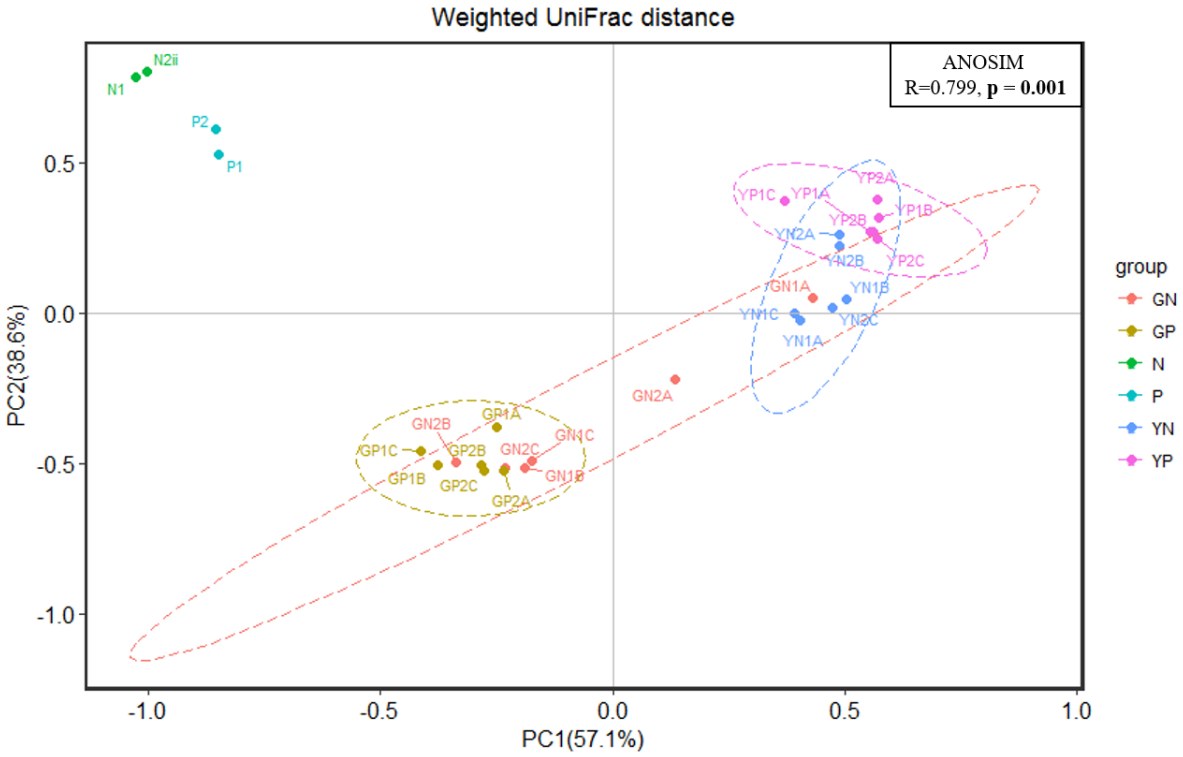



*Figure 2.5 – Weighted UNIFRAC beta diversity with inocula*

**Weighted UNIFRAC beta diversity with inocula -** PCoA plot for weighted UNIFRAC for the four fermentation groups with inocula included; inocula no-pathogen (N), inocula pathogen (P), 24-hour glucose no-pathogen (GN), 24-hour glucose pathogen (GP), 24-hour yeast β-glucan no-pathogen (YN), 24-hour yeast β-glucan pathogen (YP). The significant differences between groups were calculated by analysis of similarity (ANOSIM) tests.

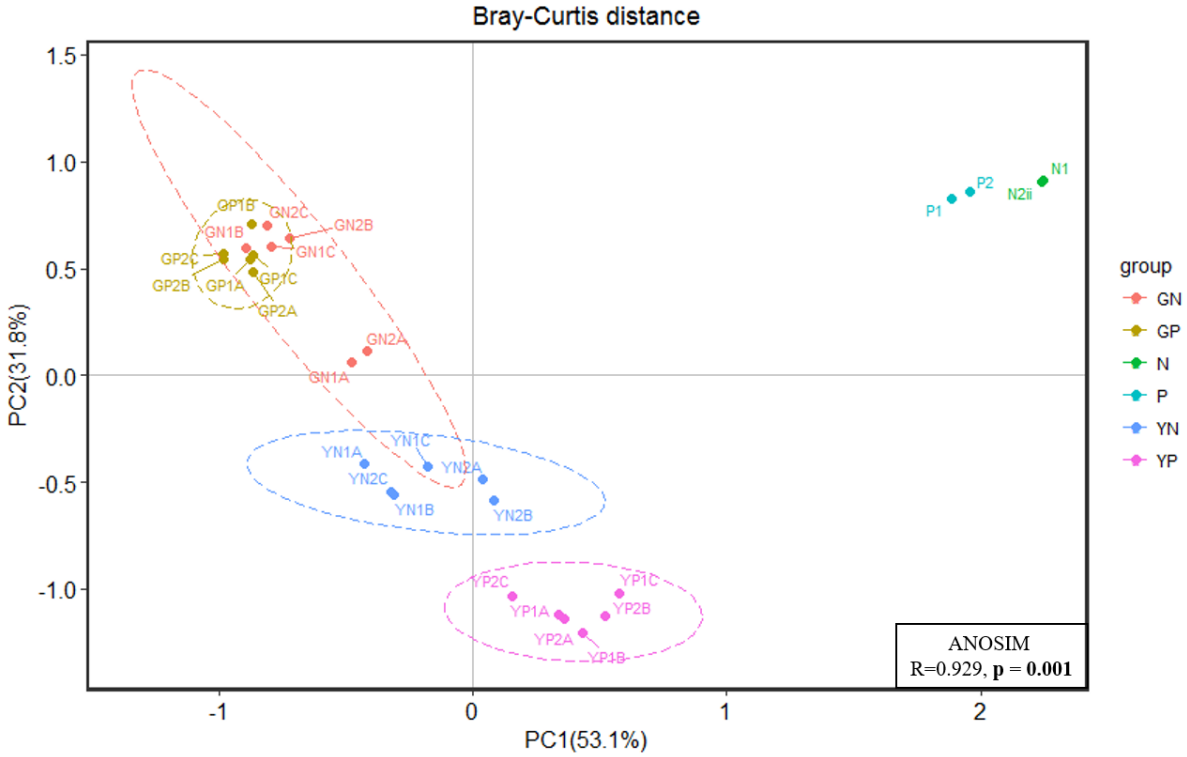



*Figure 2.6 – Bray-Curtis beta diversity with inocula*

**Bray-Curtis beta diversity with inocula -** PCoA plot for Bray-Curtis metric for the four fermentation groups with inocula included; inocula no-pathogen (N), inocula pathogen (P), 24-hour glucose no-pathogen (GN), 24-hour glucose pathogen (GP), 24-hour yeast β-glucan no-pathogen (YN), 24-hour yeast β-glucan pathogen (YP). The significant differences between groups were calculated by analysis of similarity (ANOSIM) tests.

**Taxonomic analysis**

**Phylum level**

Figure 2.7 illustrates the relative abundances of taxonomic groups at the phylum level according to the fermentation conditions. When comparing the YN and GN groups, *Bacteriodetes* (YN=24.14%; GN=4.26%; p=0.0004) and *Firmicutes* (YN=51.75%; GN=37.07%; p=0.019) were higher in the fibre treated groups. Higher abundances of *Bacteroidetes* would be expected as these groups of bacteria

are the main fibre degraders in the gut. Similarly, *Firmicutes* constitute a higher fraction in the YN group compared to the GN group, suggesting that species of *Firmicutes* are involved in the degradation of fibre and potentially of metabolites produced by the fibre fermenters. *Proteobacteria* were capable of metabolising glucose more efficiently than the fibre (YN=24%; GN=58.52% p=0.007). Low abundant members of the *Fusobacteria* phyla were higher in the GP group relative to the YP group (YP=0.01%; GP=0.1%, p=0.034).

**Phylum level**

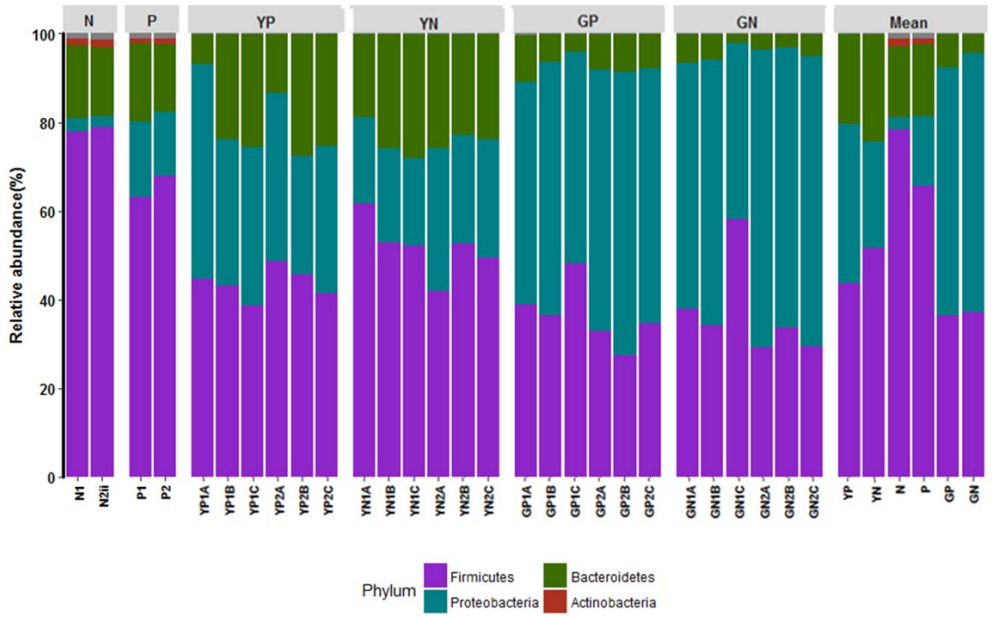



*Figure 2.7- Relative abundance of taxa at phylum level*

**Phylum level -** Relative abundance of taxonomic groups at the phylum level for the four fermentation groups (n=6); 24-hour glucose no-pathogen (GN), 24-hour glucose pathogen (GP), 24-hour yeast β-glucan no-pathogen (YN), 24-hour yeast β-glucan pathogen (YP). Inocula groups (N, no pathogens and P, pathogen spiked) and the means of the replicates are included.

**Class level**

Figure 2.8 illustrates the relative abundances of taxonomic groups at the class level according to the fermentation conditions. Comparisons of the YN and GN groups showed significantly higher *Bacteroidia* (YN=24.14%; GN=4.25%; p=0.0004) and *Clostridia* (YN=48.43%; GN=16.08%; p=0.004) in the fibre groups. Class abundances of *Bacilli* (YN=1.23%; GN=19.36%; p=0.0004) and *Gammaproteobacteria* (YN=23.41%; GN=55.55%; p=0.006) were higher in the glucose groups. Low-abundant members of the *Erysipelotrichia* class were higher in the fibre group (YN=0.33%; GN=0.06%; p=0.005) and *Betaproteobacteria* (YN=0.5%; GN=2.95%; p=0.007) in the glucose group. Only low-abundant members of the *Betaproteobacteria* (YP=0.25%; GP=4.06%; p=0.001) and *Fusobacteria* (YP=0.01%; GP=0.1%; p=0.034) were higher in the glucose groups while *Deltaproteobacteria* (YP=0.28%; GP=0.04%; p=0.016) were higher in the fibre group.

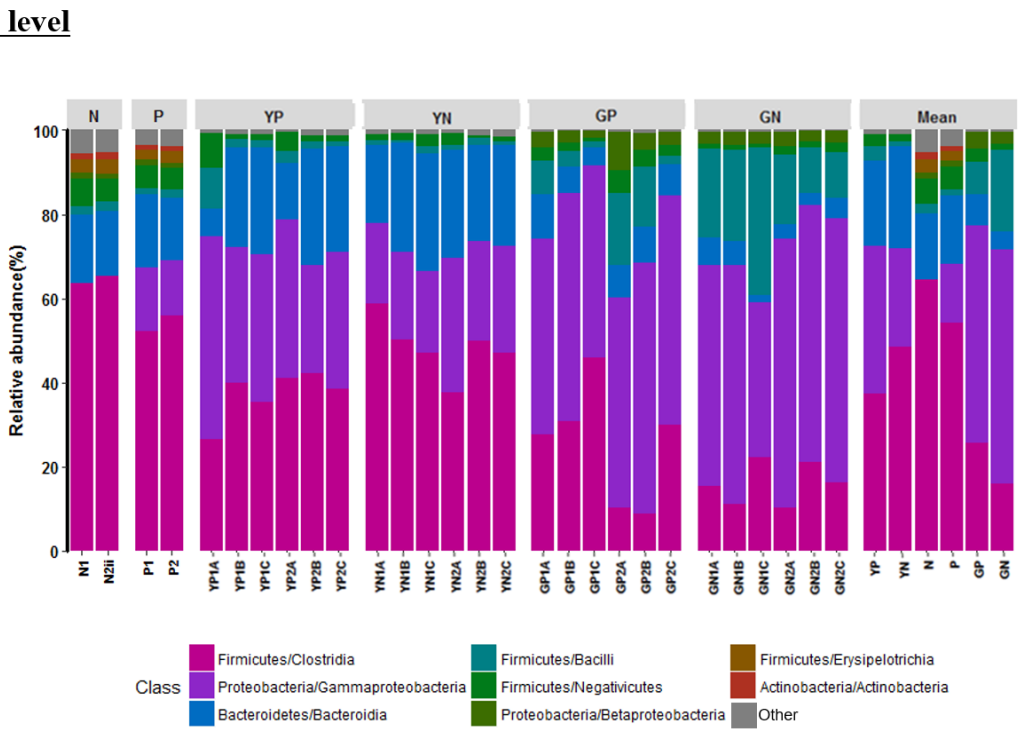



*Figure 2.8 – Relative abundance of taxonomic groups at class level*

**Class level -** Relative abundance of taxonomic groups at the class level for the four fermentation groups (n=6); 24-hour glucose no-pathogen (GN), 24-hour glucose pathogen (GP), 24-hour yeast β-glucan no-pathogen (YN), 24-hour yeast β-glucan pathogen (YP). Inocula and the mean are included.

**Order level**

Figure 2.9 illustrates the relative abundances of taxonomic groups at the order level according to the fermentation conditions. Low-abundant members of the *Burkholderiales* order were higher in the glucose groups both when no pathogens (YN=0.5%; GN=2.95%; p=0.007) and pathogens (YP=0.25%; GP=4.06%; p=0.001) were added. Orders that were significantly increased in the fibre groups were *Bacteroidales* (YN=24.14%; GN=4.25%; p=0.0004), *Clostridiales* (YN=48.43%; GN=16.08%; p=0.004), and low-abundance *Erysipelotrichales* (YN=0.33%; GN=0.06%; p=0.005).

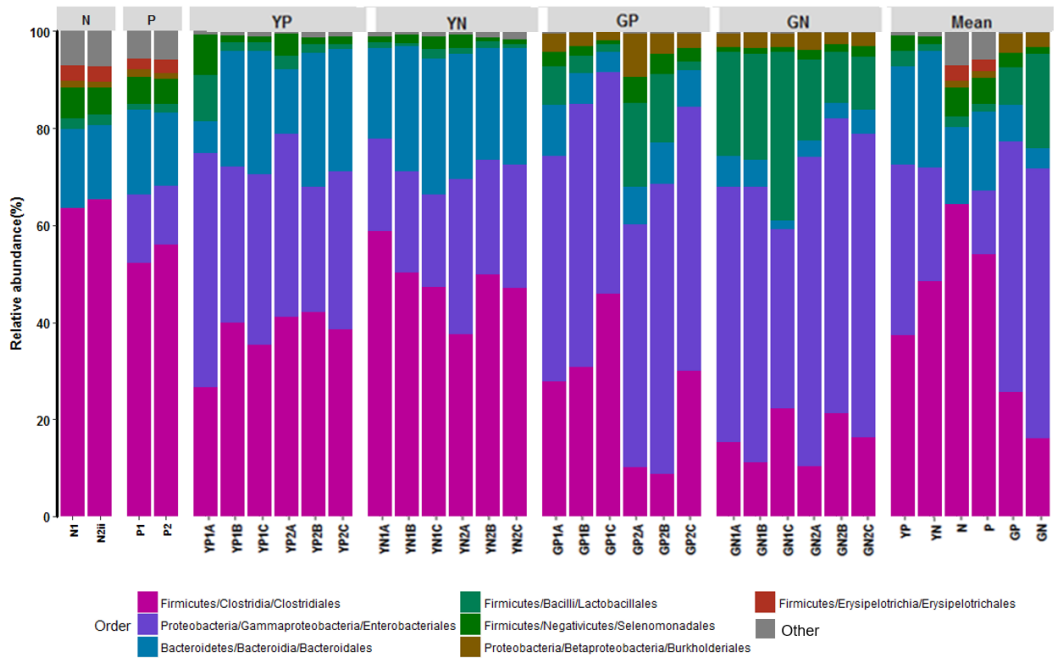



*Figure 2.9 – Relative abundance of taxa at order level*

**Order level -** Relative abundance of taxonomic groups at the order level for the four fermentation groups (n=6); 24-hour glucose no-pathogen (GN), 24-hour glucose pathogen (GP), 24-hour yeast β-glucan no-pathogen (YN), 24-hour yeast β-glucan pathogen (YP). Baseline groups and the mean are included.

**Family level**

Figure 2.10 illustrates the relative abundances of taxonomic groups at the family level according to the fermentation conditions. The main families significantly higher in the fibre groups were *Bacteroidaceae* (YN=23.85%; GN=4.08%; p=0.0004) and *Clostridiaceae 1* (YN=46.34%; GN=12.81%; p=0.005). The main family which was higher in the glucose group was the *Enterobacteriaceae* (YN=23.41%; GN=55.54%; p=0.006). Interestingly, the family *Leuconostocaceae* (YN=0.04%; GN=11.46%; p=0.017%) performed well in the presence of glucose; however, its growth was all but inhibited when pathogens were added (YP=0.002%; GP=0.54%; p=0.007). The *Sutterellaceae* were higher in the glucose groups with no pathogens (YN=0.49%; GN=2.95%; p=0.007) and pathogens (YP=0.25%; GP=4.06%; p=0.001) compared with the respective fibre groups.

**Family level**




*Figure 2.10 – Relative abundance of taxa at family level*

**Family level -** Relative abundance of taxonomic groups at the family level for the four fermentation groups (n=6); 24-hour glucose no-pathogen (GN), 24-hour glucose pathogen (GP), 24 hour yeast β-

glucan no-pathogen (YN), 24 hour yeast β-glucan pathogen (YP). Inocula groups and the mean are included.

**Genus level**

Figure 2.11 illustrates the relative abundances of taxonomic groups at the genus level for the fermentation conditions. *Bacteroides* (YN=23.85%; GN=4.08%; p=0.0005), *Clostridium sensu stricto* (YN=46.34%; GN=12.81%; p=0.005) and unclassified *Enterobacteriaceae* (YN=8.14%; GN=3.36%; p=0.043) were higher in the YN groups compared with the glucose controls. Unclassified *Enterobacteriaceae* was the only main genus significantly higher in the fibre group compared with control (YP=8.64%; GP=2.16%; p=0.01) when the pathogens were added. Low abundance members *Parabacteroides* (YP=0.45%; GP=0.19%; p=0.021), *Clostridium XIVa* (YP=0.74%; GP=0.1%; p=0.028) and *Bilophila* (YP=0.27%; GP=0.04; p=0.004) were significantly higher in the fibre group with pathogens added compared with the respective glucose groups. The genera that performed better in the presence of glucose compared with fibre were *Escherichia/Shigella* (YN=14.96%; GN=52.13%; p=0.011), *Enterococcus* (YN=0.61%; GN=3.05; p=0.035), *Leuconostoc* (YN=0.04; GN=11.46; p=0.017) and *Sutterella* (YN=0.49%; GN=2.92%; p=0.007). When the pathogens were added, we observed higher *Escherichia/Shigella* (YP=10.07%; GP=27.86%; p=0.021) and *Sutterella* (YP=0.25%; GP=4.04%; p=0.001) in the glucose treated groups.

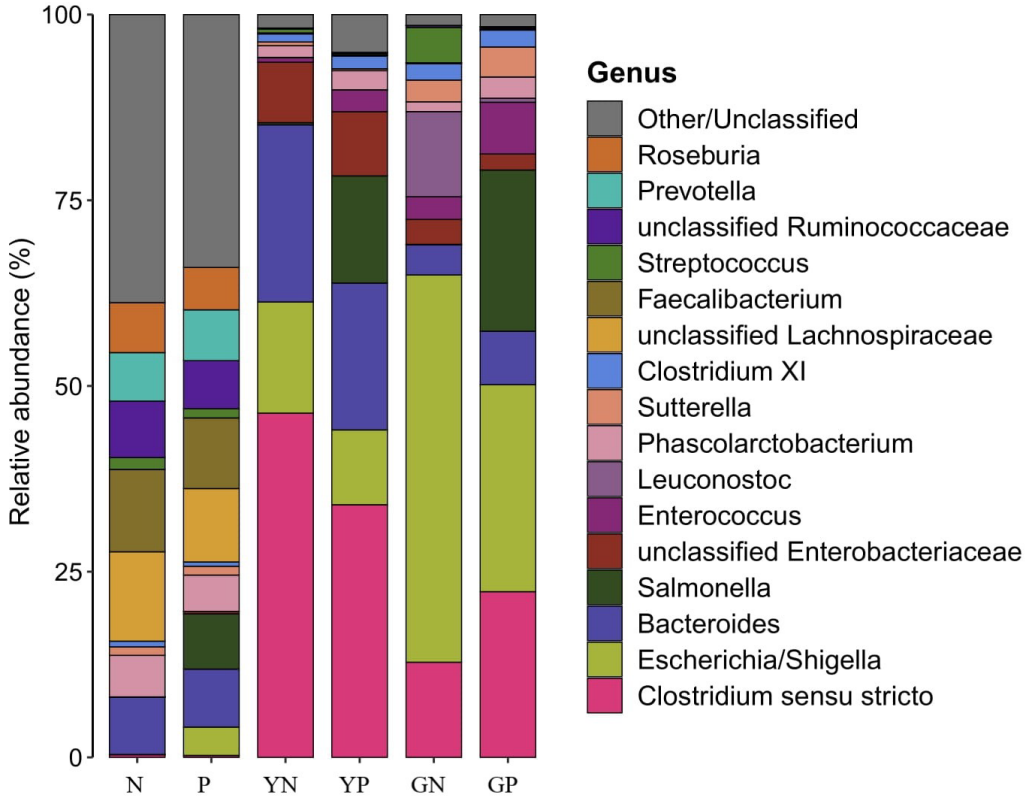



*Figure 2.11 – Relative abundance of taxa at genus level*

**Genus level -** Relative abundance of taxonomic groups at the genus level for the four fermentation groups (n=6); 24-hour glucose no-pathogen (GN), 24 hour glucose pathogen (GP), 24 hour yeast β-glucan no-pathogen (YN), 24 hour yeast β-glucan pathogen (YP). Inocula groups and the mean are included.

**Species level**

Figure 2.12 illustrates the relative abundances of taxonomic groups at the species level for the fermentation groups. We observed six species of *Bacteroides* which were significantly higher in the fibre treated groups; three of these were low-abundance members displaying minimally higher abundances (*Bacteroides intestinalis* (YN=0.04%; GN=0.01%; p=0.032); *Bacteroides salyersiae* (YN=0.23%; GN=0.02%; p=0.015); *Unclassified Bacteroides* (YN=0.42%; GN=0.04%; p=0.008)). One species performed much better in the presence of fibre compared with glucose: *Bacteroides uniformis*

(YN=20.41%; GN=3.15%; p=0.006) and two other species to a lesser extent *Bacteroides ovatus* (YN=1.6%; GN=0.5%; p=0.028) and *Bacteroides vulgatus* (YN=0.95%; GN=0.2%; p=0.007). One species of *Parabacteroides goldsteinii* was significantly higher, albeit at very low abundance, in the fibre groups without (YN=0.19%; GN=0.04; p=0.039) and with (YP=0.35%; GP=0.03; p=0.005) pathogens. A species of *Clostridium paraputrificum* performed comparatively very well in the fibre groups (YN=26.38%; GN=0.94%; p=0.015) and in the presence of pathogens (YP=14.99%; GP=0.54%; p=0.012). An unclassified *Clostridium sensu stricto* (YN=18.42%; GN=4.77%; p=0.043) was significantly higher in the fibre groups but not in both carbohydrate sources in the presence of pathogens (YP=18.76; GP=21.0%; p=0.461). Low-abundant members of *Clostridia* that were higher in the fibre groups included *Clostridium citroniae* (YN=0.23%; GN=0.03%; p=0.037)(YP=0.26%; GP=0.02%; p=0.023); *Clostridium ramosum* (YN=0.27%; GN=0.003%; p=0.006)(YP=0.12%; GP=0.003; p=0.043) and *Clostridium symbiosum* (YP=0.29%; GP=0.01; p=0.031). An unclassified *Enterobacteriaceae* was significantly higher in the fibre groups (YN=8.14%; GN=3.36%; p=0.043); including when spiked with pathogens (YP=8.64%; GP=2.16%; p=0.01).

Two unclassified *Lactobacillus* species were higher in the glucose groups compared with the fibre groups; an unclassified *Enterococcus* (YN=0.2%; GN=1.47%; p=0.04) and an unclassified *Leuconostoc* (YN=0.04%; GN=11.46%; p=0.017) (YP=0.002%; GP=0.54%; p=0.007). *Sutterella stercoricanis* (YN=0.47%; GN=2.91%; 0.013) (YP=0.19%; GP=4.03; p=0.001) was significantly higher in the glucose groups with and without pathogens added compared with the respective fibre groups. An unclassified *Escherichia/Shigella* was significantly higher in the glucose groups (YN=14.96%; GN=52.13%; p=0.011) (YP=10.07%; GP=27.26%; p=0.021) in the presence or absence of pathogens compared with the respective fibre groups. There were no significant differences in pathogen abundances between fibre and control.

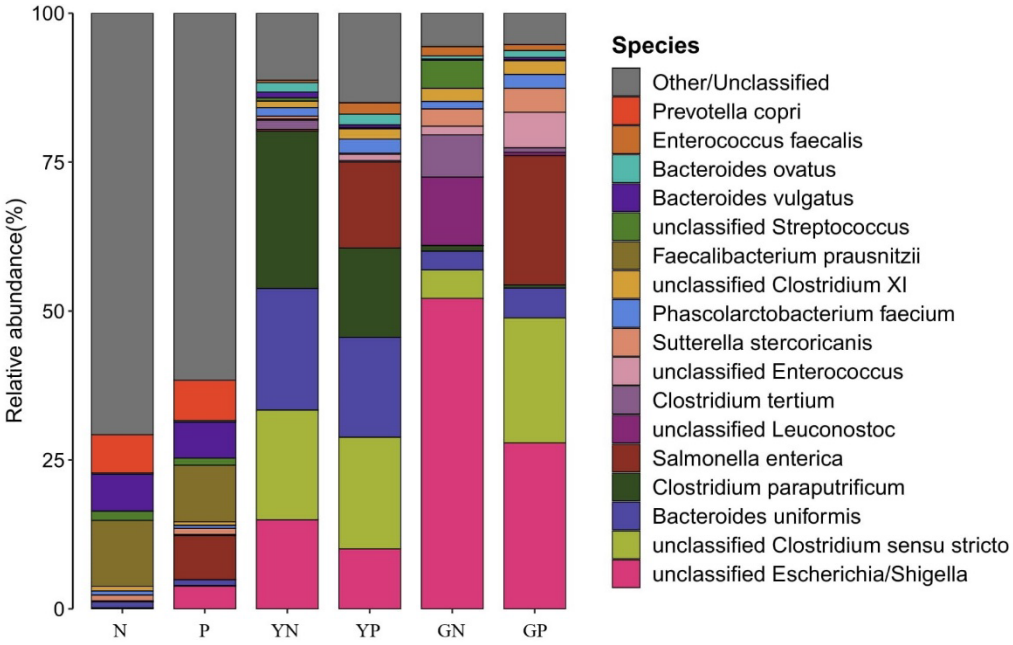



*Figure 2.12 – Relative abundance of taxa at species level*

**Species level -** Relative abundance of taxonomic groups at the species level for the four fermentation groups (n=6); 24-hour glucose no-pathogen (GN), 24-hour glucose pathogen (GP), 24-hour yeast β-glucan no-pathogen (YN), 24-hour yeast β-glucan pathogen (YP). Inocula groups and the means are included.

**Short chain fatty acid analysis**

SCFAs are produced as a result of the catabolism of dietary carbohydrates by gut bacteria. Figure 2.13 shows the SCFA and BCFA concentrations following 24-hour fermentations. Concentrations of SCFA were compared for the four fermentation conditions to determine differences in microbiota production of SCFAs by carbohydrate source and presence of pathogens. When comparing the GN and GP groups, concentrations of acetate (GN=13.65, GP=17.43, *P*=0.002), propionate (GN=1.34, GP=2.45, *P*=0.002) and butyrate (GN=0.65, GP=6.29, *P*=0.002) were significantly higher in the pathogen added group, suggesting that the spiked pathogens are contributing to the production of SCFAs, through fermentation of glucose. With respect to the production of BCFAs, isovalerate (GN=0.15, GP=0.1, *P*=0.002) and total BCFAs (GN=0.22, GP=0.17, *P*=0.009) were significantly lower when the pathogens

were added. Comparisons of the YN and YP groups showed that butyrate (YN=5.32, YP=2.86, *P*=0.004) and total SCFA (YN=24.46, YP=22.29, *P*=0.041) were significantly lower in the pathogen added group, suggesting spiking of pathogens is having a detrimental effect on the fibre-degrading and butyrate-producing commensal bacteria. With regard to BCFA concentrations, there was no significant effect on the production of BCFAs in the yeast β-glucan groups when pathogens were added.

In terms of carbohydrate source, comparisons of the GN and YN groups showed significantly higher concentrations of SCFAs; acetate (GN=13.65, YN=16.95, *P*=0.002), propionate (GN=1.34, YN=2.19, *P*=0.002), butyrate (GN=0.65, YN=5.32, *P*=0.002) and total SCFAs (GN=15.64, YN=24.26, *P*=0.002) in the YN group. Similarly, significantly higher concentrations of BCFAs were observed in the fibre group when comparing GN and YN; isobutyrate (GN=0.056, YN=0.087, *P*=0.002), isovalerate (GN=0.15, YN=0.41, *P*=0.002) and total BCFA (GN=0.22, YN=0.3, *P*=0.002). Interestingly, concentrations of isobutyrate (GP=0.055, YP=0.122, *P*=0.041), isovalerate (GP=0.1, YP=0.25, *P*=0.002) and total BCFA (GP=0.17, YP=0.39, *P*=0.004) were higher in the pathogen-spiked fibre groups, suggesting that the pathogens are contributing to the production of BCFA from fibre.

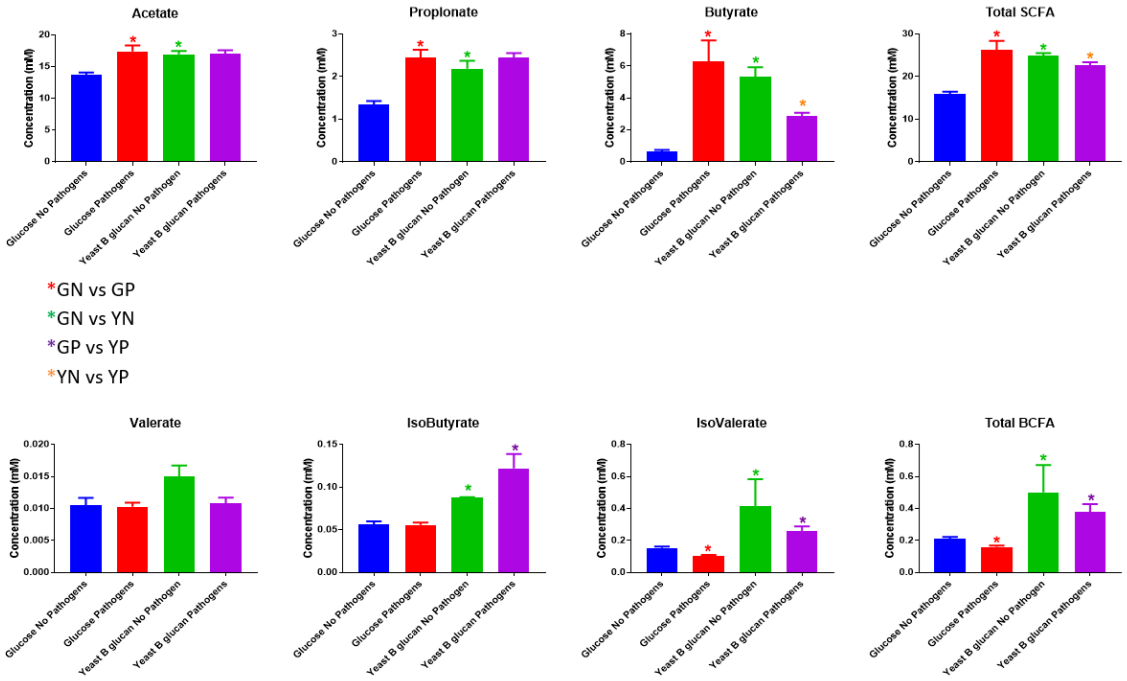



*Figure 2.13 – SCFA analysis for the four fermentation groups*

**Short Chain Fatty Acid analysis –** Short chain and branched chain fatty acid analysis for the four fermentation groups following 24-hour fermentation. 24-hour glucose no-pathogen (Blue), 24-hour glucose pathogen (Red), 24-hour yeast β-glucan no-pathogen (Green), 24-hour yeast β-glucan pathogen (Purple).

## Discussion

Dietary fibre has long been accepted as providing health benefits, such as normalising bowel movements and lowering blood cholesterol. Since the turn of this century, more attention has been focused on the impact of particular indigestible fibres on microbial gut diversity, driven by the advent of next-generation sequencing technologies. In this study, we aimed to investigate the possibility of fibre-mediated increases in gut microbial diversity in providing colonisation resistance against a cocktail of gastrointestinal pathogens. We selected a novel yeast β-glucan as our test prebiotic and compared it against glucose as negative control. We assessed the composition of the gut microbial

community in the fermentation test groups by 16S rRNA sequencing, which provides culture-independent species-level taxonomic resolution (Yarza et al., 2014).

The MicroMatrix has several advantages: it enables a high-throughput fermentation system and owing to the small working volume per vessel (5ml), it requires a low amount of faecal inoculum (250ul) and test substrate (50mg), which in many cases may be valuable compounds. The ability to set-up 24 reactions in parallel, allows for simultaneous independent highly controlled experiments. Additionally, with regard to 16S sequencing analysis, the MicroMatrix can provide higher statistical power through the ability to run many independent reactions. However, this *ex vivo* model is not without its weaknesses; for example, we detected a ~3-fold reduction in the number of observed species from baseline to 24-hour time-point. Maintenance of gut diversity in *ex vivo* models remains a challenge (Julie A.K. McDonald, 2017).The complexity of the microbial interactions between species, nutrient substrates, immune stimuli among others makes it all but impossible to replicate this ecosystem in an *ex vivo* setting. However, in our case, MicroMatrix provides an opportunity to study the impact of a prebiotic fibre on specific bacterial species such as gastrointestinal pathogens.

*Bacteroides spp.* are among the most prominent fibre-degrading commensals in the gastrointestinal tract and we detected eight species of *Bacteroides* in the baseline faecal slurry. Accordingly, we observed that in particular, *Bacteroides uniformis* and to a lesser extent *Bacteroides ovatus* were much higher in the fibre groups compared with the glucose groups. The growth of these two species in the glucose group suggested that the choice of media may provide an advantage over the other *Bacteroides* species. However, a recent study compared the degrading capacity of mixed-linkage β-glucans in 121 strains of *Bacteroides*, and found that only seven strains were capable of growing on β-glucans (Tamura et al., 2017). Interestingly, 34/34 *B. ovatus* and *33/35 B. uniformis* strains tested in that study were competent β-glucan metabolisers, which would explain the large relative abundances of these species compared with the other *Bacteroides* in the yeast β-glucan

groups. This observation further supports the nutrient niche theory, whereby a prebiotic can target particular species in a complex gut community (Pereira & Berry, 2017).

Two species of *Firmicutes* performed well in this system; both belonging to the *Clostridium senso stricto* genus. An unclassified *Clostridium sensu stricto* found in low abundance in the initial inoculum was relatively higher than the inocula in three of the fermentation groups, apart from the GN group, suggesting that the model and the choice of medium itself is well suited to this species. The abundance of this unclassified *Clostridium sensu stricto* is much lower in the GN group compared with the others; concentrations of butyrate are much lower in the GN group compared with the others. These observations combined suggest that this unclassified *Clostridium sensu stricto* may be contributing to the production of butyrate. Indeed, it is known that commensal *Clostridia* are among the main producers of butyrate in the gut (Lopetuso et al., 2013). The other species that were much higher in the fibre treated groups was *Clostridium paraputrificum.* This species is an extremely low-abundant member of the gut microbiome (inocula abundances were below the limit of detection in our study) and has been implicated in cases of bacteraemia (Shinha & Hadi, 2015). It was previously described as a chitinolytic bacterium, producing an array of chitinolytic enzymes (Simunek et al., 2012). Chitin is widely distributed in nature, including the outer shells of crustaceans, arthropod exoskeletons and fungal cell walls. The cell walls in yeast are composed of β(1-3)- and β(1-6) glucan oligosaccharides and chitin (Cabib et al., 2012). It is possible that this species is capable of degrading β-glucan oligosaccharides and/or the crude preparation of the yeast β-glucan contains residual amounts of chitin, which is driving the expansion of this species. The implication of *Clostridium paraputrificum* in sepsis, in combination with its nutrient-niche capabilities in degrading yeast β-glucan, should be considered if this novel prebiotic is to be deemed safe for human consumption.

We observed some species to be significantly higher in the presence of glucose compared with the fibre groups. Adding glucose as a 10% solution, however, may not be representative of physiological conditions, as the majority of glucose molecules in the gut would likely be liberated

slowly from fibre sources and would not come from dietary free glucose or sucrose. An unclassified species of *Leuconostoc*, a member of the *Lactobacillales* family was undetectable in our inocula samples, but was found to be higher in the GN group but not the GP group. The higher relative abundance in this species suggests that either the pathogens are exhibiting an antagonistic effect on its growth either by production of antimicrobials or through competition for glucose. Species of *Leuconostoc* have previously been shown to have a high fermentation capacity of glucose under anaerobic conditions (Dols et al., 1997), compared to other sugars; therefore, it is likely that the pathogens are out-competing the *Leuconostoc* through glucose utilisation. The high concentrations of butyrate in the GP group, but not the GN group, suggest that the pathogens may be contributing to the production of butyrate, when anaerobically fermenting glucose. A member of the *Sutterella* genus with homology to *Sutterella stercoricanis* (Greetham et al., 2004) was observed to be elevated in the glucose treated groups. Members of *Sutterella* have been associated with autism and IBD, yet more recent studies have found that they are abundant in the duodenum of healthy adults with a decreasing gradient toward the colon and display mild-proinflammatory activity (Hiippala et al., 2016), indicating they may have an immunomodulatory role.

A limitation of this study is the choice of spiked pathogens, which are all closely related members of the *Enterobacteriaceae.* A drawback of 16S rRNA sequencing is accurately determining species and strain classification owing to the similarities in the 16S gene, the depth of taxonomic resolution based on the V3-V4 region, and this is even more apparent when attempting to separate closely related members of the *Enterobacteriaceae,* both in terms of pathogenic and commensal microbiome members. Indeed, a recent study demonstrated that choosing the V2-V3 region (205bp) provided higher resolution for lower-rank taxa i.e. genera and species, compared with the V3-V4 region (443bp) (Bukin et al., 2019). Moreover, this resolution is further hampered by variability in the 16S gene within an individual bacterial strain (Vetrovsky & Baldrian, 2013). For example, our 16S data detected two species of *Cronobacter*; an unclassified species and *Cronobacter sakazakii,* both of which were absent in the inoculum with no pathogens added but present in the spiked inoculum. Owing to

the fact that we only spiked one species of *C. sakazakii*, it is possible that there is variability in the 16S gene within this particular strain, as *C. sakazakii* strains generally have seven copies of the 16S gene (Stoddard & Smith, 2015); thus our data give the impression that there is more than one *Cronobacter* strain present. Furthermore, it becomes even more ambiguous if the choice of spiked pathogens' genome is not fully sequenced, which is the case for two of the pathogens (*C. sakazakii* and *E. coli*). Other *ex vivo* models of the gut microbiota have observed high abundances of Proteobacteria growth following 24-hour fermentation (Gopalsamy et al., 2019; O'Donnell et al., 2018) reflecting that particular species perform better *ex vivo*. Moreover, faeces contain a higher proportion of dead bacteria and likely aero-tolerant species which is not representative of the living mucosal-adherent and/or luminal bacteria in the gut.

*Enterobacteriaceae* are found in low abundance in the human gut and generally use simple sugars as a carbon source (Pickard & Núñez, 2019). Western diets which are higher in fat and simple sugars, and lack complex dietary fibres, can promote the growth of pathobiont *Proteobacteria* (Agus et al., 2016). Thus, the expansion of relative abundances of *Enterobacteriaceae* in our glucose groups can be explained by their preference for simpler sugars. Prebiotics can have differential effects on pathogen colonisation (Petersen, Heegaard, Pedersen, Andersen, Sørensen, et al., 2009), of which the vast majority of mechanisms underpinning these effects are largely uncharacterised, but can be dependent on prebiotic source, structure, supplementary diets, microbiota pre-composition and its capacity to respond to the prebiotic. The use of a batch culture method of faecal fermentation, in which it is a closed system, forced us to inoculate the pathogens at timepoint zero. This may not be ideal, as more time would be required for the commensal bacteria to ferment the dietary fibres and produce our hypothetical effect on the pathogens. A continuous model of fermentation may have been more suitable, which can allow for addition of substrates or pathogens at any given time.

We did not find any significant differences in relative abundances of pathogens in the fibre or glucose groups. There could be many reasons in our experimental approach that could be leading to

the apparent lack of effect of the fibre source and some of these could be amended in future studies. The addition of the pathogens as a cocktail might be contributing to the complications seen in our experiment. Spiking the pathogens individually may be a better approach and could potentially avoid ambiguous results. Furthermore, the digested fibre substrate may not have been in a uniform state (i.e., variation in particle size etc.) prior to fermentation, and thus some of the fibre may have been digested faster.

In conclusion, these data show that yeast β-glucan can increase commensal *Clostridia* and *Bacteroides* species *ex vivo.* Commensal *Escherichia/Shigella* and *Lactobacillus* species are increased in the glucose groups. Although we did not find any significant differences in relative abundances of the microbiota on spiking with pathogens, production of SCFAs were increased when pathogens were added to the glucose medium, suggesting that these pathogens play a role in SCFA synthesis.

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# Chapter 3 - Pathogen performance in the presence of prebiotic fibres in an ex vivo model of the human gut

Notes:

Simulated Digestion, pathogen culturing, micromatrix fermentation, DNA extractions, sequencing library preparation, qPCR and statistical analysis was carried out by Ronan Strain (author).

## Abstract

Modern dietary practices, including adoption of the Western diet are associated with reduced consumption of fibre, which can modify gut microbiome ecology. There has been much focus on the link between fibre deprivation and the association of gastrointestinal diseases such as inflammatory bowel disease (IBD) and colorectal cancer. Furthermore, studies in animal models have also implicated a lack of dietary fibre with an increased risk to colonisation of invading pathogens. Here, we sought to screen the putative protective properties of a range of novel prebiotic fibres (arabinoxylan, oat β-glucan, yeast β-glucan) against pathogen proliferation in an *ex vivo* model of the human gut by means of qPCR following 24-hour faecal fermentation. We show that the various fibres had differential effects on pathogen growth. Pathogenic *Escherichia coli* exhibit proliferation in the presence of fibres, with yeast β-Glucan (5.89 x $10^6$ copies/µl) significantly higher than the glucose control (2.63 x$10^3$ copies/µl) (p=<0.05). Similarly, *Salmonella typhimurium* expanded in the presence of fibres, with yeast β-Glucan (6.15 x $10^7$copies/µl) and arabinoxylan (8.35 x $10^7$ copies/µl) significantly higher than glucose (2.33 x $10^3$ copies/µl) (p=<0.05). Conversely, vancomycin-resistant *Enterococcus faecium* growth was significantly higher in the presence of the three test fibres, yeast β-Glucan (2.24 x $10^7$ copies/µl), arabinoxylan (3.66 x $10^7$ copies/µl) and oat fibre (8.71 x $10^7$ copies/µl) compared with the glucose control (1.48 x $10^8$ copies/µl) (p=<0.05). *Listeria monocytogenes* displayed differential growth capabilities in the presence of fibres, with yeast β-glucan enhancing (1.25 x $10^6$ copies/µl) and galactoligosaccharides (4.59 x $10^3$ copies/µl) mitigating growth (p=<0.05).

Our study shows that fibre can influence the growth of gastrointestinal pathogens and sheds light on the potential protective capabilities of prebiotic fibres.

## Introduction

There is roughly as many bacterial cells as human cells in the human body, with the greatest density found in the large intestine at $10^{14}$ bacterial cells (Sender et al., 2016). The intestinal microbiota is crucial to host nutrition, immunity, colonisation resistance to pathogens and pathobiont expansion. This community of microbes is the first hurdle that gastrointestinal pathogens must circumvent in order to establish a replicative niche, before progressing to infection and disease. The mechanisms of microbiota-mediated colonisation resistance is a complicated and multifactorial process: including secretion of antimicrobial peptides (S. G. Kim et al., 2019), production of inhibitory metabolites (Jacobson et al., 2018), induction of infective prophage (Duerkop et al., 2012), competition for oxygen (Litvak et al., 2019) and nutrients (Oliveira et al., 2020).

An underpinning environmental influence that shapes the microbial composition is diet, which determines the bioavailability of nutrients for commensal bacteria. One such dietary component is fibre, which is impervious to human digestive enzymes, but is readily fermented to short chain fatty acids (SCFAs) by a myriad of fibre-consuming saccharolytic bacteria. Fibre itself is not a single substance, but rather a heterogenous group of carbohydrate polymers; the chemical structure and fermentability of which determines their biological activity. The discernible impact of dietary fibre on bacterial taxa and diversity in the gut is a much-studied topic which is reflected in the quantity of publications in this area (for a review see (Makki et al., 2018). Furthermore, a diet rich in fibre reinforces the colonic mucus barrier, a common access point for invasive gastrointestinal pathogens, and thus serves as a primary defence against these species (Desai et al., 2016).

Many gastrointestinal pathogens belong to the *Enterobacteriaceae* family; Gram-negative bacteria that include harmless symbionts, opportunistic pathobionts and “enteric” pathogens. While *Enterobacteriaceae* often comprise <1% of the microbiota in healthy subjects (Donaldson et al., 2016), their propensity to initiate inflammation and capability to survive in the resulting conditions makes them a trademark of microbial “dysbiosis” (Winter et al., 2013). Characteristic features of the *Enterobacteriaceae* that enable them to survive in the inflamed gut include aerotolerance (Rivera-

Chavez et al., 2016), ability to metabolise oxidised carbohydrates (Faber et al., 2016) and having a repertoire of iron siderophores (Kortman et al., 2012). Fibre deprivation has been implicated in reduced *Enterobacteriaceae* colonisation resistance in mice (Desai et al., 2016; Wotzka et al., 2019). Furthermore, the on-going pandemic of antibiotic resistant *Enterobacteriaceae* (extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBLE)) is driving researchers to study and develop new strategies to combat this family of bacteria.

Enterococci are Gram-positive low-abundant members of the gut microbiota comprising <1% in healthy individuals. However, their proportion can change dramatically under the influence of antibiotic treatment, particularly antibiotics e.g., vancomycin, that are effective against anaerobic bacteria (Donskey et al., 2000). The elimination of their anaerobic competitors opens up an opportunistic environmental niche, which allows enterococci to expand to high densities, and thereby facilitating their transmission. Indeed, persistent Vancomycin-resistant *Enterococcus* colonisation is a major risk factor for nosocomial infections, particularly among vulnerable patients (Ubeda et al., 2010). Studies are beginning to identify which commensal species are lost through antibiotic treatment (*Clostridial clusters IV* and *XIVa*) enabling VRE colonisation (Livanos et al., 2018). Interestingly, administration of *Clostridia* increased colonisation resistance against VRE in mice (Caballero et al., 2017). A similar niche-opportunistic pathogen, *Clostridioides difficile* has been shown to be decreased on a high fibre diet, through outgrowth of taxa that feed on the fibre intervention (Hryckowian et al., 2018b). Thus, diet has the potential to select for beneficial taxa that can outcompete antibiotic resistant pathogens, and thereby lower the chance of transmission and infection.

*Listeria monocytogenes* is a Gram-positive food-borne pathogen that is responsible for listeriosis. Specific lactic acid bacteria, which are closely related to *L. monocytogenes* can produce antimicrobial peptides called bacteriocins to inhibit *L. monocytogenes*. Bacteriocins from *Lactobacillus salivarius* (Corr et al., 2007) and *Pediococcus acidilactici* (Fernandez et al., 2016) have been identified

as promising candidates to inhibit *L. monocytogenes* growth. The microbiota plays a protective role against *L. monocytogenes*, as germ-free mice are more susceptible to infection than conventional mice (Archambaud et al., 2013) (S. G. Kim et al., 2019). Indeed, recent studies are beginning to identify specific taxa that provide colonisation resistance against *L. monocytogenes* (Becattini et al., 2017). Furthermore, certain dietary fibres have been shown to provide a protective effect against *L. monocytogenes* in animal models (Ebersbach et al., 2010). In this regard, the contribution of dietary fibre in influencing taxa that provide colonisation resistance against food-borne pathogens is an emerging field of investigation.

Gut fermentation models have been used to study the changes in the microbiota in response to many interventions, including probiotics, prebiotics and pathogens and the lack of a host means that there are fewer ethical concerns. In this study, we employed the use of an *in vitro* model of the human colon to study the effect of three prebiotic fibres (arabinoxylan, oat β-glucan, yeast β-glucan) on the gut microbiota spiked individually with four clinically relevant gastrointestinal pathogens (*Escherichia coli, Salmonella enterica, Enterococcus faecium* and *Listeria monocytogenes*). The effect of fibre on pathogen performance following 24-hours fermentation was assessed using qPCR with glucose as a carbohydrate control.

## Methods

### Faecal sample collection and processing

Faecal samples (n=8) were collected from healthy and obese consenting adults (25-50 y/o) as part of the ImmunoMET project which was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (CREC) according to the study protocol APC 055. All subjects had not consumed antibiotics for 6 months prior to sample collection. Faecal samples were collected in a sterile container and within 1 hour transferred to a laboratory and processed for freezing as described previously

(O'Donnell et al., 2016b). Eight faecal samples were pooled together in equal volumes and stored at -80$^{o}$C prior to the fermentation experiment.

**Bacterial strains, media and fibres**

*Escherichia coli* (DPC 6054; P1432) was grown aerobically overnight at 37°C in LB broth (Merck Millipore). *Salmonella typhimurium* LT2 (DPC 6048; ATCC 700720), *Enterococcus faecium DO* (APC 852; ATCC BAA-472) and *Listeria monocytogenes EGDe* (APC 154; DPC 6554; ATCC BAA-679) were grown aerobically overnight at 37$^{o}$C in Brain Heart Infusion broth (Merck Millipore). On the day of faecal fermentation, overnight cultures of pathogens were sub-cultured into fresh aliquots of their corresponding media and grown to mid-log phase (OD600 ~0.1). Each of the four pathogens was added to fresh pre-reduced basal faecal fermentation media (see below) at a final concentration of 1 x $10^5$ cfu/ml.

A no-carbon minimal faecal fermentation media (Tryptone water 2.0g/l; Yeast extract 2.0g/l; Cysteine-HCl 1.0g/l; Bile salts 0.5g/l; Tween 80 2ml/l; Vitamin k1 10ul/l; NaCl 0.1g/l; $KH_2PO_4$ 0.04g/l; $K_2HPO_4$ 0.04g/l; $CaCl_2.6H_20$ 0.04g/l; $MgSO_4.7H_2O$ 0.01g/l; $NaHCO_3$ 2.0g/l; pH 6.8; Fooks, 2003 #26) was prepared fresh on the day prior to fermentation and allowed to reduce in an anaerobic cabinet overnight (6%$H_2$, 20% $CO_2$, 74% $N_2$). Test fibres were Arabinoxylan (Bioacter A.V), Oat Fibre (Kerry Group) and Yeast β-Glucan (Kerry Group). A 10% glucose (Sigma) solution was prepared as a negative control.

**MicroMatrix *in vitro* fermentation**

An in-vitro digestion step was performed on the fibres to mimic the digestive action of the gastrointestinal tract prior to the fermentation as described previously (Minekus et al., 2014) (see Chapter 2). Applikon MicroMatrix (Applikon Biotechnology, The Netherlands) is a 24-vessel bioreactor enabling small scale faecal fermentations to be performed with individual or simultaneously

controlled parameters (O'Donnell et al., 2018). Each vessel has a 5ml total volume, consisting of 4.75ml faecal fermentation media supplemented with 0.25ml faecal slurry. Predigested test carbohydrates were added at a final concentration of 1% w/v. Each 24-vessel plate consisted of the three test fibres and glucose as control: Glucose (n=3); Arabinoxylan (n=3); Oat fibre (n=3) and Yeast β-Glucan (n=3); spiked with a test pathogen. This approach enabled two pathogen strains to be studied in triplicate for each fibre on each run. Fermentation parameters were as follows; pH 6.8; 37$^{o}$C and 300rpm.

Following 24 hours of fermentation, 2mls of fermentation product was centrifuged at 20,000g for 15 min at 4°C. The supernatant was removed and the pellets stored at -80$^{o}$C prior to DNA extraction.

**DNA extraction**

DNA from the pellets from the faecal fermentation was extracted using a repeated bead beating (RBB) method adapted from (Z. Yu & Morrison, 2004). Extracted DNA was immediately stored at -20$^{o}$C prior to analysis by qPCR. In order to generate standard curves for qPCR, pure genomic DNA of pathogens was extracted using GenElute™ Bacterial Genomic DNA Kit (Sigma) according to the manufacturer's instructions.

**Gradient PCR**

To determine the optimal annealing temperature for qPCR primers (see Table 3.1), primers were tested against genomic DNA isolated from the appropriate pathogen. Primers targeting the 16S gene of *E. faecium* TX16 were designed using PrimerBlast (Ye et al., 2012). Master mix was made to cover 24 reactions consisting of 12.5ul Biomix red (Bioline), 10ul mqH2O, 1ul of 10mM forward primer, 1ul of 10mM reverse primer and 0.5ul gDNA; per reaction. PCR was performed on an Applied Biosystems 2720 thermal cycler (Life Technologies). Annealing temperature gradient was based on the average Tm of the forward and reverse primer minus 5$^{o}$C; this value provided the median temperature over which gradient PCR was performed. No-template controls consisting of 0.5ul mqH20 in place of gDNA

were run in order to eliminate temperatures under which primer-dimers were potentially formed. Optimal annealing temperatures were chosen based on the vividness of the band of the test samples, and the absence of primer dimers in the no-template controls.

*Table 3-1 – Primers used for pathogen quantification*

| **Primer Name** | **Sequence (5'-3')** | **Product Size (bp)** | **AT (°C)** | **Target Gene (Organism)** | **Gene Accession # (Organism Accession #)** | **Reference** |
|---|---|---|---|---|---|---|
| EcH7F<br>EcH7R | GCGCTGTCGAGTTCTATCGAGC<br>CAACGGTGACTTTATCGCCATTCC | 625 | 65 | H7 antigen *(E. coli p1432)* | Not submitted | Gannon (1996) |
| Stm4497F<br>Stm4497R | GGAATCAATGCCCGCCAATG<br>CGTGCTTGAATACCGCCTGTC | 542 | 66 | STM4497 *(S. typhimurium LT2)* | NP_463356.1<br>(NC_003197.2) | Shanmugasundaram (2009) |
| PrfAF<br>PrfAR | GATACAGAAACATCGGTTGGC<br>GTGTAATCTTGATGCCATCAGG | 274 | 60 | PrfA *(L. monocytogenes* EDG-e) | 6EUT_B<br>(NC_003210.1) | D'Agostino (2004) |
| Ef16SF<br>Ef16SR | GCTTCTTTTTCCACCGGAGC | 305 | 55 | 16S *(E. faecium TX16)* | HMPREF0351_r10001<br>(NC_017960.1) | This study |

| | | | | | | |
|---|---|---|---|---|---|---|
| | CTGCCTCCCGTAGGAGT<br>TTG | | | | | |

**qPCR**

Spiked pathogens (*E. coli, S. typhimurium, E. faecium* and *L. monocytogenes*) were detected in the 24-hour fermentates using qPCR. Specific primers (see Table 3.1) were used targeting different genes that ensured specificity. Detection of pathogens was achieved using absolute quantification on a LightCycler® 96 and software (Roche) according to the manufacturer's instructions. Fibre and pathogen combinations were performed in triplicate in the same run. Quantitation cycle (Cq) values of each sample were compared with a standard curve (in duplicate) following the MIQE guidelines (Bustin et al., 2009). The standard curve was generated by diluting known concentrations of pathogen genomic DNA containing a known copy number of target gene in a five-fold serial dilution ($10^7$-$10^2$ copies/uL). No-template controls were included in duplicate consisting of mqH2O in place of DNA. An additional negative control consisting of a faecal metagenomic DNA sample without pathogen was included in each run, to ensure non-specific amplification of primers. A $T_0$ fermentate sample was included in each run, consisting of fermentation media, faecal inocula and spiked pathogen. The qPCR data were expressed as gene copy number per ul of fermentate. The Efficiency of primers (E) was estimated from the slope according to the equation: E = 10(−1/slope) (Pfaffl, 2001). The theoretical maximum PCR efficiency of 100% indicates the amount of product that doubles with each amplification cycle.

qPCR conditions for each reaction (10ul) were as follows: 5µl KAPA SYBR® fast qPCR Mastermix (Kapa Biosystems), 1µl of 10mM forward primer, 1µl of 10mM reverse primer, 3.8µl mqH2O, 1µl DNA. Reactions were incubated in a Lightcycler 96 ® for 5 mins at 94°C, then 40 cycles of 94°C for 30s, AT°C (see table) for 30s and 72°C for 30s. The ability of a substrate to selectively influence the growth of a spiked pathogen was determined by comparing incubations with the glucose control.

### Statistical analysis

One-way ANOVA analysis with Dunnet's post-hoc test was carried out comparing the fibre treatments with the glucose control. Statistical analysis was performed on SPSS (IBM) and visualised using GraphPad prism (GraphPad software). P values of less than 0.05 were considered to be statistically significant.

## Results

### Quantification of *E. coli* P1432 through targeting of the H7 antigen by qPCR

*E. coli* P1432 is a Shiga toxin (stx)-negative strain of 0157:H7, and thus contains the H7 antigen which is used as the target gene for quantification. Figure 3.1 shows the quantity of H7 antigen copy numbers following 24-hour fermentation in the presence of different carbohydrate sources. Growth of *E. coli* in the presence of glucose was limited with a mean of 2.63 $x10^3$ copies/µl. Quantities in the fibre groups were 1.85 x $10^6$ copies/µl, 3.57 x $10^6$ copies/µl and 5.89 x $10^6$ copies/µl in the yeast β-glucan, arabinoxylan and oat fibre groups, respectively. The standard curve generated through serial dilutions of purified *E. coli* P1432 genomic DNA produced a reaction efficiency of 71.26% and a $R^2$ value of 1.00 (Figure 3.2). One-way ANOVA with Dunnett's revealed a statistical significance when comparing the yeast β-glucan ($p=<0.05$) group with the glucose control. The other fibres tested were not significant.

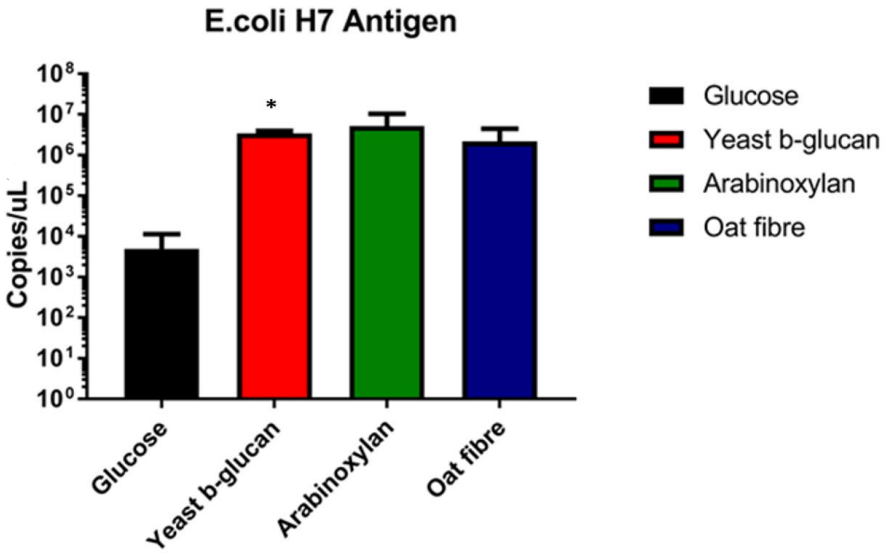



*Figure 3.1 - E. coli H7 antigen copy numbers*

*E. coli* H7 antigen copy numbers ($log_{10}$ copy numbers per µl of fermentation effluent) after 24 hours faecal fermentation with the pooled faecal inocula and test fibres as determined by qPCR. *Significant difference when comparing test carbohydrate with glucose control.

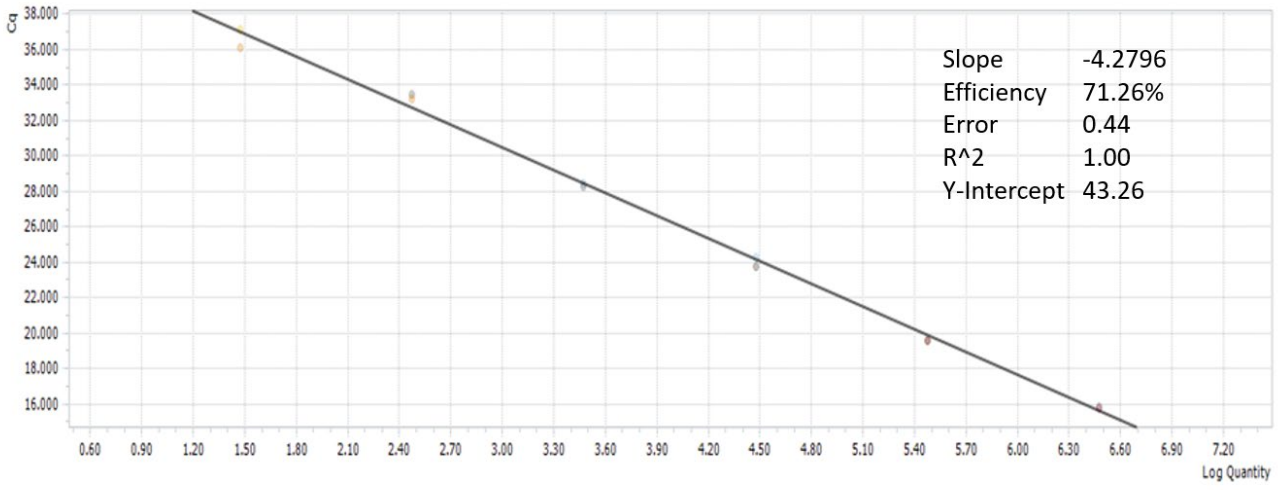



*Figure 3.2 – Standard curve for E. coli H7 gene*

Standard curve of serial dilutions of genomic DNA for H7 antigen of *E. coli* using the Lightcycler96 software.

**Quantification of *S. typhimurium* LT2 through targeting the Stm4497 gene by qPCR**

Primers targeting the *Stm4497* gene (putative cytoplasmic protein) of *S. typhimurium* LT2 enabled specific amplification of the target gene. Figure 3.3 shows the *Stm4497* copy numbers following 24-hour fermentation in the presence of different carbohydrate sources. Similar to the *Enterobacteriaceae E. coli*, growth was limited in the glucose control at 2.33 x $10^3$ copies/µl. Quantities in the fibre groups were 6.15 x $10^7$copies/µl, 8.35 x $10^7$ copies/µl and 1.86 x $10^8$ copies/µl in the yeast β-Glucan, arabinoxylan and oat fibre groups, respectively. One-way ANOVA with Dunnett's revealed statistical significance in the yeast β-glucan and arabinoxylan (p=<0.05) groups when compared with glucose. The standard curve produced through serial dilutions of *S. typhimurium* genomic DNA resulted in a PCR efficiency of 91.06% and an $R^2$ value of 1.00 (Figure 3.4).

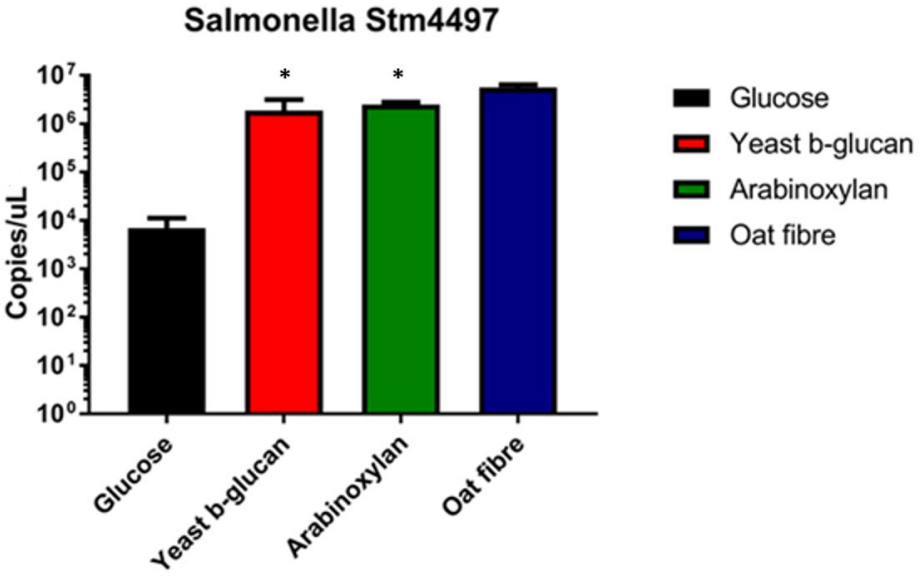



*Figure 3.3 – S. enterica stm4497 gene copy numbers*

*Salmonella stm4497* gene (Putative cytoplasmic protein) copy numbers (log10 copy numbers per µl of fermentation effluent) after 24 hours faecal fermentation with the pooled faecal inocula and test

fibres as determined by qPCR. *Significant difference when comparing test carbohydrate with glucose control *=<0.05).

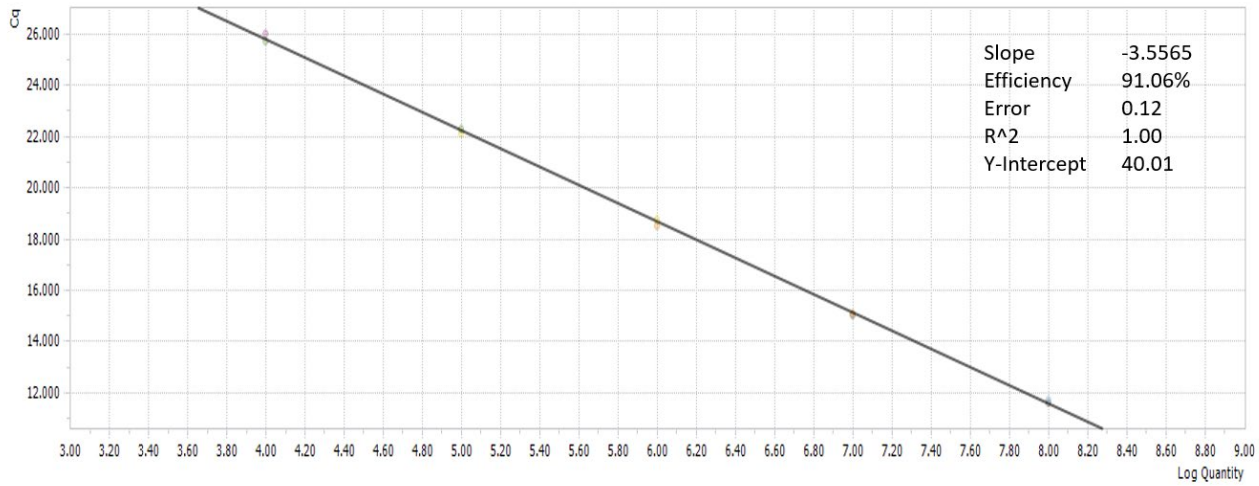



*Figure 3.4 – Standard curve for S. enterica stm4497 gene*

Standard curve of serial dilutions of genomic DNA for *Stm4497* gene of *S. typhimurium* using the Lightcycler96 software.

**Quantification of *E. faecium* TX16 16SrRNA genes through qPCR**

Primers were designed using PrimerBlast based on the 16S rRNA sequence of *E. faecium* TX16. Serial dilutions of TX16 genomic DNA produced a standard curve with a PCR efficiency of 63.18% and an $R^2$ value of 1.00 (Figure 3.6). Figure 3.5 shows the 16S rRNA copies following 24-hour fermentation with the different carbohydrate sources. The glucose control resulted in the highest *E. faecium* growth at 1.48 x $10^8$ copies/µl. Quantities in the fibre groups were 2.24 x $10^7$ copies/µl, 3.66 x $10^7$ copies/µl and 8.71 x $10^7$ copies/µl in the yeast β-Glucan, arabinoxylan and oat fibre groups, respectively. One-way ANOVA with Dunnett's revealed statistical significance in the yeast β-glucan (p=<0.01), arabinoxylan (p=<0.05) and oat fibre (p=<0.05) groups when compared with glucose.

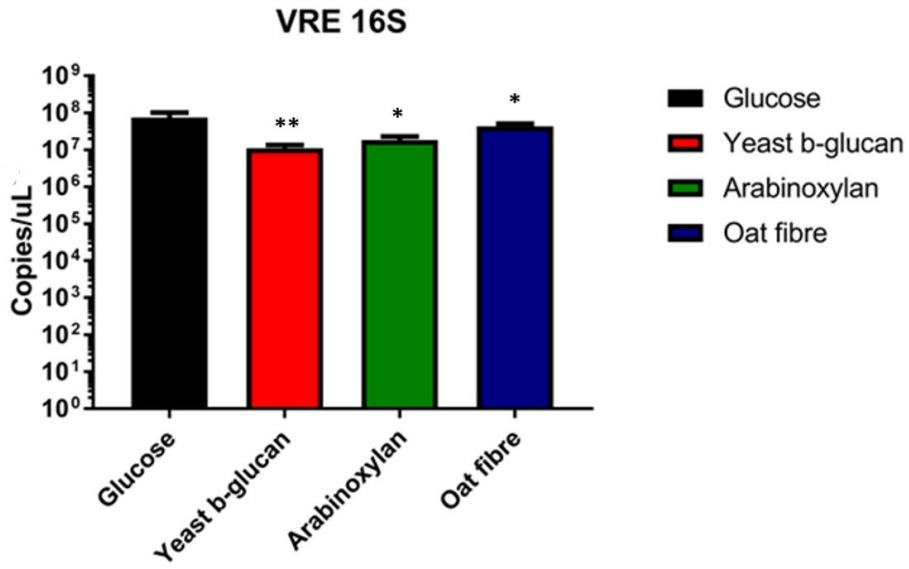



*Figure 3.5 – E. faecium 16S copy numbers*

16SrRNA gene copy numbers from *E. faecium* (log10 copy numbers per µl of fermentation effluent) after 24 hours faecal fermentation with the pooled faecal inocula and test fibres as determined by qPCR. *Significant difference when comparing test carbohydrate with glucose control (*=<0.05; **=<0.01).

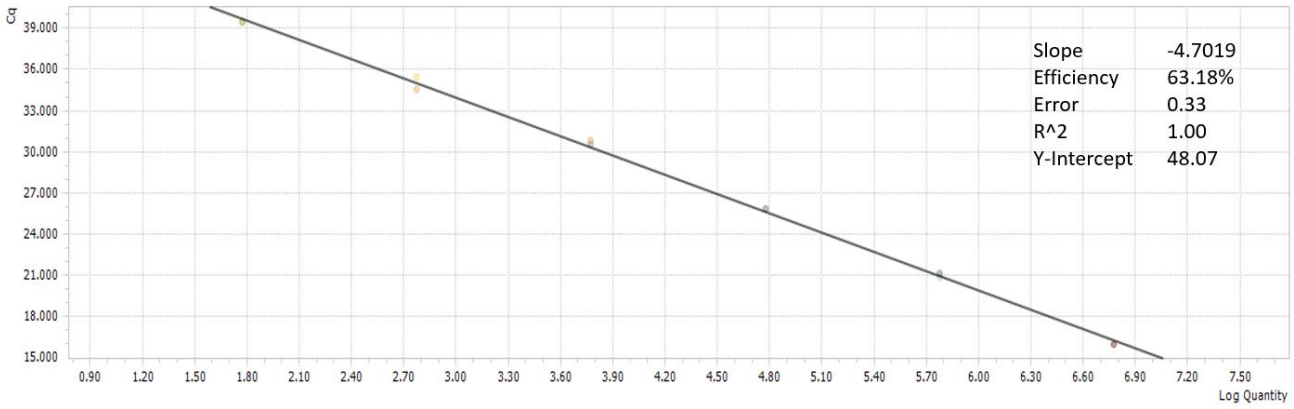



*Figure 3.6 – Standard curve for VRE 16S gene*

Standard curve of serial dilutions of genomic DNA for 16SrRNA gene of *E. faecium* using the Lightcycler96 software.

**Quantification of *L. monocytogenes* EGD-e PrfA gene through qPCR**

The PfrA gene is a single copy gene that encodes the listeriolysin transcriptional regulatory gene, a commonly used target gene that specifically detects *L. monocytogenes.* Serial dilutions of *L. monocytogenes* genomic DNA produced a standard curve with a PCR efficiency of 85.23% and an $R^2$ value of 1.00 (Figure 3.8). Quantification of PrfA copies following 24-hour fermentation in the presence of different carbohydrate sources are shown in Figure 3.7. Additional carbohydrates were tested in this run owing to space available on the MicroMatrix run. The glucose control resulted in concentrations of 3.14 x $10^4$ copies/µl. Quantities in the fibre groups were 1.25 x $10^6$ copies/µl, 7.20 x $10^4$ copies/µl, 2.15 x $10^4$ copies/µl, 7.58 x $10^4$ copies/µl and 4.59 x $10^3$ copies/µl in the yeast β-Glucan, arabinoxylan, oat fibre, cellulose and GOS groups, respectively. One-way ANOVA with Dunnet's revealed a significant difference in the yeast β-glucan and GOS groups when compared to the glucose control.

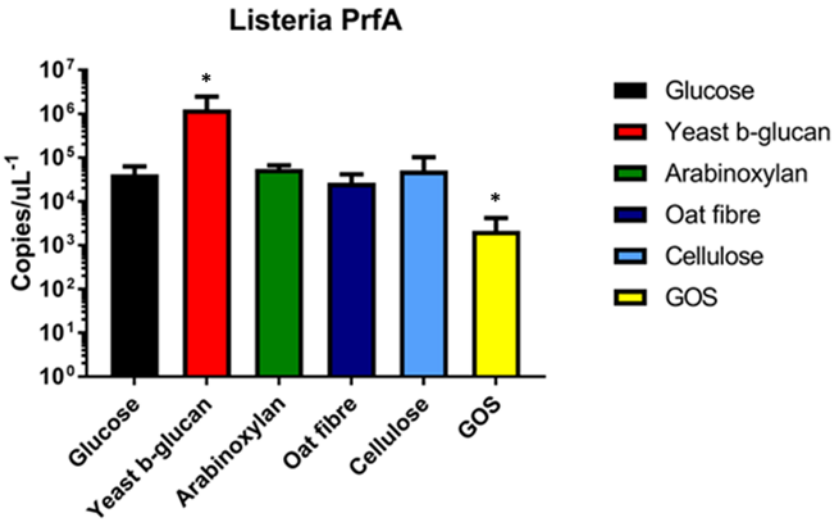



*Figure 3.7 – L. monocytogenes PrfA gene copy numbers*

PfrA (Transcription factor) gene copy numbers from *L. monocytogenes* (log10 copy numbers per µl of fermentation effluent) after 24 hours faecal fermentation with the pooled faecal inocula and test fibres as determined by qPCR. Additional test substrates were included on this run. *Significant difference when comparing test carbohydrate with glucose control *=<0.05).

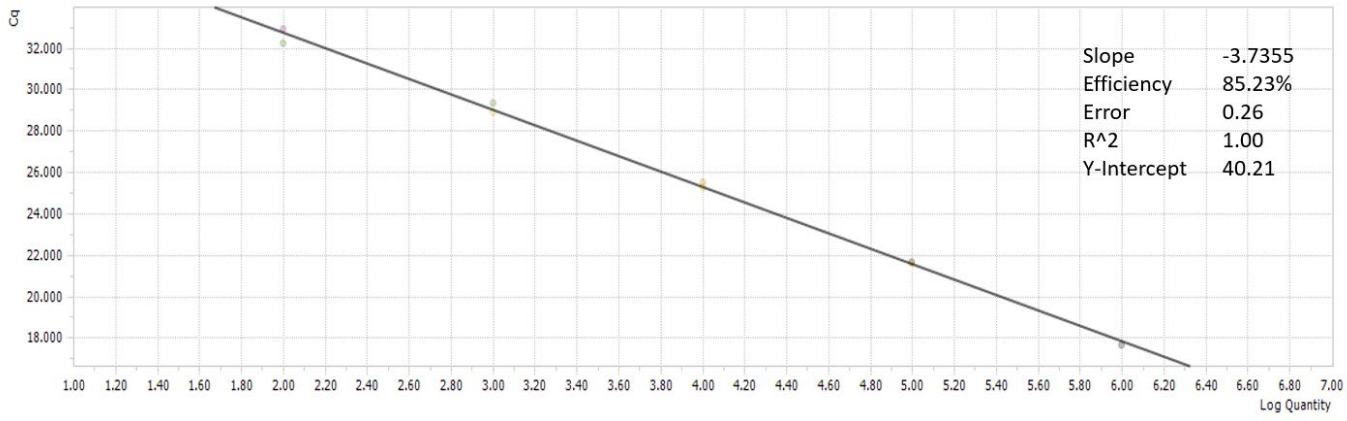



*Figure 3.8 – Standard curve for L. monocytogenes PrfA gene*

Standard curve of serial dilutions of genomic DNA for PfrA gene of *L. monocytogenes* using the Lightcycler96 software.

**Discussion**

Prebiotic fibres are substances which are selectively utilised by commensal bacteria that provide a health benefit; thus, we sought to test if prebiotics can be used to protect against gastrointestinal pathogens. Specific prebiotics such as oligosaccharides have been demonstrated to provide protective effects against pathogens both *in vitro* and *in vivo* (Frese et al., 2017). In this study the aim was to elucidate the putative protective effects of a selection of novel and commercial prebiotic fibres on pathogens in an ex vivo model of the human gut.

The quantitative real-time PCR assay is a powerful tool in its capacity to detect minute quantities of target nucleic acid against a complex, diverse background of DNA, such as those present in faecal samples. At the same time, there are issues regarding the reliability and repeatability of these results that depend on a number of parameters such as sample processing, the method of DNA extraction, choice of fluorescent dye, primers, instrument type and operator usage (Bustin et al., 2009). We employed qPCR to detect spiked pathogens in an ex vivo model of the human gut after 24 hours of fermentation, to assess the putative protective effect of prebiotic fibres on pathogen proliferation. PCR primers were designed to target unique genetic regions of each pathogen to determine abundance following fermentation.

We chose four common gastrointestinal pathogens; two members of the *Enterobacteriaceae* family (*Salmonella* and *E. coli*), vancomycin-resistant *Enterococcus* and *Listeria monocytogenes*. Surprisingly, the growth of *Salmonella* and *E. coli* was almost completely inhibited in the glucose treated group following 24 hours' fermentation (Figures 3.1 & 3.3), suggesting that commensal bacteria in the faecal inoculum were inhibiting their growth. The PCR efficiency for *E. coli* and *Salmonella* were 71.26% and 91.06%, respectively, with both calibration curves maintaining a $R^2$ of 1.00. Ideally, the PCR efficiency should be as close to 100% as possible, with 100% denoting that for each cycle the product doubles exactly (Bustin et al., 2009). The primer set used for *E. coli* targets the *fliC* gene encoding the H7 flagella of *E. coli* 0157:H7 strains (Gannon et al., 1997). There are many reasons for the low efficiency, such as primer sequence, secondary structure, ionic strength and thermocycling conditions (Bustin et al., 2009). Given that these primers were obtained from an old publication (Gannon et al., 1997), it is possible that there is some non-specific amplification in the *fliC* gene of commensal *E. coli*, leading to a lower PCR efficiency. We chose this *E. coli* strain based on its safety profile i.e., a shiga toxin absent model of 0157:H7, in hindsight, a fully sequenced *E. coli* model would have been a better choice in order to design higher specificity primers. Studies in pigs have observed a reduced *Bacteroides:Firmicutes* ratio upon infection with shiga-toxin *E. coli* (STEC) (Bin et al., 2018), suggesting that STEC can outcompete commensal bacteria. Interestingly, prebiotics such as

fructooligosaccharides have been shown to reduce STEC pathogen burden and improve gut health in pigs infected with STEC (L. Liu et al., 2020).

The stm4497 gene of *Salmonella* appeared to be a better target in the complex background DNA of the faecal inoculum, as we observed a high efficiency of 91.06%. It has been shown in previous studies that metabolites produced by commensal such as SCFAs can inhibit *Salmonella* growth (Jacobson et al., 2018) and slow the growth of T3SS-1 (type-3 secretion system 1) expressing subpopulations (Hockenberry et al., 2021). It may be possible that glucose-mediated commensal *Enterobacteriaceae* blooms are outcompeting the spiked pathogenic *Enterobacteriaceae* growth through undetermined mechanisms. A recent study demonstrated that commensal *E. coli* can limit *Salmonella* growth by up to 10,000-fold after challenge in mice compared to a commensal microbiota lacking *E. coli* (Wotzka et al., 2019). Conversely, pathogenic *Enterobacteriaceae* growth was not inhibited in the three fibre treated groups, even though these pathogens were unable to metabolise any of the tested fibres. Fibre-degrading bacteria are primarily members of the *Bacteroides/Prevotella* and *Firmicutes* genus (Makki et al., 2018) and these may not possess inhibitory properties against pathogenic *Enterobacteriaceae* or have much slower growth kinetics to produce an inhibitory effect.

*Enterococcus faecium* strain DO was subjected to the same fermentation procedure as used previously for *Salmonella* and *E. coli*. We chose this strain as it was isolated from a patient with endocarditis and represents a nosocomial lineage responsible for the majority of multidrug-resistant *E. faecium* infections (Kodali et al., 2015). Interestingly, the growth of this pathogen was not inhibited in the glucose group; however, a 1-log reduction in growth in the yeast β -glucan group was obtained, suggesting that this fibre may be a candidate for reducing *E. faecium* burden in hospitalised patients. We targeted the 16S gene for *E. faecium,* as the primers showed high efficiency in test assays. However, the working efficiency in the qPCR assay performed to a low rate of 63.18%, likely a result of non-specific amplification with commensal *Enterococcus. E. faecium* is a member of the Gram-positive *Firmicutes* phylum and it may be possible that commensal fibre-degrading *Firmicutes* are

outcompeting this pathogen's growth, such as was shown in an FMT trial that identified members of the *Lachnospiraceae* and *Clostriaceae* families involved in VRE clearance (Seong et al., 2020). To our knowledge, little research has been performed to date on the potential of dietary intervention to limit multidrug-resistant *Enterococcus,* but it remains an attractive premise to utilise in combination with FMT to accelerate multidrug-resistant *Enterococcus* clearance.

Unlike the previous selection of gastrointestinal pathogens which colonise the colon, *Listeria monocytogenes* predominantly infects in the ileum (Cossart, 2011). Prebiotics that encourage the growth of closely related lactic acid bacteria (LAB) could in theory improve colonisation resistance against *L. monocytogenes.* We included an additional test substrate GOS as a comparison to our test fibres, and an additional cellulose control, as GOS has previously been shown to inhibit *L. monocytogenes* colonisation in a guinea pig model (Ebersbach et al., 2010). We targeted the PrfA gene of *L. monocytogenes*, which is a commonly used target for detection in the food sector (D'Agostino et al., 2004) and our assay demonstrates its high specificity with an efficiency of 85.23%. Given that the natural habitat of *L. monocytogenes* is the soil, it is possible that it possesses the means to metabolise yeast β-glucan or its metabolites, as the structure of yeast β-glucan resembles that which is found in a range of fungi (Ruiz-Herrera & Ortiz-Castellanos, 2019). Our results demonstrate that yeast β-glucan can promote the growth of *L. monocytogenes*, whereas GOS can suppress growth, reaffirming the findings of an *in vivo* study (Ebersbach et al., 2010). A recent study found that commensal microbes of the *Clostridiales* order rapidly cleared *L. monocytogenes* from the intestinal lumen of mice (Becattini et al., 2017), and thus identifying prebiotic substrates that enhance the growth of commensals such as *Clostridiales* could help provide a dietary-mediated colonisation resistance against *L. monocytogenes*. Our results open up the possibility of screening other oligosaccharides, such as fructooligosaccharides (FOS) obtained from human breast milk, as a means of boosting colonisation resistance against food-borne pathogens.

Whilst the burden of gastrointestinal infections is higher in developing nations as opposed to westernised nations (S. M. Fletcher et al., 2013), with the former retaining higher fibre intake, the encroaching threat of antibiotic-resistant pathogens in the west encourages new, or in essence, old strategies to limit pathogen expansion in the gut. Our findings can provide a platform to explore this paradigm further, and inform future animal studies by means of a starting point.

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# Chapter 4 - Prebiotic potential of fibre and lipids for metabolic syndrome in an ex vivo model of the human gut

Notes:

Simulated Digestion, micromatrix fermentation, DNA extractions, sequencing library preparation and fatty acid analysis was carried out by Ronan Strain (author).

Sample collection and preparation was performed by Dr. Eibhlis O'Connor

Analysis of sequencing data was performed by Dr. Tam Tran.

## Abstract

Obesity and type 2 diabetes are metabolic disorders that are associated with a microbiota imbalance. Given that diet Influences microbial composition, we sought to assess the prebiotic potential of three β-glucans (two oat β-glucans isolated using two different extraction methods from spent brewer's grain and one yeast β-glucan), and three lipids (fish oil, palmitic acid and oleic acid) in an *ex vivo* model of the human colon. Faecal samples were collected from patients of varying levels of metabolic syndrome; obese (n=2), prediabetic (n=3) and type 2 diabetic (n=7). The samples were subjected individually to a 24-hour faecal fermentation in a 24-well MicroMatrix™ using a 6 X 4 factorial design which consisted of four β-glucan treatments, plus positive and negative controls (oat β-glucan1, oat β-glucan2, yeast β-glucan, oat β-glucan2 + yeast β-glucan, and Synergy, a commercially produced inulin-fructoligosaccharide (FOS) served as positive control fibre, while glucose served as the negative control) and three lipid treatments, plus negative control (palmitic acid, oleic acid, fish oil, and no lipid control). Effects on microbial composition and diversity were assessed by 16S rRNA gene sequencing following DNA extraction and PCR amplification and some treatments were assessed for short chain fatty acid production. All β-glucans and lipid sources significantly (p=<0.05) impacted alpha- and beta-diversity matrices. We observed a significant (p=<0.05) reduction in *Escherichia/Shigella* abundances following β-glucan supplementation compared with glucose, with a concomitant rise in relative abundance of taxa negatively associated with metabolic disease including *Faecalibacterium* and *Bacteroides.* The lipids markedly impacted a range of taxa, with fish oil exhibiting a reduction in pro-inflammatory LPS-producing *Enterobacteriaceae* and a rise in *Bifidobacterium* and *Veillonella.* The results demonstrate that β-glucans and fish oil offer dietary tools to promote diversity and potentially beneficial taxa in patients with metabolic syndrome.

## Introduction

Obesity is a global health concern that has increased over the past ~50 years, reaching pandemic levels. It is a strong risk factor for metabolic complications such as type 2 diabetes (T2D) (Lambeth et al.), driven by obesity-induced insulin resistance, with over 90% of T2D patients being overweight or obese (Okazaki et al., 2019). While development of T2D is governed by genetic and environmental factors, there is accumulating evidence that it is influenced by patients' gut microbiota, specifically through particular species abundance, diversity and subsequent metabolite production (Harsch & Konturek, 2018). Additionally, the gut microbiota is associated with modulation of immune function and inflammation, and loss of microbiota diversity has been observed in T2D patients (Qin et al., 2012). Although the exact mechanisms linking the gut microbiota and the development of T2D remain unknown, alterations of the gut microbiota (termed 'dysbiosis') observed in T2D patients strongly suggests it plays a role in the pathogenesis of T2D.

Specific food ingredients, such as prebiotics, can promote gut microbiota diversity, through colonic fermentation and offer a tool for promoting health and well-being. Prebiotics are defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). Inulin is a type of fructan compound (β[2,1]-fructans) and is a universally agreed-upon prebiotic known to exert strong bifidogenic effects i.e. promote the growth of beneficial bifidobacteria (van de Wiele et al., 2007). Novel prebiotic candidates, such as β-glucans, are soluble fibres derived from mushrooms, yeast and oats and have been receiving research attention recently. β-glucans possess many health benefits such as immune stimulatory properties and help maintain blood glucose homeostasis, among others, in part through their effects on the gut microbiota (Jayachandran et al., 2018). Thus, β-glucans have been proposed as potential pharmaceutical prebiotics in the management of diabetes (Y. W. Kim et al., 2005). Aside from the known health benefits of β-glucans, more focus has shifted recently to the direct effects of β-glucans on gut microbial composition. *In vitro* and *in vivo* studies have shown that β-glucans can promote the growth of *Bifidobacterium* and *Lactobacillus*

(Jaskari et al., 1998; Snart et al., 2006), and increase the production of short chain fatty acids (SFCAs) (De Angelis et al., 2015; Drzikova et al., 2005). Colonic fermentation of dietary fibres by the microbiota results in the production of SCFAs. The main SCFAs produced by the gut microbiota are butyrate, acetate and propionate; with a consistent finding that T2D patients possess a gut microbiota composed of species that are less adept at producing butyrate, a SCFA associated with improved insulin response (Sanna et al., 2019).

Insulin resistance is defined as a defect in the efficiency of insulin to stimulate glucose entry into its target tissues, and often precedes and predicts the development of T2D (Samuel & Shulman, 2016). Free fatty acid (FFA) concentrations are increased in obese subjects as a consequence of the expansion of adipose tissues and are linked to impaired β cell function, inflammatory processes and provoke insulin resistance (Biden et al., 2014). However, the saturated fatty acid, palmitic acid, and the monounsaturated fatty acid, oleic acid, contribute differently to the development of insulin resistance. Palmitic acid attenuates the insulin signalling pathway through numerous mechanisms and thus provokes insulin resistance, whereas oleic acid prevents attenuation of the insulin pathway and possesses anti-inflammatory properties and thus mitigates the development of insulin resistance. Furthermore, omega-3 PUFAs have been observed to correct postprandial hyperglycemia and improve insulin secretion in patients with impaired glucose metabolism (Sawada et al., 2016). The direct effect of FFAs on the microbiota is gaining interest, opening up new potential mechanisms in the development or prevention of T2D. For example, palmitic acid has been observed to select for a gut microbial community that produces more lipopolysaccharide (LPS), a known inflammatory factor (Cani et al., 2007); oleic acid was capable of impacting the gut microbiota at the genus level in obese subjects (Pu et al., 2016) and omega-3 PUFAs were shown to increase *Bifidobacterium*, *Roseburia* and *Lactobacillus* abundance in healthy subjects (Watson et al., 2018).

*In vitro* gut fermentation models are often used to complement *in vivo* animal and human studies, allowing for the study of the direct effect of a dietary intervention on the changes in the gut

microbiota community, without confounding effects on the host (Payne et al., 2012). Moreover, *in vitro* models employ a highly controlled environment enabling reproducible and stable representations of complex microbial communities, bypassing tight ethical constraints (J. A. McDonald et al., 2013). There are various gut model systems described to date, each varying in their design and complexity (Payne et al., 2012). Further, each model system comes with its own advantages and disadvantages (for a review, see (Julie A.K. McDonald, 2017)); therefore choice of *in vitro* model should correspond with the aims of the study.

The aim of this study was to assess the effect of prebiotics, novel dietary fibres and lipids, including fatty acids, on promoting gut microbiota diversity in obese and diabetic patients. Faecal samples were collected from obese, pre-diabetic and diabetic subjects and underwent *in vitro* faecal fermentation experiments using a 6X4 factorial design. The faecal samples were supplemented with 3 β-glucans (2 oat β-glucans, 1 yeast β-glucan and a combination of oat β-glucan 2 and the yeast β-glucan). Fructo-oligosaccharide enriched inulin (Synergy 1) and glucose served as the positive and negative controls, respectively. The three lipid sources were the fatty acids, palmitic acid (saturated), and oleic acid (monounsaturated), and a fish oil (containing a mix of fatty acids but predominantly the long-chain n-3 polyunsaturated fatty acids (PUFA), docosahexaenoic acid and eicosapentaenoic acid) while fermentation with no lipid served as the control. Effects on microbiota were assessed by investigating changes in microbial diversity based on 16S rRNA sequencing, and SCFA production, which was measured by GC-FID.

## Methods

### Subject recruitment and faecal sample processing

Faecal samples were obtained from consenting subjects with varying levels of metabolic syndrome under the approval of the Clinical Research Ethics Committee of the Cork Teaching Hospitals (CREC) according to the study protocol APC 055. Samples were collected from two non-diabetic obese patients, three pre-diabetic obese patients and seven diabetic obese patients (all of whom did not

consume any antibiotics in the previous six months), and the samples were immediately stored at 4$^{o}$C. The diabetic, pre-diabetic and obese were categorised based on plasma glucose concentrations. A standardised faecal inoculum for each subject was prepared as described previously (O'Donnell et al., 2016a), incorporating 60g of each patient's faecal sample and aliquoted in 2ml eppendorfs and immediately stored at -80$^{o}$C.

**Preparation of test carbohydrates and lipids**

Fibres were subjected to a simulated digestion representing the physiologically relevant conditions present in the gastrointestinal tract, in order to produce digestate that would simulate what reaches the colon and gut microbiota in vivo. Digestion was adapted for 30g of test fibre following the protocol as described previously (Minekus et al., 2014). The resulting digested fibres were lyophilised in a VirTis AdVantage freeze drier and stored at -20$^{o}$C. A 10% solution of glucose (Sigma) was prepared in Milli-Q water. Palmitic acid (Sigma, Ireland), oleic acid (Sigma, Ireland), Fish oil (Biopharma, Norway), and Synergy 1 (Orafti, Belgium) were left in their neat form. Synergy 1 is a commercially available prebiotic fibre that is free of any digestible portions and would be too small for final dialysis step to purify the indigestible fibres.

**MicroMatrix faecal fermentation**

The Applikon MicroMatrix (Applikon Biotechnology, The Netherlands) was employed to perform 5ml volume micro faecal fermentation experiments as described previously (O'Donnell et al., 2018), with minor modifications. A no-carbon faecal fermentation medium (Fooks & Gibson, 2003) was prepared and reduced in an anaerobic cabinet overnight prior to the day of the fermentation experiment. To each well of the MicroMatrix the following was added; 50mg (1% w/v) of test fibre (or 1% glucose), 50ul (1% v/v) test lipid (or no lipid), 250ul (5% w/v) test faecal inoculum and 4.75ml fermentation medium. The MicroMatrix enables 24 fermentations to be performed simultaneously, and thus each subject's faecal sample was tested against 24 fibre lipid combinations in the following orientation which provided a 6X4 factorial design to test the main effects of each of the fibre and lipid sources:

*Table 4-1 – MicroMatrix cassette layout for each fermentation condition*

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A + No Lipid | Glucose | Oat Fibre 1 | Oat Fibre 2 | Yeast β-glucan | Synergy 1 | Oat 2 + Yeast |
| B + Palmitic Acid | Glucose | Oat Fibre 1 | Oat Fibre 2 | Yeast β-glucan | Synergy 1 | Oat 2 + Yeast |
| C + Oleic Acid | Glucose | Oat Fibre 1 | Oat Fibre 2 | Yeast β-glucan | Synergy 1 | Oat 2 + Yeast |
| D + Fish Oil | Glucose | Oat Fibre 1 | Oat Fibre 2 | Yeast β-glucan | Synergy 1 | Oat 2 + Yeast |

Fermentations were run for 24 hours with the temperature kept constant at 37$^{o}$C, pH at 6.8, anaerobiosis maintained by addition of $N_2$ gas and orbiter set at 300rpm. Two millilitres of fermentate were collected and centrifuged at 16,000xg for 15 min, the pellet and supernatant retained and frozen at -80$^{o}$C.

**Bacterial DNA extraction and 16S rRNA gene sequencing**

Total bacterial DNA was isolated from faecal fermentate pellets according to the Repeated Bead Beating plus Column (RBB+C) method (Z. Yu & Morrison, 2004) in combination with a Qiagen Blood & Tissue Kit (Qiagen, Ireland). 16S V3-V4 gene sequencing was carried out according to the Illumina 16S metagenomics sequencing protocol, which incorporates an overhang adaptor using the Illumina primers F - 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and R - 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC (Klindworth et al., 2013). Indexed PCR products were cleaned with AMPure XP beads (Beckman Coulter, UK) and quantified with Qubit dsDNA HS Assay kit (Life technologies) and pooled together in an equimolar ratio. Samples were sequenced on the Illumina MiSeq Sequencing platform (Clinical Microbiomics, Denmark) using a 2 x 250 cycle kit (Illumina, UK).

**Gut microbiota composition and diversity**

Illumina MiSeq sequencing reads were quality-filtered and aligned into OTUs as described previously (Tran, Cousin, et al., 2019). Illumina overlapping paired-end sequencing reads were joined using FLASH v1.2.8 and filtered using the script 'split_libraries_fastq.py' from Quantitative Insight into Microbial

Ecology (QIIME) v1.9.1 software. The resulting quality filtered reads were combined and processed with the sequence analysis software USEARCH v6.1. The sequences were filtered by length, dereplicated and retained sequences with lengths of 350-370 bp for V4-V5 amplicon region and 373-473 bp for V3-V4 amplicon region. Reads that that did not assemble into a contig (singleton) were removed, the remaining sequences for each amplicon were clustered at 97% similarity into operational taxonomic units (OTUs). Chimeras were filtered out using UCHIME against the GOLD reference database. The quality filtered reads were then aligned against the filtered OTUs to create an OTU table. QIIME was used to calculate alpha and beta diversity metrics. The quality filtered reads were taxonomically assigned with the Ribosomal Database Project (RDP) database trainset 14 using the data processing software mothur v1.36.1 and spingo v1.3. Unclassified species were designated as sequences with a confidence score of below 50%. Four samples from each group of oat fibre 2, Synergy 1 and fibre mix were removed owing to bad quality reads obtaining n=44 in these groups. Differential abundance at the genus level in carbohydrate treatment and lipid treatment was identified using DESeq2 (Love et al., 2014), and adjusted using Benjamini–Hochberg correction.

**Short & Branched Chain Fatty Acid Analysis**

SCFA quantification was performed by gas chromatography flame ionisation detection (GC-FID) using a CP-3800 GC system (Varian, address), equipped with a Zebron ZB-FFAP column (Phenomenex, address) and a flame ionisation detector with a CP-8400 autosampler (Varian). A 2ml aliquot of fermentation liquid was centrifuged at 16,000xg and syringe-filtered (0.2um) to remove bacterial debris and subsequently subjected to gas chromatography as described previously (Cussotto et al., 2019). SCFA analysis was only performed for the no lipid and palmitic acid-carbohydrate groups. It appeared that our internal standard 2-ethylbutyric acid was reacting with the oleic acid and fish oil fermentates, thus we were unable to produce valid standard curves using these lipids.

**Statistical analysis**

Statistical analysis was performed using the R v.3.5.1 package (Team, 2016). The significant difference between alpha diversity comparisons was made using Kruskal-Wallis test with Dunn's multiple comparison test, with P-value corrected for multiple testing by Benjamini-Hochberg correction. Differences in beta diversity were examined using analysis of similarity (ANOSIM). Comparisons of differential abundance at the genus level between the treatment groups were made using DESeq2 and adjusted using Benjamini-Hochberg correction. Fatty acid analysis data are presented as mean + SEM and visualised using Graphpad Prism, with threshold for statistical analysis set at <0.05.

## Results

### Differences in microbial composition based on carbon source

#### Alpha diversity

Alpha diversity is defined as the bacterial diversity within a particular ecosystem or sample. We used five different alpha diversity metrics to assess the effect of carbohydrate source (Glucose; Fibre mix; Oat fibre 1; Oat fibre 2; Synergy 1; Yeast β-glucan) on diabetic/prediabetic microbiota. The alpha diversity indices (Phylogenetic diversity; Observed species; Chao1; Simpson index; Shannon index) are shown in Figure 4.1, with one-way analysis of variance (ANOVA) comparing linear mixed models indicated above the boxplots. We observed significant changes in observed species and Chao1 when comparing glucose and the carbohydrate treatments for all except for Oat fibre 2. All carbohydrate treatments displayed significance for Simpson and Shannon indices when compared to glucose.

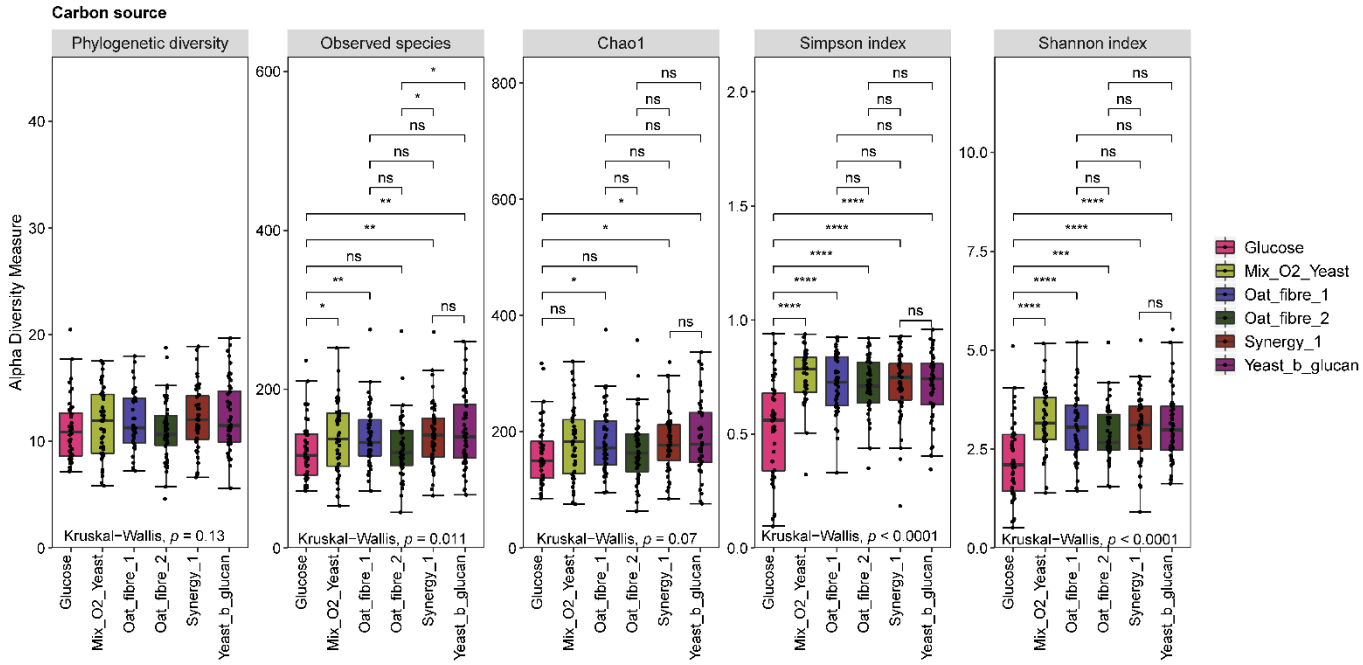



*Figure 4.1 – Alpha diversity metrics for carbohydrate source*

**Alpha diversity metrics based on carbon source for fermentation groups –** Differences in alpha diversity (phylogenetic diversity, observed species, chao1, Simpson and Shannon indexes) of microbial composition based on carbon source (Glucose (n=48); Fibre mix (n=44); Oat fibre 1 (n=48); Oat fibre 2 (n=44); Synergy 1 (n=44); Yeast β-glucan (n=48)). One-way ANOVA comparing linear mixed models for carbon source are indicated above the boxplots. Levels of significance are as follows; * = <0.05, ** = <0.01, *** = <0.005, **** = <0.001.

**Beta diversity**

Beta diversity (PCoA) analysis measures the change in diversity of species from one environment to another. Comparisons between the different fibre groups can be seen in Figure 4.2. Only weighted UNIFRAC (p=0.002) measures were found to be significant. Weighted UNIFRAC weighs the phylogenetic branch lengths by the relative abundances of the microbes and therefore emphasises dominant species. Unweighted UNIFRAC only uses presence or absence of species and therefore emphasises low-abundant species. Thus, the differences between microbial community composition from the fibre sources is due to the differences in the relative abundances of bacterial lineages rather

than the presence or absence of microbial community. Therefore, relative abundances of bacterial lineages in the fibre sources groups were significantly different when compared with the glucose control.

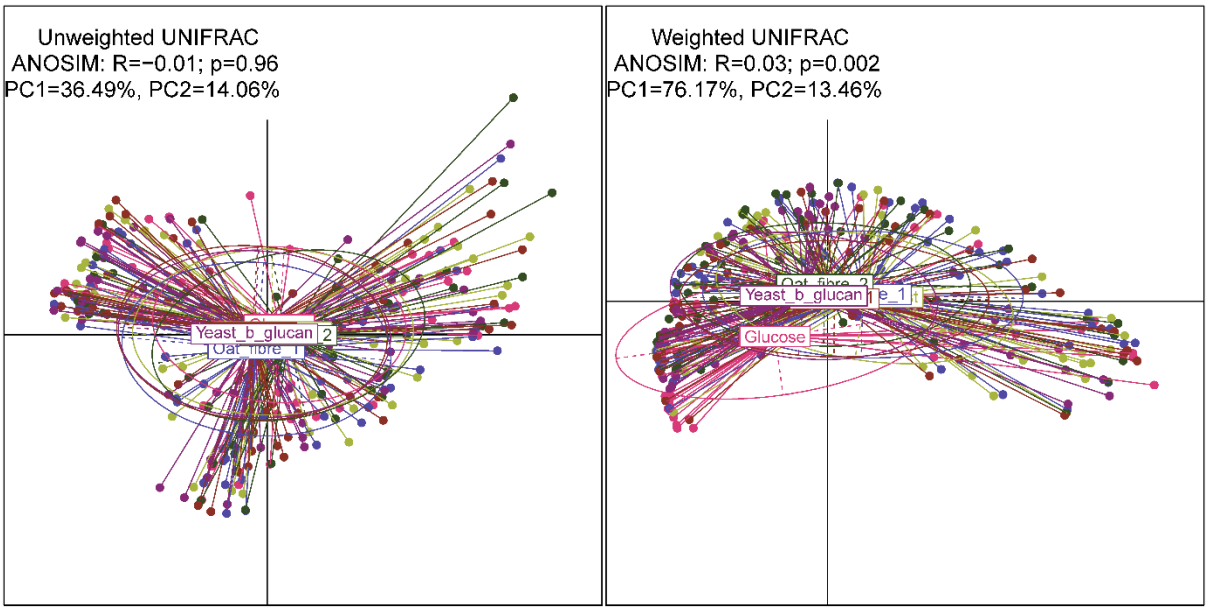



*Figure 4.2 – Beta diversity metrics for carbohydrate source*

**Beta diversity metrics based on carbon source for the fermentation groups -** Principal coordinates analysis (PCoA) based on unweighted and weighted UniFrac distances of the 16S rRNA gene showing microbial diversity based on carbon source (Glucose (n=48); Synergy 1 (n=44); Oat fibre 1 (n=48); Oat fibre 2 (n=44); Yeast β-Glucan (n=48); Oat fibre 2 & Yeast β-Glucan Mix (n=44)) following 24-hour fermentation.

**Taxonomic analysis**

The predominant families following 24-hour fermentation based on carbon source can be found in Figure 4.3. Overgrowth of *Enterobacteriaceae* was observed in the majority of patient samples regardless of carbohydrate source, and to a lesser extent *Streptococcaceae* and *Enterococcaceae*. The largest increase in *Bacteroidaceae* was observed in the fibre mix group. Members of the *Clostridiaceae 1* family were observed to be increased in some patients but not others. Other potential outliers were

observed in the families *Peptostreptococcaceae* and *Veillonellaceae*. Small increases in the *Lachnospiraceae*, *Bifidobacteriaceae* and *Erysipelotrichaceae* families were observed relative to glucose.

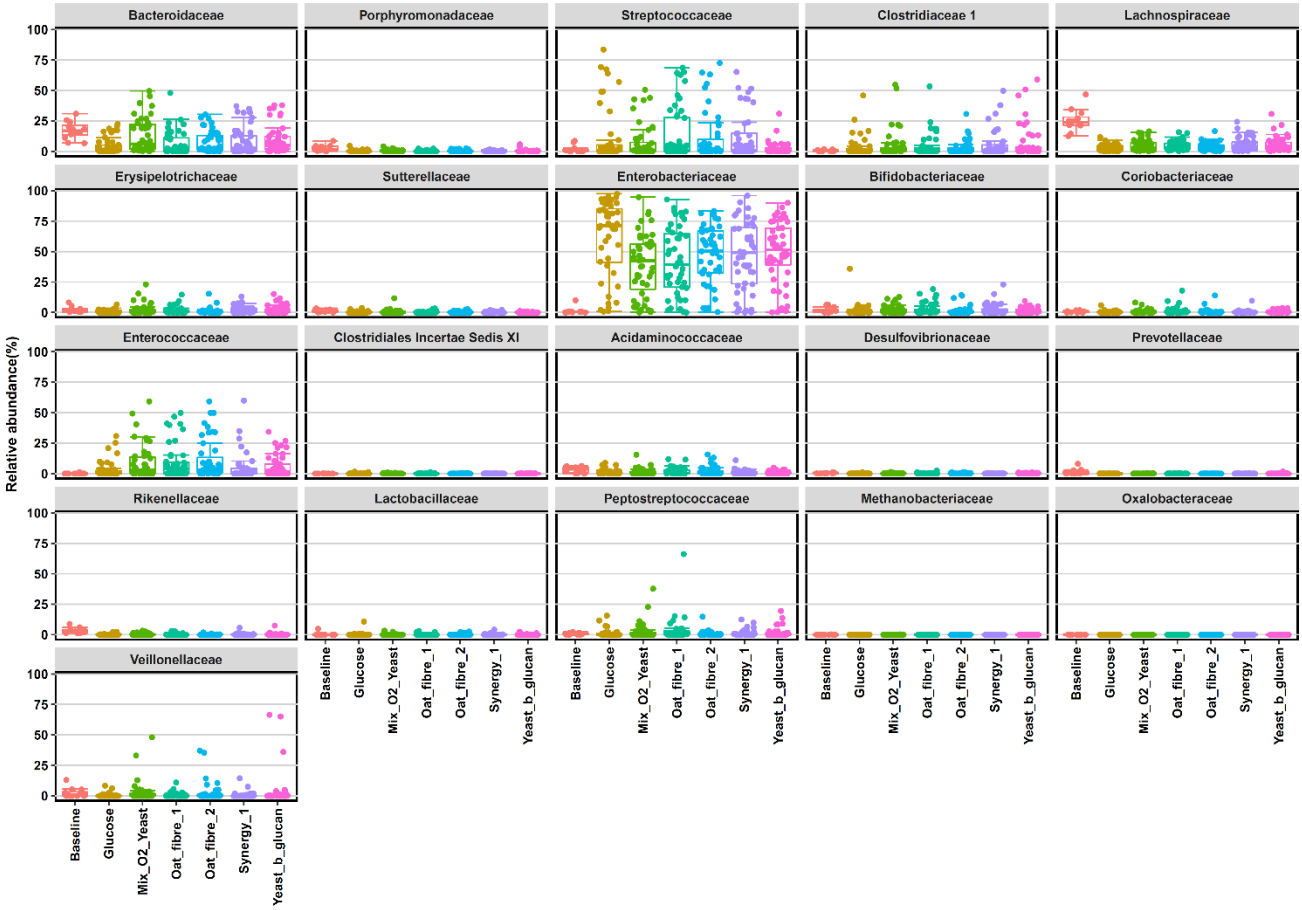



*Figure 4.3 – Box plot of taxa grouped by carbohydrate source*

**Box plot showing distribution of taxa relative abundance at family level grouped by carbon source.**

Percentage relative abundance of the major bacterial families at Baseline/timepoint zero (red), and the 24-hour fermentation groups; Glucose (brown), Yeast β-glucan & Oat fibre 2 mixture (green), Oat fibre 1 (turquoise), Oat fibre 2 (blue), Synergy 1 (purple) and Yeast β-glucan (pink).

Differential abundance testing was performed using DeSeq2 comparing relative taxon abundance between carbohydrate groups vs glucose control. The DeSeq2 test involves a negative binomial generalised linear model to obtain maximum likelihood estimates for an OTU's log-fold change between two conditions. DeSeq2 enables greater power to detect differentially abundant rare OTUs.

Figure 4.4 illustrates the log2 fold change in relative abundances between the carbohydrate test groups and the glucose negative controls.

Oat fibre 1 (n=48) exhibited a 2.03 log higher relative abundance of *Enterococcus* (p=0.003) compared with glucose (n=48). We observed a 1.25 log higher relative abundance of *Ruminococcus* (p=0.003) and a 2.13 log higher relative abundance of *Escherichia/Shigella* ($p=2.5x10^{-7}$) in the glucose group compared with oat fibre 1. Oat fibre 2 (n=44) had higher relative abundances of *Faecalibacterium* (0.91 log; p=0.04)*, Bacteroides* (1.39 log; p=0.019)*, Clostridium XIVa* (1.47 log; p=0.02)*, Bilophila* (1.47 log; p=0.003) and *Enterococcus* (2.275 log; p=0.0001) compared with the glucose group. We observed a higher relative abundance of *Holdemania* (1.96 log; p=0.009) and *Ruminococcus* (1.82 log; $p=1.72x10^{-5}$) in the glucose group (n=44) relative to oat fibre 2. Yeast β-glucan (n=48) had higher relative abundances of *Faecalibacterium* (1.23 log; p=0.018)*, Bilophila* (1.25 log; p=0.019) and *Bacteroides* (1.34 log; p=0.013) compared with glucose. In the same group, there were lower relative abundances of *Peptoniphilus* (2.35 log; p=0.027), *Streptococcus* (1.94 log; p=0.0017), *Parasutterella* (1.8 log; p=0.005) and *Escherichia/Shigella* (1.26 log; p=0.0029) compared with the glucose control. The fibre mixture group, Oat fibre 2 (50%) and Yeast β-glucan (50%) (n=44) exhibited higher relative abundances of *Bacteroides* (1.7 log; p=0.0013)*, Clostridium XIVa* (1.93 log; p=0.0014)*, Enterococcus* (2.29 log; p=0.0005) and *Veillonella* (3.37 log; p=0.00035) when compared with glucose (n=44). Conversely, the fibre mix group had lower relative abundances of *Parasutterella* (2.03 log; p=0.003), *Ruminococcus* (1.17 log; p=0.031) and *Escherichia/Shigella* (1.15 log; p=0.029) when compared with the glucose group. The positive control Synergy 1 (n=44) exhibited no significant increases in relative abundances of taxa when compared with glucose; however, a lower relative abundance of *Escherichia/Shigella* (1.45 log; p=0.008) was observed in the Synergy group compared with glucose.

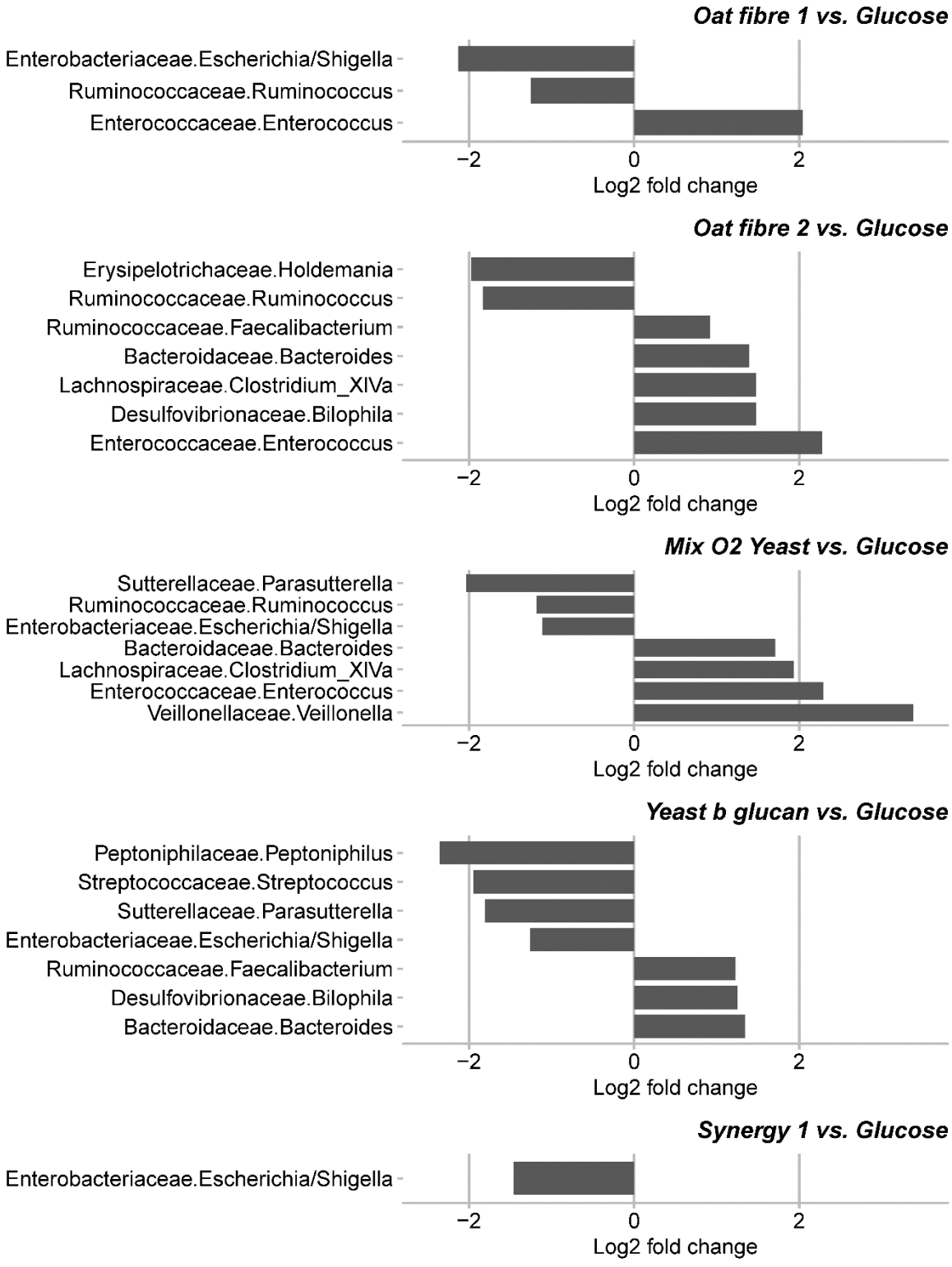



*Figure 4.4 – Deseq2 analysis of change in taxa abundance at genus level for carbohydrate source*

**Comparison of change in relative abundance of taxa at the genus level between carbohydrate groups.** Deseq2 analysis of changes in relative abundances at the genus level represented by log2 fold

changes when comparing test carbohydrates with glucose control. Adjusted p values were calculated by DeSeq2 test with Benjamini-Hochberg correction. Cut-off for adjusted p values were <0.05.

**Differences in microbial composition based on lipid source**

**Alpha diversity**

Alpha diversity metrics for the lipid groups are shown in Figure 4.5. There were no significant differences in phylogenetic diversity and Chao1 measures across the lipid groups. We observed a significantly higher abundance of observed species in the lipid groups (fish oil (p=<0.05); oleic acid (p=<0.01); palmitic acid (p=<0.05)) compared with no lipid. We observed significantly higher Simpson (p=<0.05) and Shannon (p=<0.01) indices in the fish oil group vs no lipid. There was a significantly higher Simpson index (p=<0.01) when comparing oleic acid and no lipid, but no significant difference in the Shannon index. No significant changes in Simpson and Shannon indices were observed when comparing palmitic acid and no lipid.

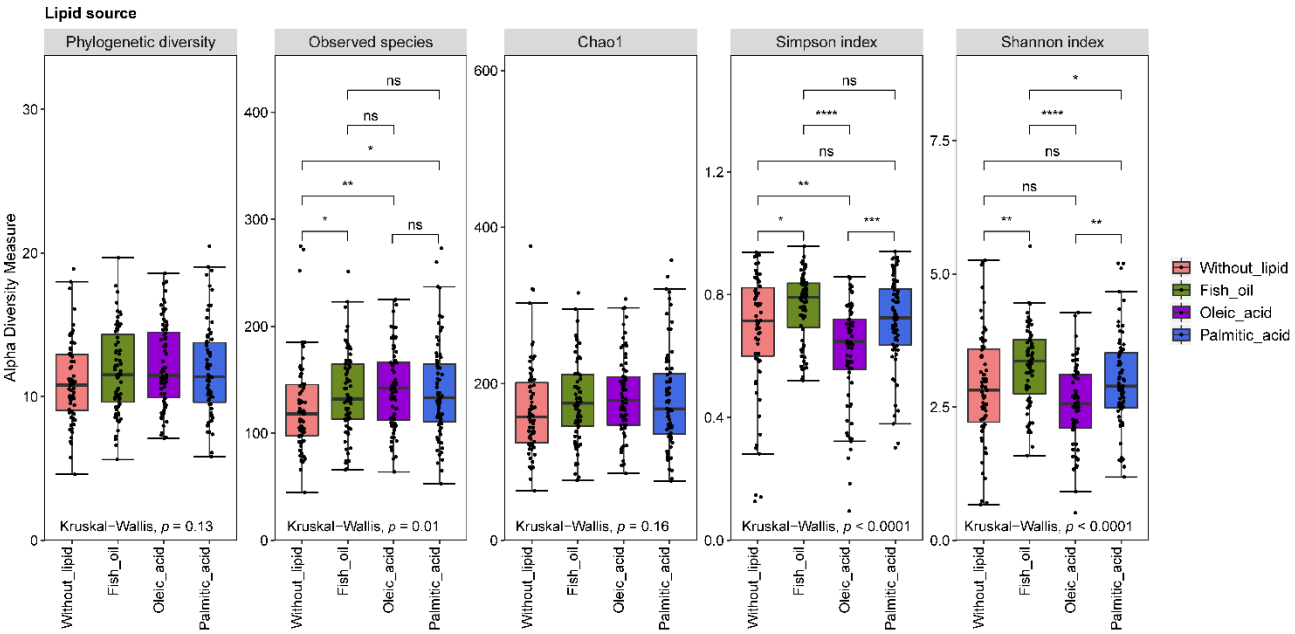



*Figure 4.5 - Alpha diversity metrics for lipid source*

**Alpha diversity metrics based on lipid source for fermentation groups –** Differences in alpha diversity (phylogenetic diversity, observed species, chao1, Simpson and Shannon indexes) of microbial composition based on lipid source (without lipid (n=66); fish oil (n=66); oleic acid (n=66); palmitic acid (n=60). One-way ANOVA comparing linear mixed models for lipid source are indicated above the boxplots. Levels of significance are as follows; * = <0.05, ** = <0.01, *** = <0.005, **** = <0.001.

**Beta diversity**

The unweighted and weighted UniFrac measures of beta diversity for the lipid groups are shown in Figure 4.6. There was a significant difference in both the unweighted UniFrac (p=0.003) and weighted UniFrac (p=0.001) when comparing the no lipid groups with the lipid groups. Thus, lipid source had a significant impact on the relative abundances of the bacterial lineages and low-abundant species compared with no lipid.

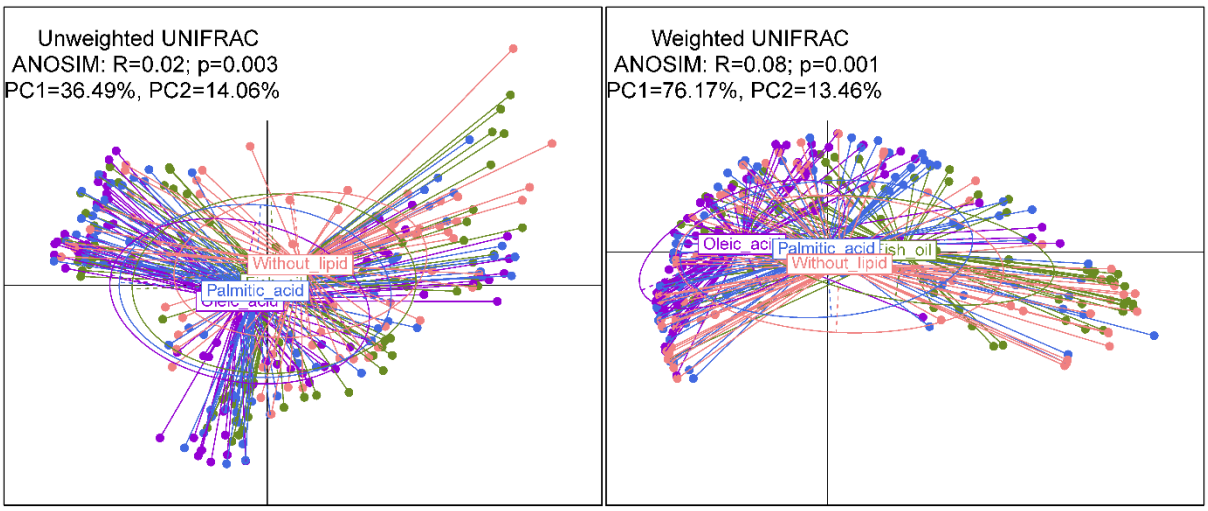



*Figure 4.6 - Beta diversity metrics for lipid source*

**Beta diversity metrics based on carbon source for the fermentation groups** - Principal coordinate analysis (PCoA) based on unweighted and weighted UniFrac distances of the 16S rRNA gene showing microbial diversity based on lipid source (without lipid (n=66); fish oil (n=66); oleic acid (n=66); palmitic acid (n=60) following 24-hour fermentation.

Differential abundance testing was performed using DeSeq2 comparing significant relative taxon abundance at the genus level between lipid sources vs no lipid and is shown in Figure 4.7. Compared with the no lipid control, fish oil fermentation resulted in a higher relative abundance of mainly *Veillonella* (1.64 log; p=0.021), *Terrisporobacter* (1.48 log; p=0.03), and *Clostridium XVIII* (1.24 log; p=0.003). Genera that were concomitantly lowered in relative abundance in the fish oil group with respect to no lipid included Unclassified *Porphyromonadaceae* (3.13 log; p=0.0032), *Escherichia/Shigella* (2.11 log; $p=6.75x10^{-8}$), *Citrobacter* (1.61 log; p=0.0007), *Parabacteroides* (1.58 log; p=0.0007), Unclassified *Enterobacteriaceae* (1.56 log; p=0.003), *Enterococcus* (1.34 log; p=0.015) and *Clostridium sensu stricto* (1.07 log; p=0.014). Fermentation of oleic acid had a more profound effect on the low abundant species, which is reflected in the alpha and beta diversity plots. Oleic acid produced a higher relative abundance of mainly *Roseburia* (2.85 log; $p=1x10^{-25}$), *Oscillibacter* (1.99 log; $p=1.2x10^{-15}$) and *Megamonas* (1.63 log; p=0.019). Comparisons of oleic acid treatment to the control without lipid exhibited a lowering in relative abundances of mainly *Clostridium XI* (7.09 log; $p=9.83x10^{-30}$), *Peptoniphilus* (6.05 log; $p=1.18x10^{-18}$) and *Anaerococcus* (5.65 log; $p=8.83x10^{-24}$). Palmitic acid fermentation resulted in a higher relative abundance of mainly *Fusicatenibacter* (1.74 log; $1.74x10^{-7}$), *Roseburia* (1.38 log; $p=2.06x10^{-5}$) and *Butyriciococcus* (1.3 log; p=0.0007). When comparing palmitic fermentation to the no lipid control, a reduction in relative abundance of mainly unclassified *Veillonellaceae* (3.52 log; $p=1.54x10^{-5}$), *Proteus* (3.49 log; $p=1.93x10^{-5}$) and *Anaerococcus* (3.26 log; $p=3.5x10^{-7}$).

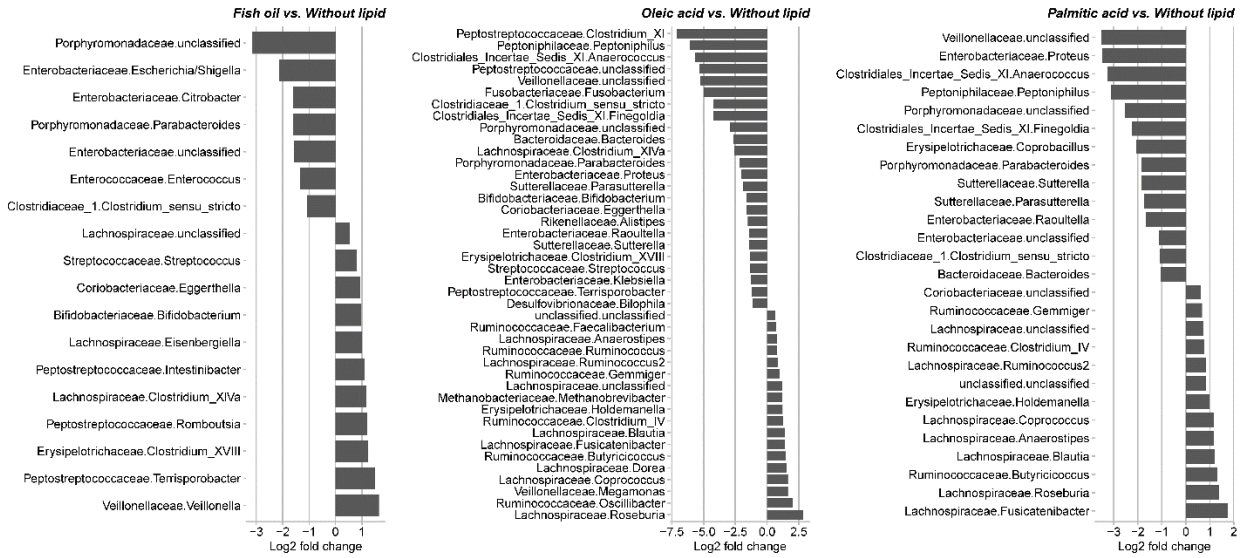



*Figure 4.7 - Deseq2 analysis of change in taxa abundance at genus level for lipid source*

**Comparison of change in relative taxa at the genus level between lipid groups -** Deseq2 analysis of changes in relative abundances at the genus level represented by log2 fold changes when comparing lipid source and no lipid.

**Taxonomic analysis**

Figures 4.8, 4.9 and 4.10 show the relative abundances of taxonomic groups at the phylum, family and genus levels, respectively, for carbohydrate and lipid source. These figures provide a clearer visualisation of the effect of carbohydrate and lipid sources on microbial taxa, with lipid sources exhibiting a greater impact on microbial species compared with carbohydrate sources.

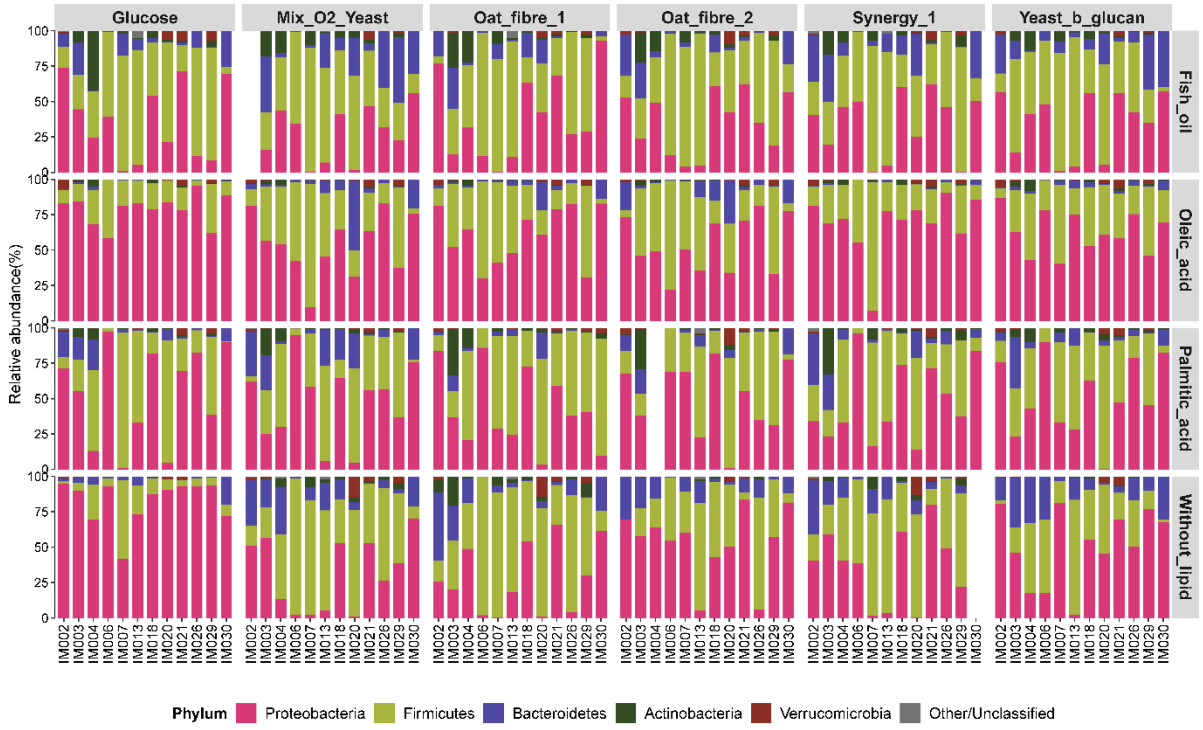



*Figure 4.8 – Relative abundances of taxa at the phylum level based on MicroMatrix cassette layout*

**Relative abundances of taxonomic groups at the phylum level –** Microbial abundances at the phylum level resulting from 24-hour fermentation based on the MicroMatrix cassette layout described in Table 4-1 for each subject tested.

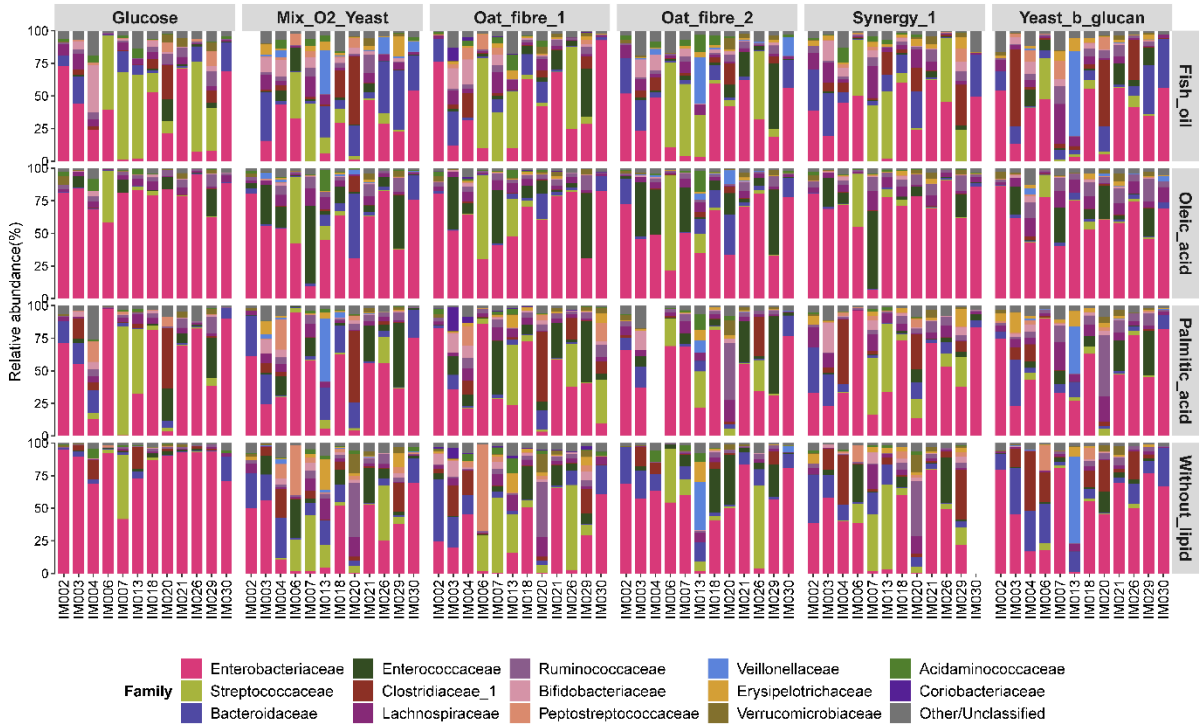



*Figure 4.9 - Relative abundances of taxa at the family level based on MicroMatrix cassette layout*

**Relative abundances of taxonomic groups at the family level –** Microbial abundances at the family level resulting from 24-hour fermentation based on the MicroMatrix cassette layout described in Table 4-1 for each subject tested.

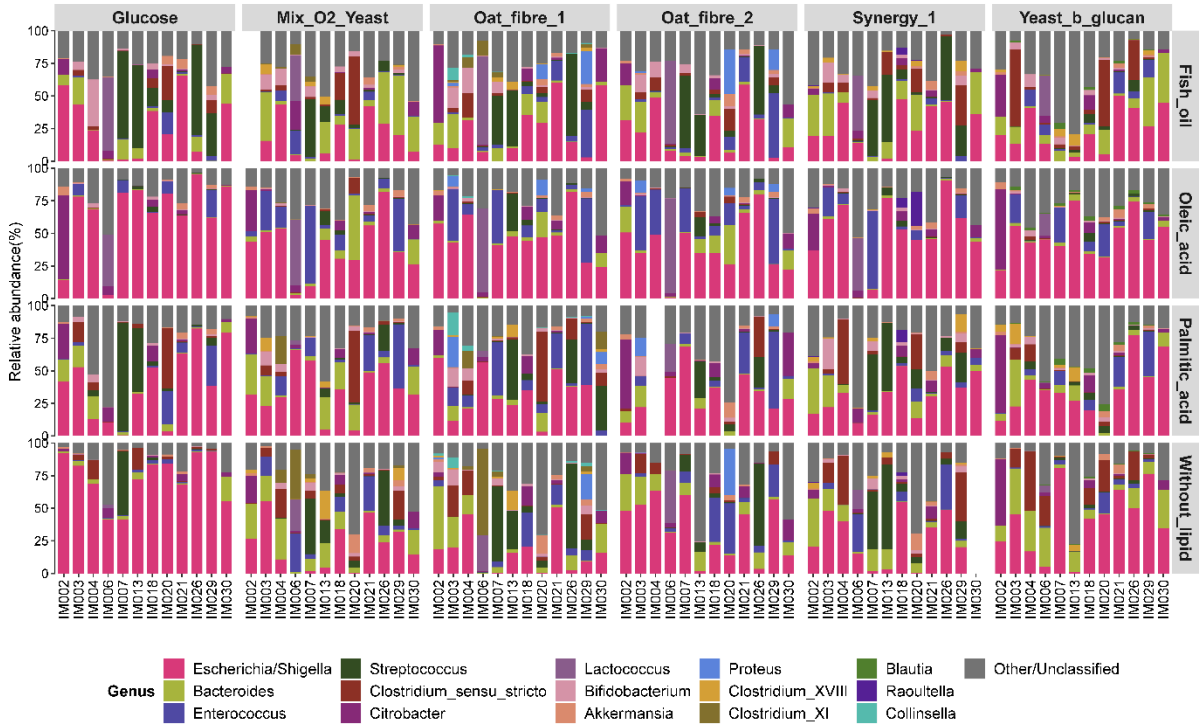



*Figure 4.10 - Relative abundances of taxa at the genus level based on MicroMatrix cassette layout*

**Relative abundance of taxonomic groups at the genus level –** Microbial abundances at the genus level resulting from 24-hour fermentation based on the MicroMatrix cassette layout described in Table 4-1 for each subject tested.

**Fatty acid analysis**

Short and branched chain fatty acid (SCFA, BCFA) analysis was performed on the fermentates of the carbohydrate groups containing no lipid and palmitic acid only. This approach was owing to the fact that the GC-FID internal standard, 2-ethylbutyric acid, was reacting with the oleic acid and fish oil in the fermentation supernatants, resulting in inaccurate standard curves. Figure 4.11 shows the SCFA and BCFA concentrations of the mean of all the subjects for the no lipid carbohydrate groups (n=12). There were no significant differences in fatty acid concentrations between any of the carbohydrate groups, likely owing to the large variation between subjects. Figure 4.11 shows the comparisons of fatty acid concentrations between the combined carbohydrate groups for no lipid and palmitic acid.

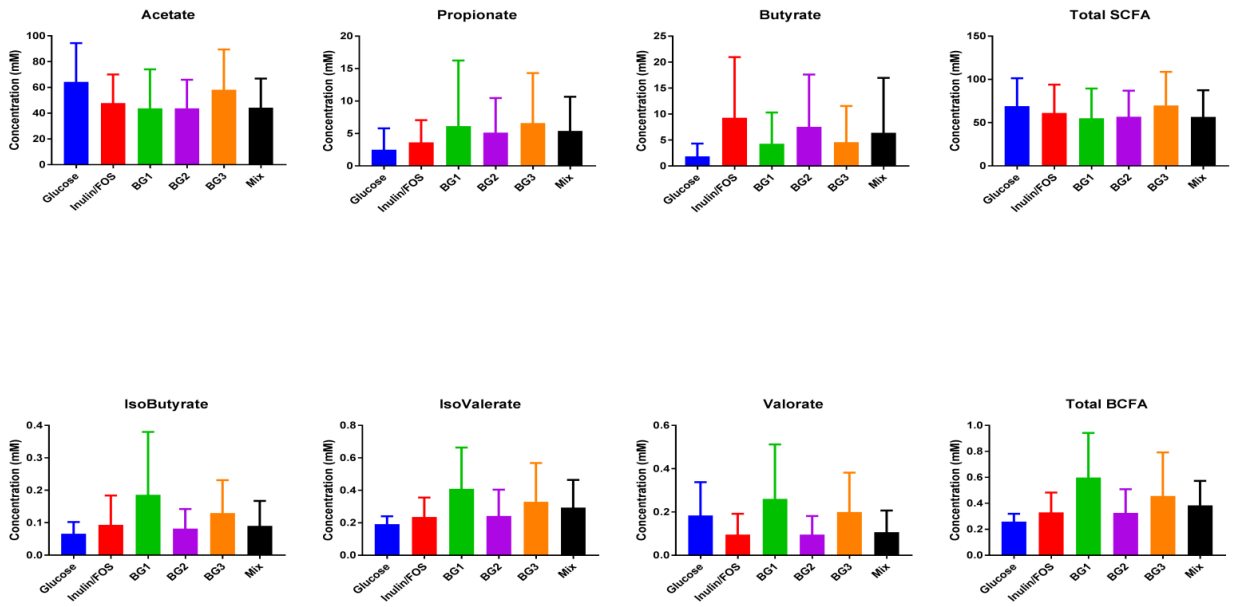



*Figure 4.11 – Mean fatty acid analysis based on carbon source*

**SCFA & BCFA concentrations for the test carbohydrates in the no lipid group.** GC-FID data for SCFA and BCFA. Carbohydrates are shown along the x axis; Glucose (n=12; blue); Synergy 1/inulin/FOS (n=12; red); Oat fibre 1/BG1 (n=12; green); Oat fibre 2/ BG2 (n=12; purple); Yeast β-glucan/BG3 (n=12; orange); Fibre mix (n=12; black). Data is shown as mean ± SD.

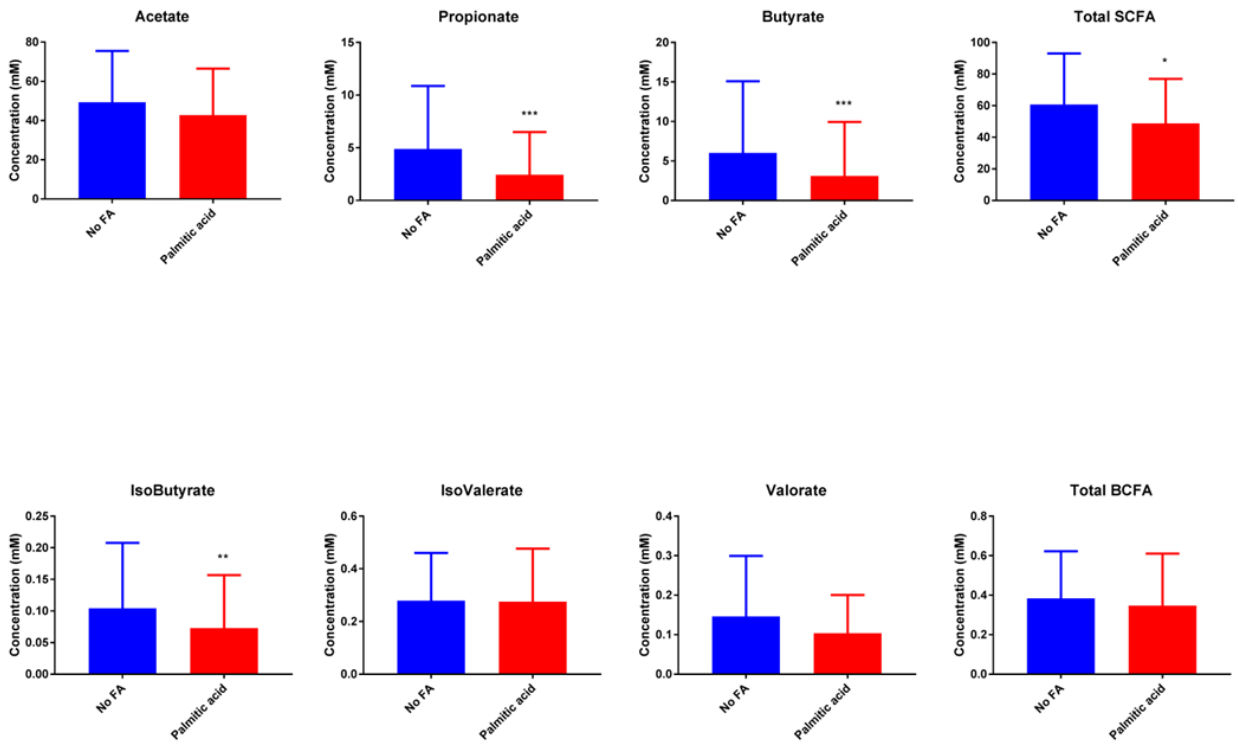



*Figure 4.12 – Mean fatty acid analysis comparing no lipid and palmitic acid*

**SCFA & BCFA concentrations following 24-hour fermentation with no lipid and palmitic acid.** GC-FID results for SCFA and BCFA concentrations comparing no lipid and palmitic acid. The no lipid group (no FA) was the combined mean of all the carbohydrate groups (n=72) and no significant effect was observed for any of the SCFA or BCFA concentrations. The combined mean of the carbohydrate groups containing palmitic acid (n=72) revealed significantly lower propionate (p=<0.001), butyrate (p=<0.001) and total SCFA (p=<0.05) concentrations. A significantly lower concentration of the BCFA Isobutyrate (p=<0.01) was observed in the palmitic acid group, compared with the combined mean of the no lipid group. The data is shown as mean ± SD.

## Discussion

In this study, an *ex vivo* faecal fermentation experiment was performed using faecal samples from patients with varying levels of metabolic syndrome. However, the bioinformatics analysis did not

differentiate between the various levels of metabolic syndrome. A factorial design approach was used to study the main effects of potential prebiotic substrates and lipid sources on microbial community diversity, richness and composition. The data showed that lipids, rather than the prebiotic substrates, had a more profound effect on overall community composition, promoting the growth of a wide range of specific taxa, reflected in the unweighted and weighted UniFrac beta diversity plots. The form of lipid (saturated, unsaturated FFAs and polyunsaturated from fish oil) determined differences in specific taxa compared with the no lipid control. The fibre groups had a greater effect on alpha diversity and a statistically significant effect only on weighted UniFrac beta diversity, suggesting that fibre source has a larger impact than lipid on bacterial lineages and overall composition rather than the presence or absence of a microbial community. Each fibre promoted the growth of similar taxa, with only minor differences in specific taxa between fibre types, which contrasts the more pronounced effect observed between lipid types.

Glucose was used as control for comparisons with the fibre test substrates, based on the premise that β-glucans are complex carbohydrates composed of monomeric glucose molecules. Glucose promoted the growth of the *Enterobacteriaceae* family, particularly members of the *Escherichia*/*Shigella* genus, in some samples constituting >90% relative abundance. Glucose also promoted the growth of other genera belonging to *Ruminococcus, Holdemania, Parasutterella, Peptoniphilus* and *Streptococcus.* Members of *Enterobacteriaceae* are a Gram-negative family that contain many pathogenic genera including *Citrobacter*, *Escherichia, Klebsiella, Proteus, Shigella, Salmonella, Serratia* and *Yersinia* which possess LPS in their cell membrane. The *in vitro* system used in this study seemed to favour the growth of *Enterobacteriaceae,* which were at low relative abundance in the faecal inocula, but successfully thrived across all carbohydrate groups, in particular glucose. This growth could be because of the aerotolerant nature of this family, the choice of media suiting their growth or the resilience of this family to survive the faecal inoculum processing step compared with less hardy species. Other studies have indicated that obese and type 2 diabetic patients possess a higher abundance of *Enterobacteriaceae* (Ahmad et al., 2019; Lambeth et al., 2015;

Qin et al., 2012), with a more recent study particularly implicating *Escherichia/Shigella* (Thingholm et al., 2019) and higher serum LPS concentrations (Amar et al., 2011), compared with healthy subjects. *Ruminococcus* has been reported to be positively correlated with pre-diabetes and type 2 diabetes (Allin et al., 2018; Zhang et al., 2013); *Holdemania* has been correlated with blood cholesterol and impaired glucose and lipid metabolism (Lippert et al., 2017); *Peptoniphilus* and *Streptococcus* are associated with skin colonisation, with the former implicated with impaired healing of diabetic foot ulcers (Min et al., 2020). *Parasutterella* is a member of the *Betaproteobacteria*, a class of bacteria that has been enriched in diabetic patients compared with healthy controls (Larsen et al., 2010). The association of the genera *Parasutterella* with diabetes has not yet been established; one study has implicated a negative association between high-fat diet and associated metabolic phenotypes with the abundance of *Parasutterella* (Kreutzer et al., 2017). Taken together, the β-glucans appeared to lower species involved in the pathophysiology of type 2 diabetes relative to the glucose group.

Oat β-glucan 1 and 2 exhibited similarities but also some differences in the growth of bacteria they promoted. The source of these β-Glucans was the same, spent brewer's grain; however, differences in the extraction methods between β-Glucan 1 and 2 likely led to variations in the chain linkages, which in turn, enabled variations in the bacteria that could be supported. Both oat β-glucan 1 and 2 promoted the growth of *Enterococcus*, which generally constitute ~1% of the microbiome (Dubin & Pamer, 2014). Little is known of the impact of *Enterococcus* on the pathophysiology of type 2 diabetes; however, growth of *Enterococcus* is promoted by antidiabetic drugs such as alpha-glucosidase inhibitors (Su et al., 2015) and sodium glucose cotransporter 2 inhibitors (Lee et al., 2018). Whether or not these bacteria play a role in type 2 diabetes or in maintaining healthy gut homeostasis remains to be seen. While oat β-glucan 1 only promoted enterococcal growth, oat β-glucan 2 promoted a wider range of taxa, including *Faecalibacterium, Bacteroides, Clostridium XIVa* and *Bilophila. Faecalibacterium prausnitzii* is considered one of the most important bacterial indicators of a healthy gut and has been negatively associated with diabetes in four out of five human case control studies (Gurung et al., 2020). Moreover, oral treatment with *F. prausnitzii* in diet-induced metabolic

disease in mice resulted in an improvement in liver fat inflammation and hepatic function (Munukka et al., 2017). Members of the *Desulfovibrionaceae*; *Bilophila,* are sulphite-reducing pathobionts, found to be increased in response to a high saturated fat diet which promotes inflammation in mice (Devkota et al., 2012), with a detrimental impact on glycaemic control and hepatic function (Natividad et al., 2018). Yeast β-glucan promoted the growth of the same aforementioned genera as oat β-Glucan 2, besides *Clostridium XIVa.* This cluster of *Clostridium* constitutes a normal butyrate-producing constituent of a healthy gut microbiome found to be decreased in morbidities such as IBD and type 1 diabetes (de Goffau et al., 2014). The role that these bacteria play in type 2 diabetes is unknown, but their 'anti-inflammatory' nature and butyrate producing capabilities may contribute to normal gut homeostasis and glycaemic control, with one study pointing to a strong negative association with plasma glucose concentrations in patients with prediabetes (Allin et al., 2018). No significantly higher taxa were found in the positive control, synergy 1, which is a commercially produced inulin-fructoligosaccharide fibre. This finding conflicts with other studies which have identified a clear bifidogenic response (Fehlbaum et al., 2018) to inulin--fructoligosaccharide. Bifidobacteria are consistently negatively associated with type 2 diabetes in patients. To our knowledge, this is the first *in vitro* study using faecal samples from patients with metabolic syndrome, and, therefore, perhaps the low-abundance of bifidobacteria present in these patients may be responsible for the lack of comparable bifidogenic responses observed in healthy patients.

We observed a much more pronounced effect when examining comparisons between lipid groups. This effect would be expected as the negative control used in this case was no lipid, and thus the lipid-containing groups provided an expanded food source for the bacteria. Regardless, our experiment can still provide an indication of lipid-degrading taxa in patients with metabolic syndrome. Fish oil promoted the growth of three members of the *Lachnospiraceae* family, two *Peptostreptococcaceae* family members, one *Coriobacteriaceae* family member, one from the *Streptococcus* genus, one from the *Bifidobacterium* genus, one from *Clostridium XVIII* genus and one from the *Veillonella* genus. Fish oil concomitantly reduced the relative abundances of a range of LPS-

producing pathogenic Gram-negative bacteria. *Peptostreptococcaceae* and *Veillonella* are generally considered members of the oral microbiome, but also components of the gut microbiome. A *Peptostreptococcus anaerobius* species was found to be significantly enriched in type 2 diabetics following weight loss (Remely et al., 2016) and *Veillonella spp.* were found to possess performance-enhancing properties in marathon runners, owing to *Veillonella spp.* preference to utilise lactate as its sole carbon source (Scheiman et al., 2019). It may be possible that these bacteria play crucial roles in glycaemic control, either through direct or indirect effects on plasma glucose. Relative abundance of a *Coriobacteriaceae* family (*Eggerthella* genus) was higher in the fish oil group, and bacteria from this family have been found to be positively associated with type 2 diabetes remission following Roux-en-Y gastric bypass (Liu et al., 2018), highlighting their potential as mediators of glucose metabolism. Finally, *Lachnospiraceae* and *Erysipelotrichaceae* (*Clostridium XVIII* genus) have been positively associated with metabolic syndrome in some studies (Lippert et al., 2017) but not others (Chavez-Carbajal et al., 2019), leaving the role of these microbes in type 2 diabetes open to speculation. However, the high capacity of these microbes to produce SCFAs suggests they may be either beneficial or harmful contributors to SCFA homeostasis in the gut. Bacterial species involved in the utilisation of fish oil are largely uncharacterised, however, one study in mice identified higher abundances of *Akkermansia, Lactobacillus* and *Bifidobacteria* in mice on a fish oil diet (Caesar et al., 2013). Overall, the data show that fish oil promotes the growth of taxa involved in type 2 diabetes remission, while lowering the abundance of pro-inflammatory LPS-producing bacteria.

Oleic acid had a more equivocal effect on taxa, promoting/inhibiting both potentially beneficial/harmful species. This unsaturated fatty acid encouraged the growth of a number of *Ruminococcoceae* and *Lachnospiraceae* families, with one from each of the *Erysipelotrichaceae*, *Methanobacteriaceae,* and *Veillonellaceae* families. Oleic acid promoted the growth of *Coprococcus, Megamonas, Oscillobacter* and *Roseburia* which are not reported to have causal association with type 2 diabetes; and *Anaerostipes, Blautia, Dorea* and *Faecalibacterium* which are only modestly associated with a lower risk of type 2 diabetes (Yang et al., 2018). Bacterial processing of oleic acid is primarily

performed by *Lactobacillus, Butyrivibrio* and *Megasphera* (Schoeler & Caesar 2019). Interestingly, we observed higher abundances of *Methanobrevibacter*, a bacterium which has been negatively associated with BMI (Million et al., 2013) and *Fusicatenibacter* which has been correlated with lower plasma cholesterol concentrations in mice (Prieto et al., 2018). The expansion of a range of *Lachnospiraceae* mirrors a recent human intervention study, in which oleic acid supplements following a western run-in diet, promoted the growth of similar *Lachnospiraceae* genera and these were inversely associated with cardiovascular risk factors (Tindall et al., 2020). Oleic acid inhibited a variety of families, including *Enterobacteriaceae, Peptostreptococcaceae, Streptococcaceae, Porphyromonadaceae, Sutterellaceae, Desulfovibrionaceae* and *Fusobacteriaceae* which are generally negatively associated with type 2 diabetes. However, oleic acid also inhibited the growth of some 'beneficial' bacteria including *Bifidobacterium* and *Bacteroides.* While oleic acid clearly promoted the growth of SCFA producers, we do not have the SCFA data for oleic acid for comparison and to confirm that the increase in SCFA producers results in greater SCFA than the no lipid control. We could not accurately determine the fatty acid concentrations for the oleic acid and fish oil treated groups owing to an unforeseen chemical reaction with the internal standard, 2-ethylbutyric acid, which hindered quantification.

The fermentation of the saturated FA palmitic acid produced a similar profile of bacteria to those observed for oleic acid, which promoted the growth of a variety of *Lachnospiraceae* and *Ruminococcaceae.* Similarly to oleic acid, palmitic acid inhibited a mix of 'benefical' and 'harmful' bacteria including *Enterobacteriaceae, Sutterellaceae, Veillonellaceae, Parabacteroides* and *Bacteroides.* The expansion of *Lachnospiraceae* and *Ruminococcaceae* in the palmitic acid group would suggest an increased production of SCFA relative to no lipid; however, we observed significantly less butyrate, propionate and total SCFA production following palmitic acid fermentation. We observed a larger fold change reduction in potential SCFA producers including unclassified *Veillonellaceae* and *Porphyromonadaceae;* or intermediate metabolite producers such as the succinate producer *Parabacteroides* (K. Wang et al., 2019). The reduction in anti-inflammatory SCFAs following

fermentation of the pro-inflammatory palmitic acid warrants further investigation and may be contributing to proinflammatory effect of palmitic acid.

Surprisingly, we observed no significant difference in SCFA and BCFA concentrations between the carbohydrate groups. However, it is known that prebiotic responders and non-responders exhibit differential alterations of the gut microbiota (Y. Liu et al., 2020), and thus differential production of SCFAs. These alterations may explain our observations in the SCFA data; non-significant concentrations resulting from large variations between subjects.

A limitation of this study is the choice of glucose as negative control, with which all of the fibre comparisons were made. Microbial exposure to glucose varies along the gastrointestinal tract, with some estimates that >95% of glucose is reabsorbed in the small intestine (Di Rienzi & Britton, 2019). The high administration of 1% w/v of glucose used in the current study is not representable of the physiological concentrations present in the large intestine and this may have caused an expanse of glucose 'opportunists', skewing our results and producing an overestimation of the fibre-degrading taxa. Moreover, one would expect glucose to collapse microbial diversity, reflected in strong overgrowths of *Escherichia/Shigella* in the glucose treated groups, and thus diversity comparisons for the fibre groups will in many cases be significant, but may not be biologically important i.e., would β-Glucan supplementation produce higher diversity *in vivo*? Similarly, in this *in vitro* study the lipids used may not be representative of those found in the physiological environment of the intestine. Owing to the fact we could not pre-digest the lipids in the same way (the lipids are too small for the final dialysis step in the extraction procedure) we could the test β-Glucans, combined with the use of a 'blank' no lipid negative control (vs glucose in the β-Glucan group), it is difficult to make direct comparisons between the differences observed in the lipid and β-Glucan groups. An emulsification step of the lipids may produce a more physiological representation of those that reach the colon. In relation to our DeSeq2 data, it may be more appropriate to consider the p value over log-fold change, which would give a better indication of fibre-degrading taxa across the general population.

This study also has some strengths. Given the inherent variability of inter-patient microbiomes, the application of 12 individual faecal samples to the fermentation experiment provides us with a larger effect size, and thus the magnitude of differences resulting from fibre supplementation would be greater than those obtained by pooling faecal samples. The advantage of employing a factorial design experimental approach is that it allows us to assess both fibre and lipid sources in one experiment, saving time and cost.

In conclusion, this study provides evidence for the potential of β-glucans and fish oil to promote gut microbiota diversity and species associated with providing health benefits in patients with metabolic syndrome. The *in vitro* findings warrant further investigations and should inform dietary intervention studies to explore the possibility of administering novel prebiotic and lipids to improve the health status of T2D patients. These findings can complement mechanistic studies identifying potential probiotic species involved in glycaemic control and gut homeostasis.

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# Chapter 5 - A pilot study exploring the relationship between fish consumption and the gut virome in human volunteers

Notes:

Culturing of bacteriophage standard, viral nucleic acid extractions and library preparation was performed by Ronan Strain (author).

Sample collection was performed by Dr. Marie Conway

Bioinformatics analysis was performed by Dr. Tom Sutton

## Abstract

Oily fish intake is a prominent component of a 'Mediterranean' diet and has been associated with a reduction in an array of diseases from rheumatoid arthritis and IBD. The exact mechanisms underpinning these health benefits remain undiscovered; however, more recently, focus has shifted towards the gut microbiota as a possible mediator in influencing human well-being. An appreciation of a largely unexplored component of the microbiota, the virome, has been implicated in certain gastrointestinal disorders. How specific dietary ingredients influence the gut virome may shed light on understanding this component of the microbiota and its affiliation with human health. In this pilot study, we obtained faecal samples from a randomised controlled trial (originally designed to examine the effects of fish type (sardine and tuna) and quantity (0,1 and 2 portions) on plasma lipid profile to assess how oily fish intake correlates with viral load and diversity, in order to inform future dietary intervention studies on the gut virome. We found that oily fish intake can influence viral diversity; however, our analysis is hampered by inter-individual variation and the lack of viral matches with the global database.

## Introduction

Large metagenomic sequencing projects of microbes and their associated gene content has identified core microbiota members that may provide beneficial functions and form part of our understanding of what constitutes a healthy gut microbiome i.e., there is a distinct clustering of bacterial species in healthy subjects and a dissimilar bacterial clustering in subjects with gastrointestinal disorders. Diet sculpts the gut microbiome by directly modulating the abundance of bacterial species and their associated individual or collective functions. However, little is known regarding the impact of diet on the gut virome. The predominant members of the gut virome are bacteriophage (phage), bacteria-specific viruses which exist at approximately $10^{10}$ Virus-Like Particles (VLPs) per gram of faeces

(Shkoporov & Hill, 2019). Phage proceed in two life cycles, virulent and temperate. The virulent life cycle involves phage infection of their bacterial hosts, replicating therein and lysing the host, releasing phage progeny to infect new hosts. Temperate phage can inject their genome into cells and may enter a symbiosis with their bacterial hosts, forming a unit called a lysogen. The lytic-lysogeny outcome, in all likelihood, evolved from a trade-off between the opportunistic costs of lysogeny (intracellular reproduction) and lysis (extracellular survival) (Touchon et al., 2016). Owing to this paradigm, and the observation that lysogeny is prevalent in the microbiota (M. S. Kim & Bae, 2018), the interests of phage and host are somewhat aligned, because phage rely on the bacterial host for replication (Goldhill & Turner, 2014). Thus, we can postulate that the impact of diet on the bacteriome should have similar implications for the virome. Appreciating how specific dietary ingredients influence bacteriophage populations can shed light on understanding how this largely uncharacterised community contribute to microbiome structure and human health.

Interpretations of gut virome studies are hampered by the lack of full genome sequences available; the National Centre of Biotechnology Information (NCBI) contains only 10,462 viral genome sequences (as of February 2021), a minute fraction of the global diversity (Liang & Bushman, 2021). Indeed, this lack of data is reflected in viral metagenomics, in which the majority of newly identified phage sequences do not have counterparts in the online databases, often referred to as "viral dark matter" and can comprise 65%-95% of the total viral genomes (Callanan et al., 2021). Moreover, prophage induction is driven by environmental stressors, for the strictly anaerobic commensal bacteria containing lysogens in the excreted faeces, freezing of faecal samples or the nucleic acid extraction procedure itself may encourage prophage induction. The end result, therefore, is that sequencing of faecal VLPs may not reflect the phage population/community *in vivo*. The choice of library preparation kit also has a profound effect on end-point sequencing data. Traditionally, multiple displacement amplification (MDA) was employed to improve nucleic acid yield. However, this method is known to preferentially amplify ssDNA phage (K. H. Kim & Bae, 2011) and may not represent true virome composition. Newer technologies, such as the adaptase technology (Swift Biosciences) which

attaches special adaptors to each DNA fragment and thereby minimising potential amplification biases, are available to overcome this problem. Overall, the caveats in virome processing will undoubtedly lead to disparities between research groups. Accurate interpretations of virome studies will require more controlled protocols going forward.

A high degree of inter-individual variation is observed in the human virome, resembling what is seen in bacterial and fungal microbiome studies. Recent evidence points to associations between virome communities and certain disease pathologies, such as Inflammatory Bowel Disease (IBD)(Clooney et al., 2019), ulcerative colitis (Pérez-Brocal et al., 2015), rheumatoid arthritis (Mangalea et al., 2021) and type 2 diabetes (Y. Ma et al., 2018). The most notable change is an increase in *Caudovirales*, an order of phage associated with Crohn's disease and ulcerative colitis (Norman et al., 2015). While these cannot infect eukaryotic cells, there is evidence that some phage may stimulate the immune system (Tetz et al., 2017). However, a causal relationship between virome and disease state has yet to be defined or to what extent inter-individual variations in phenotypes are attributable to the virome (Liang & Bushman, 2021). Interestingly, neonates are first colonised by prophage from "pioneer" bacteria and subsequently by potentially detrimental eukaryotic viruses; the latter can be modulated by breastfeeding (Liang et al., 2020). This phenomenon underscores the importance of diet in early life and provides direct evidence that diet can maintain a protective bacteriome and virome. Minot et al. (2001) were one of the first groups to investigate the impact of diet on the human gut virome. While the work noted that the virome in individuals on the same diet (high fat/low fibre & low fat/high fibre) converged, this study had limitations in sample size and out-dated methods. Furthermore, virome studies of monozygotic twins show that the virome is analogous between twins during infancy and it becomes more dissimilar as they age, suggesting that environmental factors such as diet, play a driving force in its configuration (Moreno-Gallego et al., 2019). Interestingly, in studies involving the fructose-metabolising gut symbiont, *Lactobacillus reuteri*, which often contain active prophages, a high-fructose diet has been demonstrated to induce prophage (Oh, Alexander, et al., 2019), which can provide a competitive advantage by killing off competitor strains in its intestinal

niche (Oh, Lin, et al., 2019). Uncovering diet-phage interactions, therefore, may provide a means to comprehending the "viral dark matter" enigma.

Diet is considered to be an environmental factor that is associated with the onset and course of IBD (Ananthakrishnan et al., 2018). Given the associations between the virome and this gastrointestinal disorder, with evidence that certain anti-inflammatory dietary components can reduce symptoms and flares (Campmans-Kuijpers & Dijkstra, 2021), little is known regarding the direct influence of these dietary components on the gut virome. Oily fish is rich in the n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have long been known to have anti-inflammatory properties in several chronic inflammatory disorders such as rheumatoid arthritis and IBD (Yates et al., 2014). We hypothesised that a diet rich in these components may promote a healthy gut virome, either by stimulating the growth of beneficial bacteria and their associated lysogens or repressing the growth of bacteria and their immune-stimulating lysogens. In this pilot study, we used faecal samples from volunteers in a randomised controlled trial (RCT) which was designed to investigate how dietary fish consumption of sardine and tuna can influence the plasma fatty acid profile compared with those not consuming fish. We performed viral metagenomics to assess changes in the virome in response to the fish diet intervention in human volunteers, with the aims of investigating important parameters in the design and implementation of dietary interventions for future human virome studies. The data show that fish intake produces a viral signal across the experimental cohorts; however, our limited dataset in this pilot study cannot determine if this signal is attributable to inter-individual variation or dietary intervention *per se*.

## Methods

### Study design and faecal collection

Samples were sourced as part of the "iFish Study" (registered at www.clinicaltrials.gov (NCT03765580)), an eight-week randomised controlled trial investigating the impact of fish consumption (sardine and tuna) in women of childbearing age. Healthy females of childbearing age (18-45 years old) were selected and suitable participants were those who were low consumers of fish (<2 portions of fish per week), willing to consume 1, 2 or no portions of fish per week, non-consumers of supplements and had no seafood allergies. All subjects provided written consent which was approved by Ulster University Research Ethics Committee (REC/16/0077). Research was performed in agreement with the 1964 Declaration of Helsinki and its amendments.

A total of 45 women were included in this study, with faecal samples which were collected at baseline and at the end of the eight-week intervention period and were immediately frozen at -80°C. Participants were randomised into an intervention group of either no fish, one portion or two portions of fish per week; given in the form of a lunch dish. Each lunch dish contained a 140g portion of fish according to the intervention group. The no-fish group participants were given the option of choosing between a salad, baked potato or sandwich. Similar energy and macronutrient composition were calculated to ensure each subject was given similar amounts across the intervention groups, with leftovers weighed and recorded to measure compliance. Four subjects were subsequently excluded as it was not possible to obtain enough VLPs for the sequencing protocol, leaving a convenience sample of 41 subjects for virome analysis.

**Propagation and Quantification of Bacteriophage Q33**

To assess the total faecal phage loads obtained by viral nucleic acid extractions, an exogenous *Lactococcus lactis* bacteriophage stock was propagated and quantified prior to spiking in the nucleic acid extraction. Lactococcal phage Q33 (Mahony et al., 2013) was propagated on *L. lactis* SMQ-86 at 30°C in M17 broth (Oxoid) supplemented with 0.5% glucose (GM17). In brief, 100µl of a fresh culture of *L. lactis* SMQ-86 was subcultured into a fresh 10ml GM17 broth and grown to an OD600 of 0.1-0.2.

To this, 100µl of 1M $CaCl_2$ solution and 100ul of lactococcal phage Q33 was added, incubated at 30°C, and the phage was allowed to propagate for ~5 hours, without agitation. The phage lysate was then centrifuged at 4000xg at 4°C for 15 min. The supernatant was then passed through a 0.2um filter to remove bacterial debris, and the resulting phage lysate was stored at 4°C.

The concentration of phage in the lysate was determined by plaque assay using the double agar method. First, Q33 phage stock was serial diluted in SM buffer to $10^{-8}$ dilutions. One hundred microliters of diluted phage were added to 100µl of a fresh overnight culture of *L. lactis* SMQ-86, and the phage was allowed to bind to the host for 5 min at room temperature. A GM17 solution containing 0.7% agar and 100mM $CaCl_2$ was heated to 50°C, and then 4 ml of this was added to the phage-host mixture and quickly poured onto a 1.5% agar GM17 plate. Phage dilutions of $10^{-5}$, $10^{-6}$, and $10^{-7}$ were prepared in this way, in triplicate. Plates were incubated aerobically overnight at 30°C. Plaques were counted the following day to determine phage concentration, and the concentration expressed as plaque forming units (pfu)/ml.

**Gut virome nucleic acid extractions**

Extraction of viral nucleic acids from faecal samples was based on an extraction method published by Reyes et al (2013). Five hundred milligrams of faecal sample were added to 10ml SM buffer (0.05M Tris-HCl (pH 7.4); 0.1M NaCl; 0.01M $MgSO_4$) in a 15ml falcon tube and homogenised by vigorous vortexing for 5 min. Homogenates were then centrifuged in a swing bucket centrifuge (Sorvall) for 10 min at 4000xg pre-chilled to 4°C, and the supernatant was added to a new 15ml falcon tube. An additional centrifugation step (as above) was performed to further remove bacterial cells and large particles and the supernatant was retained. The supernatant was subsequently filtered twice through 0.45µM pore polyethersulfone (PES) membrane filters and SM buffer was added to the filtrates to reach a final volume of 10ml per sample. Bacteriophage standard Q33 was added at final concentration of 2 x $10^6$ pfu/ml. 1.0ml of a 5M NaCl solution and 1.1g of Poly(ethylene glycol) – 8000

(PEG-8000) (Sigma) were added to the filtrates to give a final concentration of ~0.5M and 10% w/v, respectively. The PEG-8000 was dissolved by gentle repeated inversion until the solution became clear. Samples were then incubated overnight on ice at 4°C to enable sufficient precipitation of viral-like particles (VLPs).

Following overnight precipitation, samples were centrifuged in a pre-chilled swing bucket centrifuge (Sorvall) at 4000xg for 20 min at 4°C, to collect the precipitated VLPs and the supernatant was discarded. Tubes were left inverted on tissue paper for 5 min to dry the pellet. Pellets were then resuspended in 400µl SM buffer and transferred to a 1.5ml Eppendorf. To this, 400µl chloroform was added, vortexed for 30s and centrifuges at 2500xg at room temp for 5 min using a benchtop centrifuge. Following centrifugation, the aqueous phase was carefully aspirated into a 1.5 ml Eppendorf. Forty microliters of a 10x DNase buffer (1x final); 4ul (20U final) Turbo-DNase (Ambion) and 2ul (10U final) RNase I (Fisher scientific) was added and incubated at 37°C for 1 hour to ensure digestion of nucleic acid contaminants. Enzymes were subsequently inactivated by incubation at 70°C for 10 min. VLPs were dissociated by addition of 20µl sterile 10% (w/v) SDS solution and 2µl proteinase k (40µg final) and incubated at 56°C for 20min. Finally, VLPs were lysed by addition of 100µl of a phage lysis buffer (4.5M guanidinium isocyanate; 44mM sodium citrate pH 7.0; 0.88% sarkosyl; 0.72% 2-mercaptoethanol) and incubated at 65°C for 10 min.

Viral nucleic acids were purified by addition of an equal volume (540µl) of Phenol/Chloroform/Isoamyl Alcohol 25:24:1 (Fisher scientific), vortexed for 30s and centrifuged at 8000xg for 5 min at room temperature. The aqueous phase was carefully aspirated into a 1.5ml Eppendorf and the Phenol/Chloroform/Isoamyl Alcohol step was repeated. The aqueous phase was then passed through a Qiagen Blood & Tissue purification kit (Qiagen) following the manufacturer's instructions, with an additional AW1 and AW2 wash step, and viral nucleic acids were eluted in 50µl final volume. Extracted VLPs were quantified using Qubit (Biosciences) utilising the High Sensitivity Quantitation Assay.

**Virome Library Processing**

Extracted VLPs were subjected to a reverse transcription step to transcribe RNA phage to cDNA. Reverse transcription (RT) was performed using Superscript IV first strand system (Thermo Scientific) in a 2x reaction (40ul final) using random hexamer oligonucleotides according to the manufacturer's instructions. A 22-microliter aliquot of VLPs were added to 2ul of 50ng/µl random hexamers and 2µl 10mM dNTP mix in a PCR tube, components were mixed and spun down before being placed in an Applied Biosystems 2720 thermal cycler (Life technologies). Primers were annealed to RNA by heating the mixture to 65$^{o}$C for 5 minutes then placed immediately on ice for 1 minute. Reverse transcriptase mix consisting of (per reaction); 8ul 5x SSIV buffer, 2ul 100mM DTT, 2ul Ribonuclease Inhibitor, 2µl Superscript IV reverse transcriptase enzyme was prepared, and subsequently added to the annealed RNA. The combined reaction was heated to 23$^{o}$C for 10 mins; then 55$^{o}$C for 10 mins and the reaction was inactivated by heating to 80$^{o}$C for 10 mins. RT reactions were immediately frozen at -20$^{o}$C prior to DNA fragmentation.

Treated VLPs were subjected to DNA fragmentation using a Covaris M220 focused-ultrasonicator. Approximately 13 microliters of low-EDTA TE buffer were added to 40µl of the treated VLPs, mixed well, and 52.5µl of this was added to a microTUBE-50 (Covaris). DNA shearing was performed on a Covaris M220 focused-ultrasonicator using the following settings; peak power of 50W; duty factor of 20%; 200 cycles per burst; total duration of 35s. Following DNA shearing, samples were concentrated using a DNA Clean & Concentrator kit (Zymo research) according to the manufacturer's instructions. In brief, 52.5µl of the sheared DNA was added to a 1.5ml Eppendorf containing 255µl DNA Binding Buffer (5:1 ratio), mixed briefly by vortexing and transferred to a Zymo-spin column in a collection tube. Columns were centrifuged at 16,000xg for 30s and the supernatant was discarded. DNA Wash Buffer (200 microliters) were added to the column, centrifuged for 30s at 16,000xg, flow-through discarded and this wash step was repeated. An 18-microliter aliquot of DNA Elution Buffer

was added directly to the column matrix and incubated at room temperature for 1 min. Column was transferred to a 1.5ml Eppendorf and centrifuged at 16,000xg for 30s to elute the DNA. Samples were immediately stored at -20°C prior to library preparation.

Library preparation was carried out using Accel-NGS 1S plus kit (Swift Biosciences) according to the manufacturer's instructions. Some 15 microliters of fragmented DNA sample were transferred to a PCR tube and placed in a thermocycler programmed at 95$^{o}$C for 2 min and cooled on ice for an additional 2 min prior to the adaptase step. The denatured ssDNA substrate was combined with 25µl of the pre-assembled adaptase reaction mix consisting of; 11.5µl low EDTA TE; 4.0µl buffer G1; 4.0µl reagent G2; 2.5ul reagent G3; 1.0µl enzyme G4; 1.0µl enzyme G5; 1.0µl enzyme G6. The combined mixture was mixed by pipetting, centrifuged at x g and placed in a thermocycler programmed at 37$^{o}$C for 15 min; 95$^{o}$C for 2 min and held at 4$^{o}$C prior to the extension step. A 47-microliter aliquot of the pre-assembled extension reaction mix (18.5µl low EDTA TE; 2.0µl reagent Y1; 7.0µl reagent W2; 17.5µl buffer W3 and 2.0µl enzyme W4) was added to the 40µl of the adaptase reaction mix, mixed by pipetting, centrifuges and placed in a thermocycler programmed at 98$^{o}$C for 30s; 63$^{o}$C for 15s; 68$^{o}$C for 5 min and held at 4$^{o}$C. Removal of small unwanted fragments and oligonucleotides was performed by using AMPure XP (Beckman Coulter) beads following a 0.8 DNA/AMPure v/v ratio and freshly prepared 80% ethanol to select for fragments of 350bp. VLPs with concentrations of >1ng/µl were cleaned with beads once and eluted in 20ul low EDTA TE buffer. VLPs with concentrations <1ng/µl were cleaned twice, the first clean-up samples were eluted in 50µl; and the subsequent second bead clean-up samples were eluted in 20µl low EDTA TE buffer. Purified samples were stored at 4$^{o}$C prior to the ligation step.

A 20-microliter aliquot of the eluted adaptase-extension reaction were combined with 20µl of the pre-assembled ligation reaction mix (4µl low EDTA TE; 4µl buffer B1; 10µl reagent B2 and 2µl enzyme B3), mixed by pipetting, centrifuged and placed in a thermocycler set at 25$^{o}$C for 15 min and held at 4$^{o}$C. The ligation reaction was washed once in a 0.8 DNA/AMPure v/v ratio using 80% ethanol

and eluted in 20µl low EDTA TE. The ligated fragments were indexed using the 1S Plus Combinatorial Dual Indexing Kit (Swift Biosciences). Unique combinations were assigned to each sample and 2.5µl of each index was added to the ligated samples. Some 25 microliters of the pre-assembled indexing PCR reaction mix (10µl low EDTA TE; 4µl reagent W2; 10µl buffer W3 and 1µl enzyme W4) were added to the ligated samples containing indexes and mixed by pipetting and centrifuged. Samples were grouped based on the initial VLP DNA concentration to determine the required PCR cycles (50-100ng 7 cycles; 10-50ng 9 cycles; 1-10ng 12 cycles; 0.6-1.0ng 15 cycles and 0.1ng-0.6ng 18 cycles). Samples were placed in a thermocycler programmed at 98$^{o}$C for 30s; in cycles of 98$^{o}$C for 10s, 60$^{o}$C for 30s and 68$^{o}$C for 60s; followed by a 4$^{o}$C hold. Samples were cleaned with AMpure XP beads twice as described previously, followed by an additional bead clean-up step using a 1:1 bead:sample ratio to ensure removal of indexing PCR primer carryover.

Libraries were quantified on an Agilent 2100 Bioanalyzer (Agilent) using an Agilent High Sensitivity DNA Kit (Agilent) following the manufacturer's guidelines and electropherograms were visualised and fragment size determined using the Agilent Bioanalyzer 2100 expert software. Samples were once again quantified by Qubit, and converted to nM using the following formula:

(ng/ul from Qubit) x (106) / (Avg frag size) x (660)

Samples were subsequently pooled in an equimolar (10nM) fashion and sequenced using 2x150 nt paired-end sequencing run on an Illumina HiSeq 4000 platform at Genewiz, Germany.

**Bioinformatics analysis**

FASTQC format was applied to raw sequence data and filtered using the Trimmomatic (Bolger et al., 2014) employing the parameters; SLIDINGWINDOW: 4:20, MINLEN: 60 HEADCROP: 15; CROP 225. The sequence taxonomic classification software Kraken (v.0.10.5) (Wood & Salzberg, 2014) was used to remove human sequences, based on version 38 of the human genome. Contigs were assembled from

reads using metaSPAdes (Nurk et al., 2017), using default parameters. SPAdes meta was recently identified as a top performer in accurate assembly programs (Sutton et al., 2019). Assembled contigs larger than 1Kb were subsequently pooled and retained. An in-house script was employed (Shkoporov et al., 2018) to parse alignments based on an all versus all BLASTn search. This search was used to calculate redundancy and 90% identity over 90% length (of the shorter) retaining the largest contig in each instance. Alignment comparisons between two contigs with an e value of $1e^{-5}$ were summed together, with removal of the overlaps between the alignments. Combined alignments length was then given as a percentage of the shorter contig.

An inclusion criterion was applied to viral contigs to remove bacterial contamination. Contigs that fulfilled the following criteria were selected; 1) Categories 1-6 from VirSorter when run with default parameters and Refseqdb (–db 1) (Roux et al., 2015). 2) Circular. 3) a minimum of 2 Prokaryotic Virus Orthologous Groups (pVogs) with at least 3 per 1kb sequence (Grazziotin et al., 2017) 4) In-house CrAssphage database was used for BLASTn alignment with an e value threshold $1e^{-10}$ (Guerin et al., 2018). 5) Larger than 3Kb contigs with no BLASTn alignments to the Genbank nucleotide database (e value threshold $1e^{-10}$) 6) BLASTn alignments to the viral Refseq database (v.89) (e value threshold $1e^{-10}$) and 7) <3 ribosomal proteins predicted using the Clusters of Orthologous Groups of proteins (COGs) database (Tatusov et al., 2000). Hidden Markov Model Scan (HMMscan) was employed to predict the protein sequences on the virus-like sequences using an e value threshold of $1e^{-5}$, with the top hit chosen in each instance.

The resulting filtered reads were aligned using bowtie2 (Langmead & Salzberg, 2012) against the reference set of viral sequences. Bowtie2 alignments with a 75% breadth of coverage filter were excluded, calculated by generating a count table using SAMtools (H. Li et al., 2009). Sequences which did not exhibit read coverage of at least 1 over 75% of the total sequence length were set to 0. Virus-like sequences that were classified as exogenous Q33 bacteriophage were excluded from further diversity analysis.

**Clustering of viral contigs**

The gene prediction software Prodigal (Hyatt et al., 2012) was used to predict the protein sequence and clustered using vConTACT2 (Bin Jang et al., 2019) with parameters set to default aside from a pc-inflation and vc-inflation of 1.5 and a pcs-mode set to MCL, enabling viral clustering. Cluster count tables were subsequently generated by adding all the counts from the previous table in each cluster.

**Statistical analysis**

R (v3.5.1) was used to generate all the figures and statistics. Alpha and beta diversity plots were determined using phyloseq (v.1.26). Beta-diversity plots were generated each for Jaccard, Spearman and Bray-Curtis dissimilarity metrics. PERMANOVA was employed using adonis from the Vegan R software package (v.2.5-3) to measure the degree of variation and significance in the beta-diversity plots. The Wilcoxon test was used to calculate significance in the alpha-diversity tables. Statistical significance was deemed at p value less than 0.05 and adjustments made using Benjamini-Hochberg where applicable. Estimated viral loads were calculated based on the initial spiked concentration of endogenous phage Q33, assuming that overall viral genomes have an average length of 31.1Kb.

## Results

### Viral load quantification through spiking of endogenous bacteriophage

To assess total viral counts from each subject and timepoint, we spiked the faecal phage suspension with a known quantity of exogenous *L. lactis* bacteriophage Q33, which is rare in human subjects. Based on the assumption that the average viral genome size is more or less equal to Q33 genome size (31.1kb), this allowed for a rough estimation of total viral counts. Viral loads ranged between $1x10^8$

and 1x10$^9$ total counts (Figure 5.1), which is consistent with other studies using the same methods (Shkoporov et al., 2019). The data suggest that for some people, the virome is stable over time and/or dietary intake and for others, viral load changes over time and/or dietary intake.

Reads per kilobase of transcript per million reads Mapped (RPKM) was applied to correct for differences in both sample sequencing depth and gene length (Figure 5.2). Interestingly, tuna consumption was associated with a non-significant trend of increases in viral load over time. The sardine group also appeared to increase over time but seemed to be highly variable among individuals as there was no statistical trend apparent.

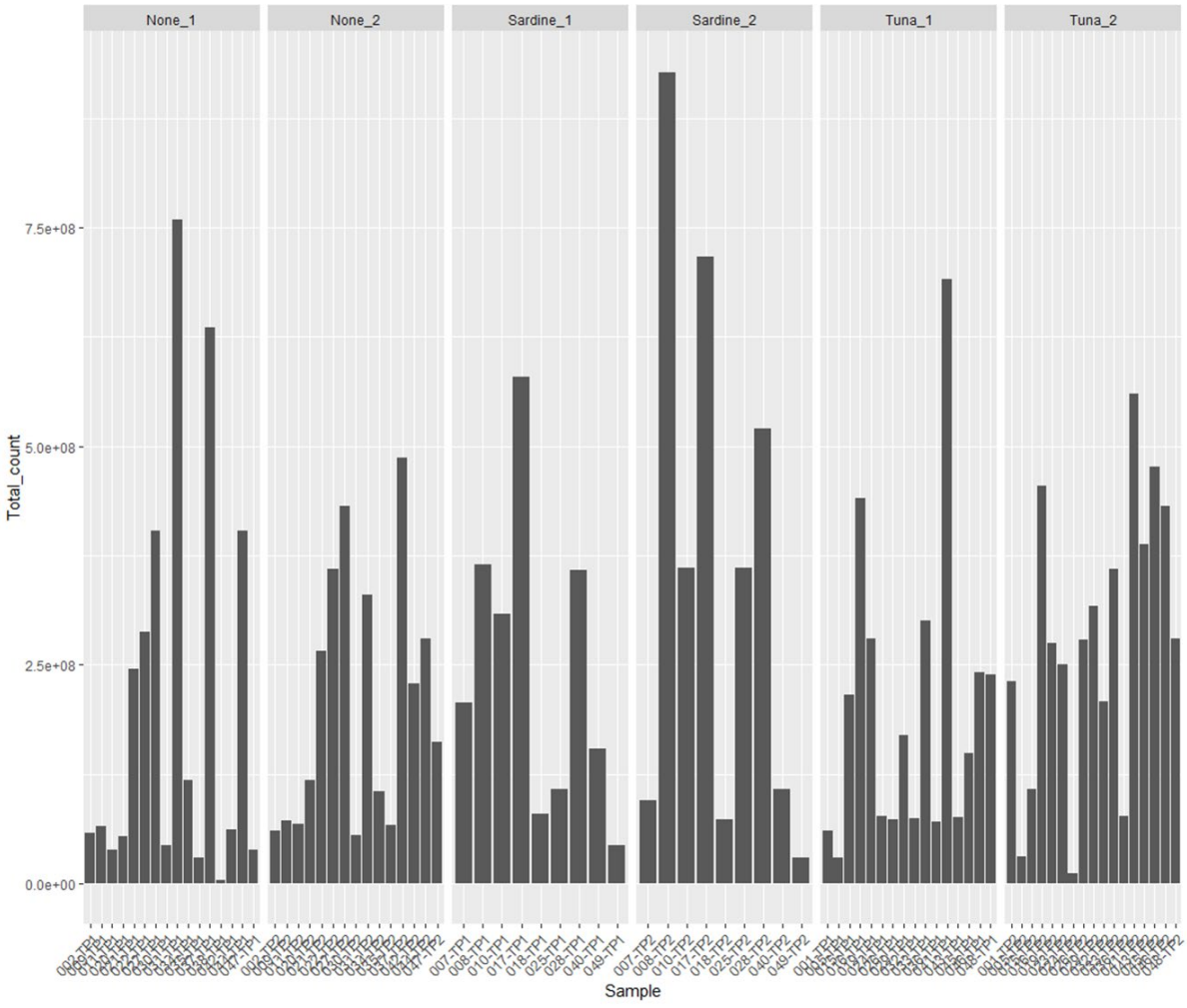



*Figure 5.1 – Viral load for each intervention group and timepoint*

Bar chart of viral load estimates using spiked *L. lactis* phage Q33 as reference for each group and timepoint; No dietary intervention, Sardine and Tuna. Subject number is indicated on the x-axis, with

none_1 (no fish timepoint 1), none_2 (no fish timepoint 2), Tuna_1 (Tuna diet group timepoint 1), Tuna_2 (Tuna diet group timepoint 2), Sardine_1 (Sardine diet group timepoint 1) and Sardine_2 (Sardine diet group timepoint 2).

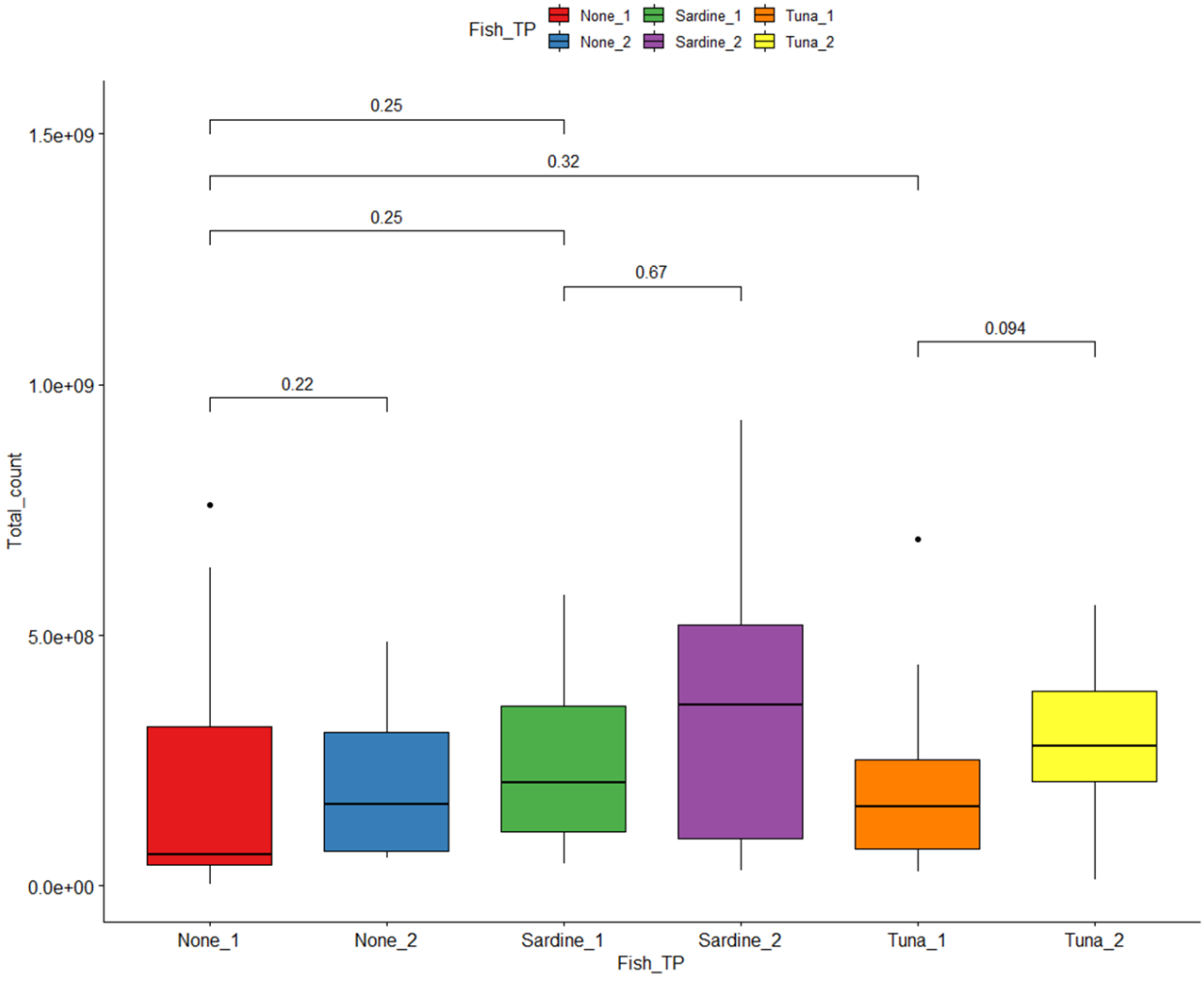



*Figure 5.2 – Boxplot of pooled viral load estimates for each group and timepoint*

Box plot of within group viral load read counts; none_1 (no fish timepoint 1) (red), none_2 (no fish timepoint 2) (blue), Sardine_1 (Sardine diet group timepoint 1) (green) and Sardine_2 (Sardine diet

group timepoint 2) (purple), Tuna_1 (Tuna diet group timepoint 1) (orange), Tuna_2 (Tuna diet group timepoint 2) (yellow).

No dietary intervention, Sardine and Tuna, for timepoints 1 & 2

**Beta diversity**

Principal coordinate analysis (PCoA) based on beta diversity metrics; Jaccard, Spearman and Bray-Curtis were performed to test for differences between the two timepoints for each treatment. The Jaccard presentation indicates that there was no effect of dietary fish intake on beta diversity. Similarly, there was no influence of dietary fish intake on Spearman and Bray-Curtis metrics.

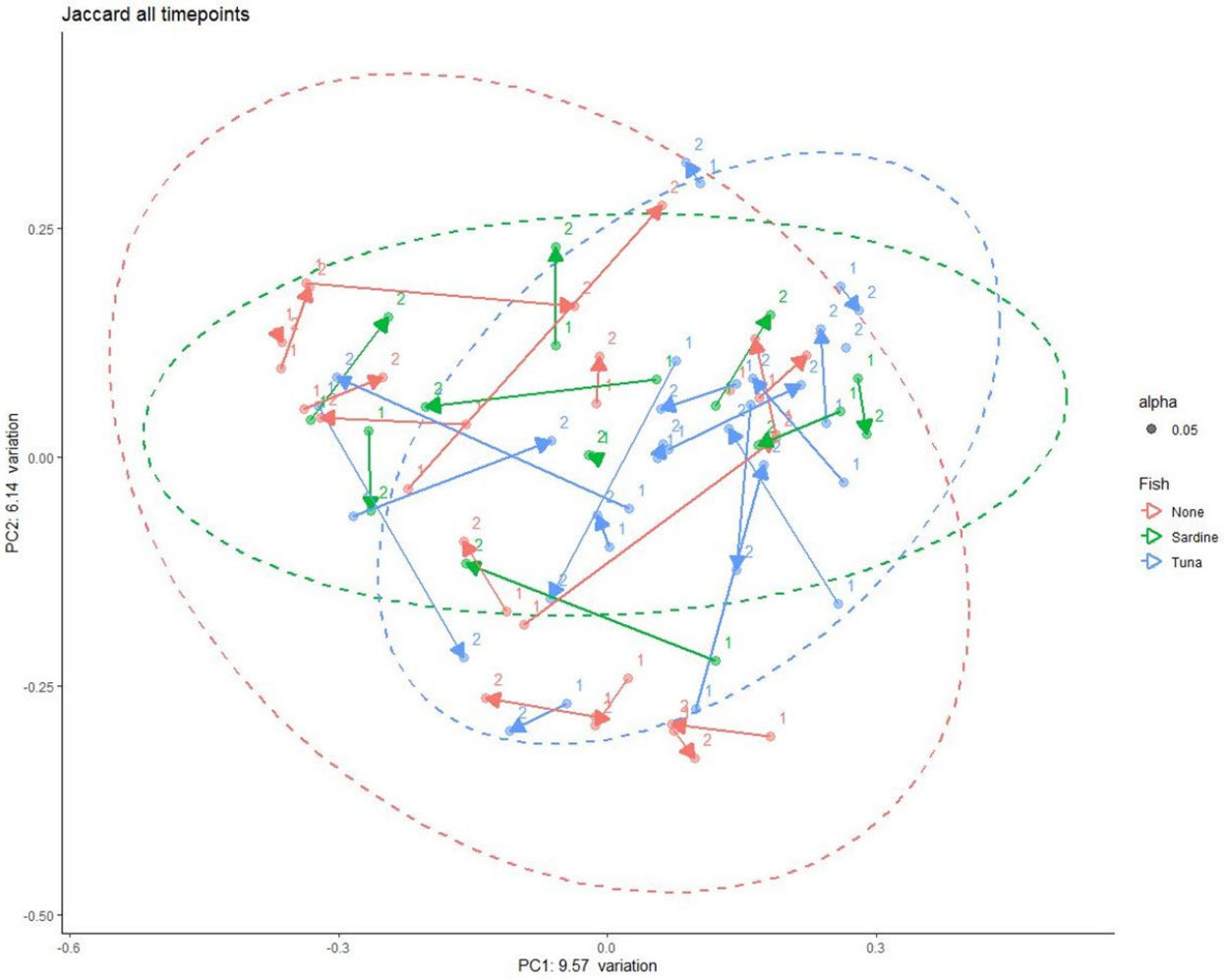



*Figure 5.3 – Change in beta diversity for each subject using Jaccard similarity metrics*

Beta diversity analysis using Jaccard similarity metrics for each intervention; No diet intervention (red), Sardine (green) and Tuna (blue). ($R^2$=0.0412 p=1e-04). The change in beta diversity for each subject is indicated by the timepoints 1 & 2.

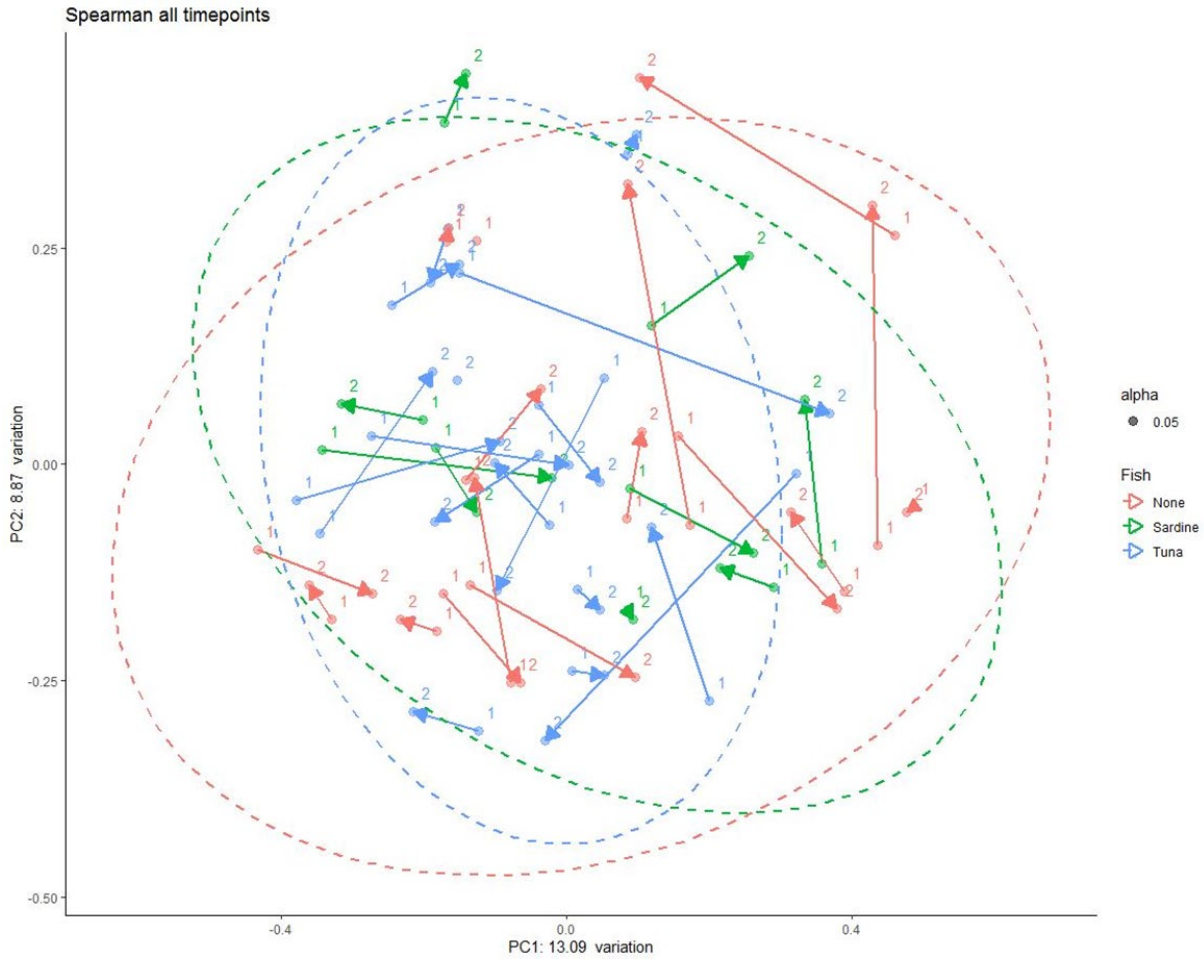



*Figure 5.4 – Change in beta diversity for each subject using Spearman metrics*

Beta diversity analysis using Spearman diversity metrics for each intervention; No dietary intervention (red), Sardine (green) and Tuna (blue). ($R^2$=0.04293, p=0.0021). The change in beta diversity for each subject is indicated by the timepoints 1 & 2.

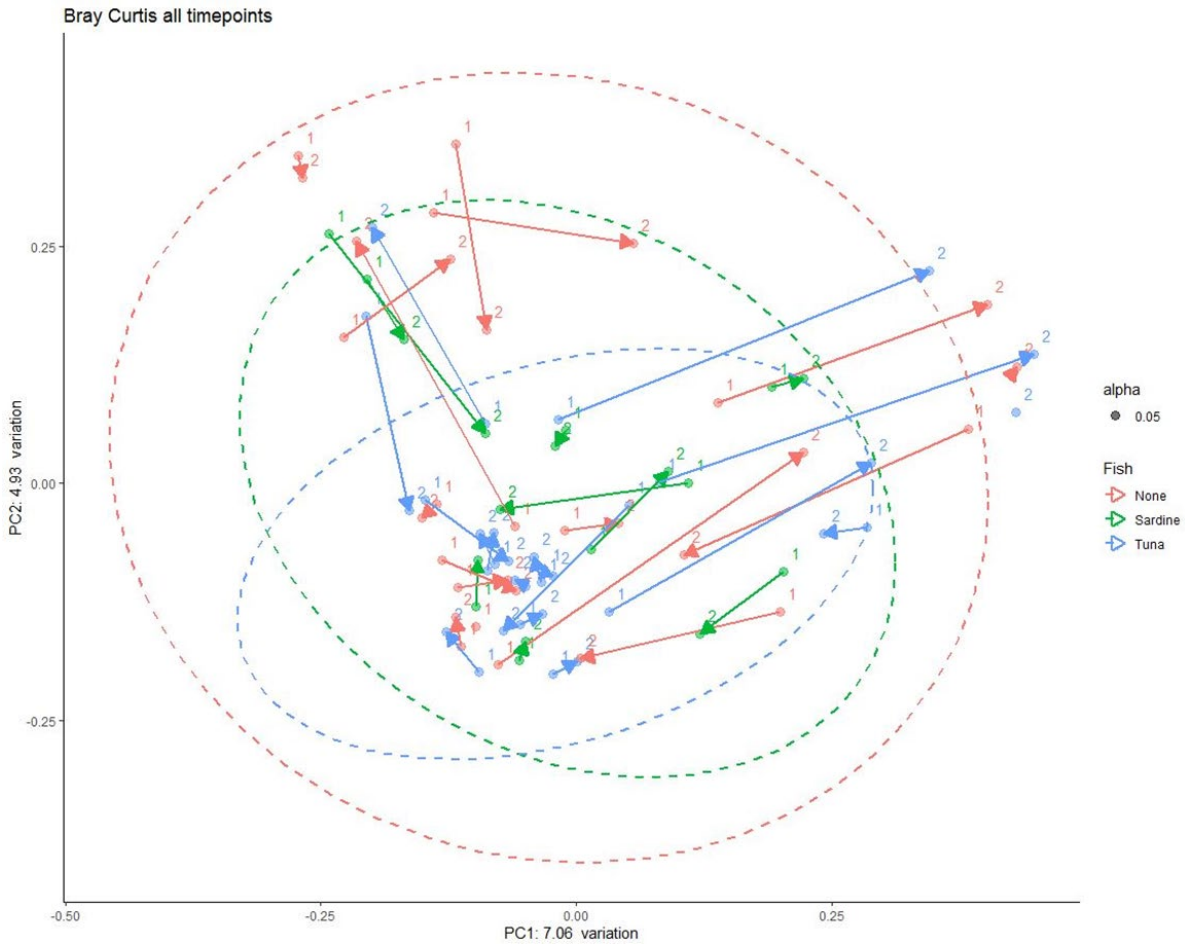



*Figure 5.5 – Change in beta diversity for each subject using Bray-Curtis metrics*

Beta diversity analysis using Bray-Curtis diversity metric for each intervention; No dietary intervention (red), Sardine (green) and Tuna (blue). ($R^2$=0.03991 p=1e-04). The change in beta diversity for each subject is indicated by the timepoints 1 & 2

## Alpha diversity

Alpha diversity for each intervention was calculated using the set of Operational Taxonomic Units (OTUs) abundances and visualised using the Shannon (Figure 5.6) and observed species (Figure 5.7) metrics. We observed a significant trend when comparing no dietary intervention and one portion of tuna, in both Shannon and observed species metrics, but no significance when comparing no

intervention and two portions of tuna. These findings suggest that inter-individual differences are accounting for this trend and not dietary intervention.

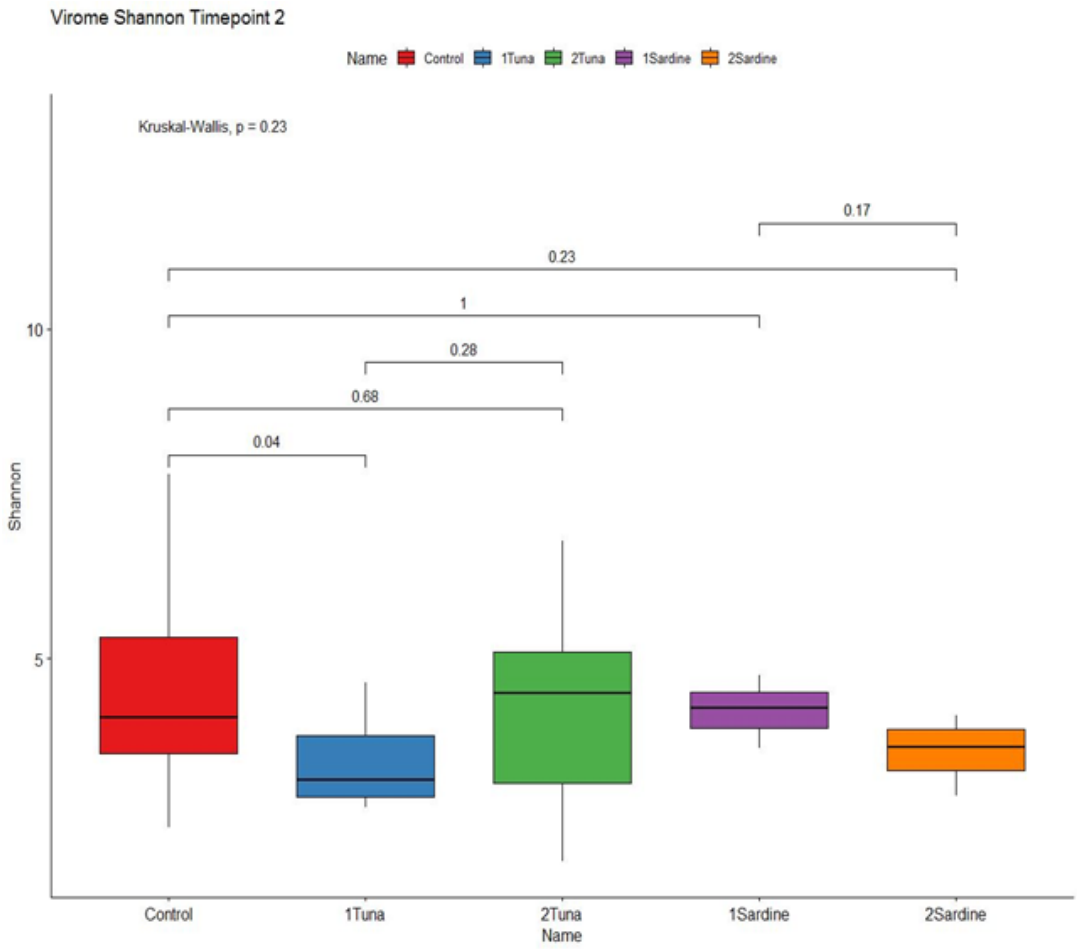



*Figure 5.6 – Shannon alpha diversity for each test group and timepoint*

Alpha diversity using Shannon index for; no intervention timepoints 1 and 2 combined (red), 1 portion tuna timepoint 2 (blue), 2 portion tuna timepoint 2 (green), 1 portion sardine timepoint 2 (purple), 2 portion sardine timepoint 2 (orange).

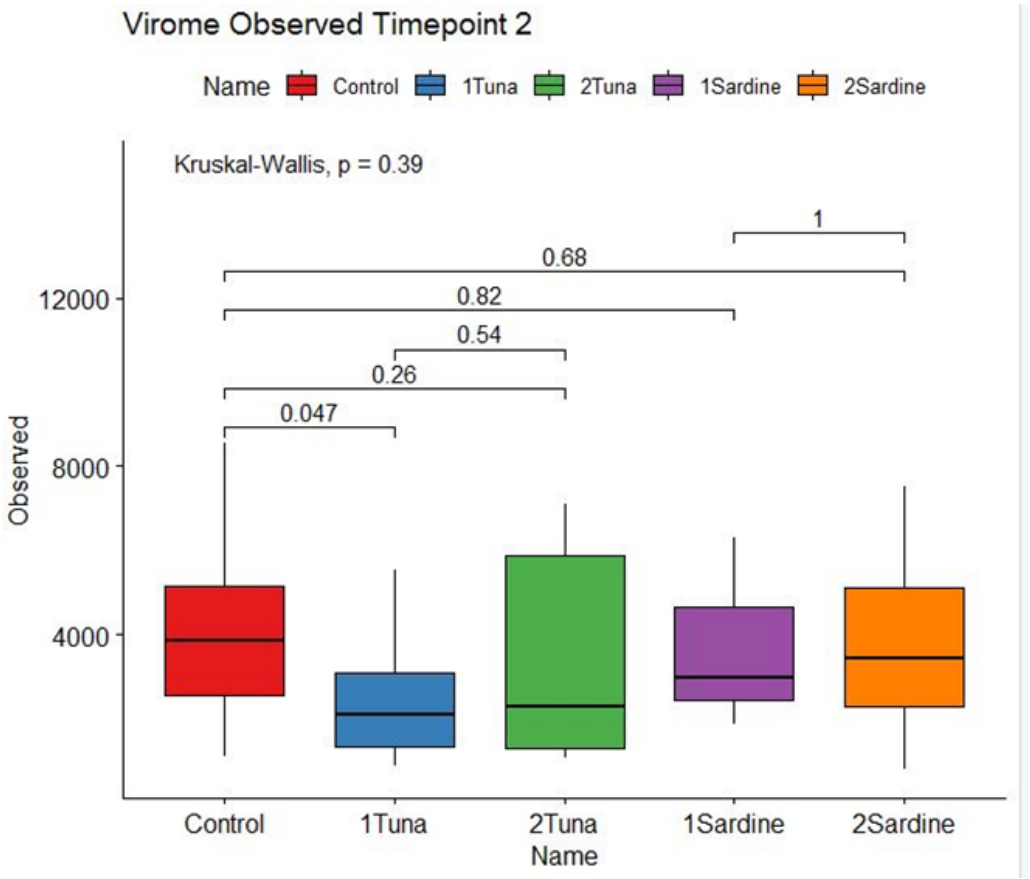



*Figure 5.7 – Observed species alpha diversity for each test group and timepoint*

Alpha diversity using observed species for; no intervention timepoints 1 and 2 combined (red), 1 portion tuna timepoint 2 (blue), 2 portion tuna timepoint 2 (green), 1 portion sardine timepoint 2 (purple), 2 portion sardine timepoint 2 (orange).

## Discussion

The faecal samples used in this pilot study on the human virome were from volunteers who took part in an intervention to investigate the effects of fish type (sardines/tuna) and amount (0/1/2 portions per week) for eight weeks on serum n-3 PUFA profile. The aims of this pilot study were to define the criteria needed to design and implement dietary interventions for future human virome studies.

Variance (measured as SD) is an important criterion to assess in any pilot study for a future intervention. In the current study, the variability in faecal viral load is shown for each individual at the

beginning and end of the intervention in Figure 5.1 and the combined viral load for each intervention group in Figure 5.2. Figure 5.1 shows that for some individuals, the viral load was stable over time, whereas for others, it varied independently of intervention group. Figure 5.2 shows that the viral load in both fish groups appeared to increase during the intervention period and in the tuna group this increase almost reached significance ($p=0.094$). Viral load should complement taxonomic assignment and/or bacterial annotation, based on relative abundance of reads to give a crude measure of virome mass. How this measure corresponds to virome fitness is unknown and can be difficult to interpret; however, it has been speculated that increased virome abundance and richness is linked to gastrointestinal diseases such as IBD (Norman et al., 2015), contrary what is observed in the bacteriome.

Variability of the most suitable outcome measure is important because it determines how many volunteers will be needed in each arm of the intervention to ensure that a type 2 error (false negative) does not occur. Our approach was to assess a number of measures of diversity as is usually done in studies on the bacteriome, wherein evidence suggests a 'healthy' bacteriome is defined by increased richness and diversity, owing to the inverse correlation of these measures in individuals with chronic disease and metabolic dysfunction (Cotillard et al., 2013). Previous bacteriome studies found that sardine intervention decreased the Firmicutes/Bacteriodetes ratio and increased the Bacteroides-Prevotella genus compared to baseline (Balfegó et al., 2016); thus, it could be expected that fish may have a similar effect on the virome.

To examine beta diversity, we used Jaccard, Spearman and Bray-Curtis metrics to measure the change between the two timepoints for each dietary intervention. Jaccard dissimilarity distances are based on the presence/absence of viral taxa and are presented on PCoA plots in Figure 5.3, wherein PC1 distances accounted for 9.57% of the variation and PC2 distances accounted for 6.14% of the variation. Interestingly, data from the no fish group (control) were less clustered (indicated by the dotted circles) compared to both the diet intervention groups, suggesting that diet is having an effect

on selecting for some specific viral taxa ($R^2$=0.0412, p=0.00014). However, this decreased clustering may be an artefactual signal, resulting from inter-individual differences and the fact that the no-diet intervention had a larger sample size as opposed to the diet intervention.

Spearman correlation coefficients were calculated for relationships between the relative abundances of identified viral taxa and dietary intervention (Figure 5.4). The Spearman correlation coefficient measures the monotonic (increasing/decreasing) relationship between paired data. The dietary intervention groups were more clustered than the control group (PC1 variation=8.87, PC2 variation=13.09), suggesting that dietary fish intake may be increasing specific viral taxa ($R^2$=0.04293, p=0.0021). However, the fact that the control group attained significance, suggests that these increases/decreases of viral taxa could be a consequence of any type of dietary ingredient i.e., it may be difficult to pick up a viral signal based on such a relatively low intake of fish. However, given that very few people in Ireland consume as much as two portions of fish per week, such a dietary intervention may influence virome composition and improve chances of detection against the complex microbiota background.

We next performed Bray-Curtis analysis as a measure of beta diversity, which provides a quantitative measure of viral taxa dissimilarity between the two timepoints for each intervention (Figure 5). Interestingly, the fish groups were much more clustered than the control intervention group ($R^2$=0.03991, p=0.0001), suggesting that out of the identified taxa, the fish intervention groups were less dissimilar in viral taxa (PC1 variation=4.93, PC2 variation=7.06) as opposed to the no fish control intervention. These findings suggest that fish intake may be selecting for certain viral taxa either through diet-mediated microbiome manipulation or viruses within the fish themselves. Plant viruses have been identified in dietary virome studies (Mihindukulasuriya et al., 2021), likely derived from plant dietary substrates. However, marine viruses would be unlikely to survive the cooking process, although it may be possible that intact nucleotide fragments from marine viruses can persist and be sequenced.

Unfortunately, we were unable to explore two important outcomes for a dietary experimental intervention: to assess if the stability of the virome can be altered by diet and whether an oily fish diet can select for phage that are associated with a healthy gut virome. These questions can only be answered through taxonomic analysis and/or host assignment, which were missing in our analysis, owing to constraints with respect to assignment of contigs to known phage in the database i.e., ~99% of contigs were of unknown viral origin, which is consistent with other studies (Breitbart et al., 2003), (Nayfach et al., 2021). We used sized filtration to enrich for extracellular viruses which allows for detection of low abundant phage (Shkoporov et al., 2018), compared to whole metagenomic sequencing, which primarily captures bacteria and associated prophage. Using this method and utilising the latest technology for unbiased viral metagenomics, these would have contributed to uncovering a myriad of undiscovered viruses. Furthermore, we applied a reverse transcriptase step to our protocol, in order to sequence RNA viruses. Recent advances in identifying these untapped viruses has led to an appreciation to the extent of how little is known about these viruses (Callanan et al., 2020), and may have contributed to the large viral 'dark matter' in our dataset.

Assignment of taxonomic groups to the virome under dietary intervention may bridge the gap between the microbiome and health. For example, it has long been assumed that oily fish intake is associated with lower prevalence of rheumatoid arthritis (RA) (Rosell et al., 2009) (Minamino et al., 2021) and a recent study identified a phage biosignature in individuals at risk of developing RA (Mangalea et al., 2021). Perhaps in the future, when we have a more improved understanding of the viral 'dark matter', we can assign taxonomy to these unknowns leading to personalised nutrition interventions tailored towards at risk individuals for diseases such as RA. Moreover, inter-individual variation of the virome may explain why some probiotic bacteria colonise the gut in some individuals but not others, owing to the presence of potentially predatory phage that can target probiotic bacteria. For example, the probiotic bacteria *Lactobacillus brevis* KB290 used in the treatment of IBS has yielded inconsistent results (Murakami et al., 2012) and this may be explained by an increase of

*Lactobacillus* virus LBR48 in patients with IBS (Mihindukulasuriya et al., 2021), a phage which can infect *L. Brevis*.

These data suggest that diet can transiently impact the gut virome. Whether this finding is solely attributed to inter-individual variation remains a possibility, given our limited dataset of diversity analysis and viral load. Taxonomic grouping based on viruses and/or host assignment may shed light on answering this impasse. Virome studies are in their infancy and advances in this understudied field may help reduce disparities in determining how viruses impact human health.

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# General Discussion

This thesis sought to provide a unique angle into characterising diet-microbe interactions through novel *in vitro*, *in silico* and *in vivo* techniques. It also aimed to bestow a scientifically rigorous approach to an *in vivo* microbiome study, through application of a randomised controlled trial to study the effects of food ingredients on an overlooked proportion of the human microbiome. Arguably, diet is the most impactful external factor affecting the gut microbiota structure and function. This is unsurprising when one considers that gut microbes, akin to their human hosts, utilise ingested foodstuffs as fuel for fundamental biological processes. The collective genome of the microbiota comprises approximately >150x genes in comparison with the human genome, enabling physiological functionalities and capabilities not present in our own genome (Qin et al., 2010). The ability to degrade complex carbohydrates and lipids is the underpinning mechanism that enables bacteria, and indeed, their intracellular parasites, bacteriophage, to survive, flourish and influence physiological processes in the gastrointestinal tract.

It has long been known that antibiotic perturbations to the microbiota can increase susceptibility to enteric infections (Bohnhoff & Miller, 1962); thus, one can speculate on similar dietary perturbations having an analogous effect. The *in vivo* impact of prebiotics may be multifactorial and include multispecies, and thus translating *in vitro* effects to living organisms should be approached with caution. However, *in vitro* studies can be invaluable tools to inform animal models going forward. It is likely that the most bioactive microorganisms are strictly anaerobic, which also adds to the difficulties when translating *in vitro* studies, wherein many of these microorganisms can be lost in the collection, preparation and fermentation stages. Moreover, the intricate interactions between immune responses, commensal bacteria and bacteriophage are lacking *in vitro,* and they no doubt play a pivotal role in accomplishing colonisation resistance.

In Chapter 1, we explored the literature examining the performance of pathogens in response to diet and metabolites in the gut microbiota. Findings from the review indicated that the success of

pathogen colonisation is highly variable and dependent on the pathogen in question. The preferred site of infection for the pathogen, the associated dietary substrates and metabolites located at these sites, the interplay between commensals, the mucus layer and immune responses, ultimately influence the prosperity of an invading pathogen in establishing itself in the gut microbiota. Identifying protective taxa associated with particular diets may provide alternative approaches to decreasing the risk of gastrointestinal conditions such as traveller's diarrhoea or chronic diseases such as IBD. Geographical comparisons between human microbiota composition and food consumption in regions of e.g., low IBD or high traveller's diarrhoea prevalence, could provide a non-invasive approach and potentially uncover associations of studying diet, taxa and colonisation resistance in human subjects. Although largely speculative, it is hoped that the literature review can guide future research into this unique approach to protection from enteric pathogen infection.

Chapter 2 was designed as a platform to inform animal models of the choice of both pathogen and indeed, prebiotics for the study of colonisation resistance. We screened a selection of gastrointestinal pathogens for their ability to metabolise novel prebiotics using a growth media devoid of carbon, to ascertain if the pathogens could metabolise the test prebiotics as a carbon source. Given that none of the pathogens could degrade yeast β-glucan, we chose this prebiotic as our test substrate for the faecal fermentation experiment using the MicroMatrix bioreactor (O'Donnell et al., 2018). We decided to add four pathogens as a cocktail to determine which would be most strongly inhibited. In hindsight, this was perhaps a bad decision and not something we would experience *in vivo*, as it would be incredibly unlikely that a human subject would face infection with four pathogens simultaneously. Our choice of control carbohydrate, glucose, was perhaps not appropriate either, as it would be metabolised rapidly by commensal bacteria, and a non-digestible carbohydrate, i.e., cellulose, may have been a more comparable control substrate. The yeast β-glucan group selected for the growth of commensal Bacteroides and Clostridia, whereas the glucose control selected for *Escherichia/Shigella* and *Lactobacillus*. We observed no significant difference in the pathogen relative abundance between the fibre and glucose group; however, the results may have been hampered by discrepancies in the

16S analysis, with it being difficult to ascertain the difference between commensal Enterobacteriaceae and pathogenic Enterobacteriaceae. We did, however, find significant differences between fatty acid production between the test groups. The relative abundances of the pathogens were skewed by the lack of specificity through using universal illumina primers i.e., it was difficult to separate commensal *Enterobacteriaceae* from the spiked pathogenic *Enterobacteriaceae.* Moreover, the timing of spiking of pathogens at T0 may have not given the commensal bacteria in the faecal inoculum enough time to metabolise the prebiotic fibre and thus provide a desired protective effect. Other gut fermentation models may be more suitable to test our hypothesis, such as the M-SHIME (Venema et al, 2013), which allows for addition of substrates/cultures mid-fermentation and houses different vessels representing various compartments of the intestinal tract. For example, pathogens that preferentially colonise the small intestine can be added following fibre fermentation to the vessel representing the small intestine. Overall, the experimental design may not have been sufficient to answer our hypothesis that prebiotic fibres can boost colonisation resistance *in vitro.*

In chapter 3, we sought to correct the mistakes of the previous chapter by repeating the experiment but adjusting a few different methods to conform more fully with our hypothesis. Considering the problems with quantifying the pathogens in the faecal fermentation with 16S sequencing, we employed qPCR to detect unique genetic regions of the pathogen to assess growth following 24-hours fermentation. The MicroMatrix fermentation unit consists of a 24-well "cassette", each well with a 5ml volume to perform miniature, controlled experiments in unison. This unit eliminates bias that would be observed using traditional large-scale bioreactors, wherein difficulties would arise when performing 24 of these simultaneously. The fragility of many species of the microbiota are apparent following many freeze-thaw cycles and by carrying out the fermentation by limiting these cycles as much as possible is of the utmost importance. The fast replication kinetics of pathogens requires swift addition to the wells to ensure that each well contains the same cfu per experiment. Furthermore, by having the test carbohydrates pre-weighed out and in each well prior to adding the media and faecal inoculum is an advantage using this approach. Thus, the MicroMatrix

system enables a much more controlled approach that would not be seen with other traditional fermentation procedures.

We selected four, fully sequenced pathogens and designed primers based on their genome sequence using PrimerBlast (Ye et al., 2012). Each of these primer sets were tested on extracted DNA from healthy human subjects, in order to determine if any of them had off-target specificity with commensal bacteria. It was decided to include two unrelated pathogens in this experiment, to assess if they behaved differently to *Enterobacteriaceae* in response to carbohydrate treatment. A pathogenic *Enterococcus faecium* strain which is used as a model of Vancomycin Resistant *Enterococcus* (VRE), to examine if prebiotic fibres have the potential to inhibit VRE. Interestingly, the novel test prebiotic fibre, yeast β-glucan, seemed to mitigate VRE growth compared to the other carbohydrates tested. While this effect was quite transient, with only a reduction of 1-log difference compared to the glucose control, this opens up the capacity to utilise prebiotics to suppress VRE growth in hospitalised patients and thus, potentially reducing the risk of VRE nosocomial infections. To our knowledge, little research has been performed to date on using prebiotics to combat VRE colonisation. The final pathogen tested was the common food-borne pathogen *Listeria monocytogenes*. Interestingly, *L. monocytogenes* asymptomatic carriage is relatively common in humans (Hafner et al, 2021) and is associated with a particular microbiota signature that limits its virulence. Thus, identifying food ingredients that encourage this protective microbiota signature can improve listeria colonisation resistance in humans. The novel prebiotic fibre, yeast β-glucan, encouraged the growth of *L. monocytogenes in vitro* and a galacto-oligosaccharide inhibited its growth. While these results are preliminary, they open up a new avenue of research to support the use of various prebiotic compounds to provide colonisation resistance in animal models and the human gut.

Colonisation resistance is one potential functional mechanism of the microbiota that influences the health of the host, while at present, the data supporting this is primarily limited to

animal models. Owing to the fact that the microbiota is an underpinning environmental factor that affects energy harvesting and energy storage from diet (Bäckhed et al., 2004), another functional mechanism with much more evidence in humans is the appreciation that the microbiota is intrinsically linked to metabolic health (J. L. Sonnenburg & Bäckhed, 2016). Alterations in microbial profiles are observed when comparing healthy, obese and diabetic patients (Thingholm et al., 2019), with notable differences in diversity and microbial functional capacity. These differences are likely driven by diet, and, therefore, we wanted to explore the potential of prebiotic and lipid food components that can improve microbial diversity and increase taxa associated with favourable metabolic health. Chapter 4 takes a different approach to the intersect of diet and health, using similar techniques implemented in Chapters 2 and 3. We performed an extensive *ex vivo* study using the MicroMatrix fermentation system using faecal material collected from obese (n=2), prediabetics (n=3) and type 2 diabetic (n=7) patients. Our findings suggest that prebiotic fibres and lipids can significantly impact alpha and beta diversity matrices when compared with glucose as control. β-glucan supplementation reduced *Escherichia/Shigella* abundance, groups of bacteria associated with a dysbiotic gut state, while concomitantly promoting the growth of *Bacteroides* and *Faecalibacterium*, taxa which are negatively associated with metabolic disease. Our lipid compounds also impacted a range of gut taxa, with fish oil reducing *Escherichia coli* abundances and increasing *Bifidobacterium* and *Veillonella*.

Metagenome-wide association studies have identified microbial taxa associated with type 2 diabetes (T2DM) (Qin et al., 2012) (X. Wang et al., 2017) and some pointing towards a causal relationship between taxa and metabolic diseases (Sanna et al., 2019) (Thingholm et al., 2019). Our ex vivo study provides a basis for elucidating the microbial responses to diet at the polysaccharide and lipid level. These *ex vivo* findings suggest β-glucans and fish oil can improve microbiome imbalances observed in patients with metabolic syndrome. The main limitations of this study were the use of glucose as control carbohydrate and that this is an *in vitro* study, which may not encapsulate the conditions observed in the gastrointestinal tract. It is possible that the species that responded well to fibre treatment are overrepresented when compared to the glucose control; however, the data are

supported by those from *in vivo* studies (Zhao et al., 2018). Our analysis for lipids was compared to the no lipid treatment group, which is likely a better comparison for accurate microbial responses to dietary intervention. However, these results should be approached with caution, as the lipids were not subjected to the same pre-digestion procedure as was done for fibres, so the lipids may not truly represent what reaches the colon. These data can inform future human dietary intervention studies on the application of dietary fibres and lipids for improving gut health in patients with metabolic syndrome. The taxa enriched in the sequencing data could be isolated, cultured and tested as functional and next-generation probiotics, as has been done for *Faecalibacterium* (Martin et al, 2017), *Bacteroides* (Tan et al, 2019).

While there are data supporting a causal link in humans between certain bacterial taxa and metabolic syndrome, the evidence in respect to autoimmune disorders such as inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) is weaker. The pertinent question remains whether the link is correlation or causation, as the hostile environment of an inflamed gut may have a detrimental effect on delicate commensals. There are some indications that human microbiota crossovers into animal models exacerbate immune pathology suggesting a questionable capacity that the microbiota is somewhat culpable (Britton et al., 2019). The same may be said for the human gut virome, with increased viral richness and diversity observed in ulcerative colitis (Zuo et al., 2019) and IBD (Norman et al., 2015). One possible explanation for these observations is the adverse surroundings of an inflamed gut is inducing prophage from commensals (Clooney et al., 2019). Environmental factors, especially diet, may influence the predisposition to IBD development (Marion-Letellier et al., 2016), and thus identifying diet-microbiome signatures can inform future explorations into the potential diet-based therapies for autoimmune disease prevention and treatment. The mechanisms in intestinal fibrosis are believed to be similar to those observed in extra-intestinal fibrosis (Marion-Letellier et al., 2021), such as those in RA. Polyunsaturated fatty acids (PUFAs) have been shown to possess anti-inflammatory properties and may help in alleviating IBD-induced inflammation (Ibrahim et al., 2011) and play a protective role in RA development (Gioia et al., 2020). Interestingly, a recent study

identified a bacteriophage signature associated with RA (Mangalea et al., 2021), providing the first evidence that the gut virome may have a position in RA pathogenesis. Furthermore, phage have been shown to stimulate the immune response against bacteria in non-human hosts (Sweere et al., 2019) (Jahn et al., 2019), with phage belonging to the *Caudovirales* order and infecting *Lactobacillus, Escherichia* and *Bacteroides* stimulating IFN-y and exacerbating ulcerative colitis (Gogokhia et al., 2019). Collectively, these data indicate that phage can alter mucosal immunity to impact mammalian health. Increases in phage belonging to the *Caudovirales* have been observed in UC and Crohn's disease (Gogokhia et al., 2019) and have been shown to translocate across the mucosal epithelial barrier (Núñez-Sánchez et al., 2020); therefore linking dietary substrates to viral taxonomic rankings and/or their bacterial hosts can help consolidate knowledge on diet-virome interactions and their relationship with autoimmune disorders.

Given the beneficial effects of PUFAs in autoimmune disorders, we sought to test if a diet rich in PUFAs can impact an underappreciated proportion of the microbiota, the gut virome. We performed a pilot study using convenience samples from a randomised controlled trial involving an oily fish diet intervention to examine the effects on the human gut virome. On the grounds that the virome is largely uncharacterised, in combination with our advanced approach to metagenomic sequencing, our dataset obtained from this study was overwhelmingly comprised of undiscovered viruses. This hampered our analysis in that we could not assign viral taxa or viral hosts to the majority of our sequences. The only analysis we could perform was diversity and viral load measurements, which we would not expect to be radically changed from a small dietary intervention. While we could observe a transient effect of diet on the virome diversity, this effect may be attributed to inter-individual variation. The data from this pilot study can inform future diet-virome studies that a more extensive dietary intervention may be required to detect a pronounced effect. We appreciate that given the large unknowns in our dataset throughout has hindered answering our hypotheses. However, we hope our findings will inform studies as a starting point for future explorations. Given that more research will be made at characterising the viral dark matter in terms of viral hosts, function

and composition as a whole, we can revisit our dataset to see if an oily fish diet has an effect on virome composition.

In conclusion, this thesis utilises the latest in *in vitro* and *in silico* technologies in an attempt to uncover novel insights into diet-microbiome interactions in the human microbiota. These findings have contributed to our understanding of the impact of fibre and lipid substrates on the gut microbiota and its associated metabolites. Future explorations utilising sequencing and culture-based gut microbiota surveys encompassing the bacteriome, virome, mycobiome and archeome can shed light on the impact of dietary substrates in the microbiome. The findings need to be explored further to elucidate how the relationships between diet and the microbiome, including the virome translate to human health.

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