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Modulation of the human gut microbiome in order to promote host health and well-being

Thesis presented for the Degree of Philosophiae Doctor
By

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In candidature for the Degree of Philosophiae Doctor

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SUMMARY

Background

Numerous studies into the effect of probiotic supplementation in the infirm have been carried out. However, research into the effect of long-term probiotic supplementation in healthy individuals is lacking. With this in mind the PROHEMI study, a randomized, double-blinded, multi-centre and long-term (6 months) probiotic feeding study in healthy males was designed and carried out. Through the use of varied culture dependent and independent techniques, the effects of long-term probiotic consumption were researched. In addition, a study into the effect of freezing faecal material on its bacterial composition was also carried out.

Results

Through a community fingerprinting technique and next generation sequencing it was shown that the distal gut bacterial community is unaffected by probiotic supplementation. Functional screening of faecal material showed a reduction in bacteria expressing protease activity when probiotic supplementation began. In addition, bacteria expressing β -galactosidase and β -glucuronidase activity increased during probiotic supplementation. Metabonomic analysis showed no difference in metabolite profiles attributable to probiotic supplementation. However, differences between the gut bacterial community, metabonomic profiles, and bacteria expressing functions were observed between the two study centres. Freezing of faecal material at -20°C detrimentally affected its bacterial composition between 2 weeks and 3 month storage time-points. Significant reductions in the abundance of the *Bacteroidetes* phylum observed following 6 months of storage at -20°C .

Conclusions

Long-term probiotic administration in healthy individuals did not seem to affect the distal gut bacterial community in these individuals and did not affect metabonomic profiles. However, some functions expressed by the resident distal gut bacterial community were significantly affected during probiotic supplementation. DNA extraction from faecal material should ideally be carried out from fresh samples. Failing this it is not recommended to store samples at -20°C for longer than 2 weeks prior to DNA extraction.

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LIST OF ABBREVIATIONS

4-NQO	4-nitroquinoline-N-oxide
AAD	antibiotic associated diarrhoea
AE	atopic eczema
AGNES	agglomerative nested clustering
Amp	ampicillin
ARISA	automated ribosomal intergenic spacer analysis
BPA	buffered protease agar
BSH	bile salt hydrolase
CD	Crohn's disease
CDAD	<i>Clostridium difficile</i> associated diarrhoea
cDNA	complementary DNA
choA	cholesterol oxidase
DGGE	denaturing gradient gel electrophoresis
EFSA	the European Food Safety Authority
ESF	European Social Fund
FOS	fructo-oligosaccharides
GeIE	gelatinase enzyme
GF	germ-free
GIT	gastro-intestinal tract
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IgE	immunoglobulin E
IL	interleukin
iTOL	interactive tree of life

KEGG	Kyoto Encyclopaedia of Genes and Genomes
KESS	Knowledge Economy Skills Scholarship
LAB	lactic acid bacteria
LHPCR	length heterogeneity PCR
MMS	methyl methanesulfonate
MRS	de Man, Rogosa and Sharpe
NEC	necrotising enterocolitis
NGS	next generation sequencing
nMDS	non-metric multidimensional scaling
NMR	nuclear magnetic resonance
NPPARs	normalised proportional peak area ratios
ONPG	<i>o</i> -nitrophenyl- β -galactoside
OTU	operational taxonomic unit
PCA	principal component analysis
PCoA	principal co-ordinate analysis
PCR	polymerase chain reaction
PDAI	pouchitis disease activity index
qPCR	quantitative PCR
RDP	ribosomal database project
RISA	ribosomal intergenic spacer analysis
RR	relative risk
rRNA	ribosomal RNA
SCFA	short chain fatty acid
SD	standard deviation from the mean
SMEs	small and medium enterprises
Th1	T helper cell type 1
TNF-α	tumour necrosis factor α
t-RFLP	terminal fragment length polymorphism
UC	ulcerative colitis
VLBW	very-low birth weight
WHO	World Health Organisation

“Well, there we are then”

Michael Evans

1 INTRODUCTION

1.1 The human gut microbiome

Human associated microbes referred to as our microbiome, have been extensively studied in recent years with a particular focus on bacteria. The Human Microbiome Project (HMP) (Turnbaugh *et al.* 2007) aims to fill in gaps of knowledge from the Human Genome Project. The cells, which make up our microbiome, significantly outnumber our own cells by a factor of 10 and in this sense we can consider ourselves more microbial than human. Through the significant increase in our gene pool, which is provided by these organisms, we can think of humans as super-organisms (Lederberg 2000). There is an intimate association between us and our co-evolved microbiome. The human gut, with an approximate area of a tennis court, harbours most of our associated bacteria (Whitman *et al.* 1998) containing approximately 10^{14} bacterial cells (Ley *et al.* 2006a). The gut of new-born babies was initially thought to be sterile and becomes colonised at birth by bacteria from the mother's vagina and environment. Succession of microbial consortia follows until a fairly uniform microbial community is established (Mändar and Mikelsaar 1996; Palmer *et al.* 2007). However, in a more recent study it has been shown that the meconium, the first stool passed by new-born babies, is not sterile. It contains bacteria, fungi, viruses and *Euryarcheota* (Koenig *et al.* 2011). It has been argued by Koenig and co-workers that there are four steps in the development of an adult gut in infants. The first step is characterised by an abundance of the number of lactose/galactose metabolising genes due to breast milk feeding. The steps are separated by life events with events such as fever separating steps 1-2. The introduction of solids separates steps 2-3. Finally the commencement of an adult diet in conjunction with antibiotic therapy separates steps 3-4. By step 4 the gut microbiota and genes expressed resemble an adult gut. With regards to the adult gut, there is a general consensus that a core gut microbiome is shared amongst adults. Analysis of the distal microbial community of 3 adults (43, 50 and 50 years old respectively) showed that the major phyla were the *Firmicutes* and *Bacteroidetes* (Eckburg *et al.* 2005). Other phyla such as the *Proteobacteria* and *Actinobacteria* were present at a lower abundance. While the abundance of phyla remained relatively uniform across the subjects, there were inter-person differences at lower taxonomic levels, showing that there is variation from person to person in terms of their distal gut community. In a more recent analysis of the distal gut community, Turnbaugh and colleagues strengthened the notion of inter-personal differences in the distal gut community of individuals

(Turnbaugh *et al.* 2009). However, it was suggested that a core microbiome is present but at the gene level rather than at a taxonomic level and gives rise to functional redundancy. In a short review Turnbaugh and Gordon suggest that changes to the core microbiome and specifically the genes expressed can give rise to obesity in individuals, as genes responsible for energy harvest may be more abundant in these individuals (Turnbaugh and Gordon 2009).

1.1.1 The human gut microbiome in health

Within the gut, services are provided by our microbiota such as vitamin B synthesis (Burkholder and McVeigh 1942), the importance of which may have been overlooked previously, as it has been shown that carrier mediated absorption of riboflavin occurs in the rat colon (Yuasa *et al.* 2000). Our gut microbiota provides protection against pathogens (O'Hara and Shanahan 2006). This protection is further highlighted in a study showing that the human gut microbiota decreases Shiga toxin 2 (Stx2) production in entero-haemorrhagic *Escherichia coli* (EHEC) O157:H7 (de Sablet *et al.* 2009). The human gut microbiota also exert immunomodulatory effects (Round and Mazmanian 2009). The gut microbiome has been shown to play a pivotal role in intestinal homeostasis where it has been suggested that the intestinal microbiota is a selective regulator of nuclear receptors (Arulampalam *et al.* 2006). This regulation in turn gives rise to anti and pro-inflammatory responses within the gut. The importance of the gut microbiota is now being understood in terms of its effect on our endocrine system. There is an increasing volume of research in this area and has recently been reviewed (Evans *et al.* 2013). For instance, the gut produces a high level of dopamine and norepinephrine. Murine models show that this occurs through the cleavage of their conjugated inactive forms by bacterial β -glucuronidases in the gut lumen (Asano *et al.* 2012). The concept of gut commensals and pathogens driving the correct development of the immune system has been researched for some years, especially since the advent of the "hygiene hypothesis" concept (Strachan 1989, 2000). However, it has been shown in a more recent study that correct intestinal immune system maturation occurs through host specific gut commensal microbes and not just any gut commensal (Chung *et al.* 2012). This was shown through the use of germ-free (GF) mice, mouse specific microbiota and human specific microbiota. Colonisation of the GF mouse with human specific microbiota resulted in an immature intestinal immune system. The human microbiota associated GF mice showed lower levels of T-lymphocytes and lower levels of dendritic cells in the small intestine. Furthermore, when challenged with *Salmonella typhimurium* Serovar Typhimurium the human microbiota offered the GF mice worse protection than the mouse specific microbiota. Higher loads of the

pathogen were observed four days post-infection. There was a highly significant ($p < 0.001$) increase in the level of pathogen translocation to the spleen and inflammation of the cecal wall observed in the human microbiota GF mice. This research highlights the role that a host's closely evolved gut microbiota has upon the development of its own immune system. The gut microbiota also drives the development of the gut as an organ. It has been shown that transplantation of a normal murine cecal microbiota into GF mice drives vascular remodelling in the small intestine (Reinhardt *et al.* 2012). The authors highlight a novel mechanism whereby the colonisation drives an increase in the localisation of tissue factor TF. This localisation in turn leads to coagulation cascade activation and leads to vascular remodelling through angiopoietin-1 (Ang-1). Remodelling is achieved through a series of steps which involve the phosphorylation of the cytoplasmic domain of TF by protease-activated receptor 1 (PAR1). Changes in the morphology of the gut have also been observed in GF pigs, where it was observed that GF and mono-associated pig guts had long villi and shorter crypts than conventionally raised pigs (Shirkey *et al.* 2006). Germ free animals also exhibit smaller Payer's patches, a decrease in the amount of lamina propria and the development of a mega caecum in gnotobiotic rodents (Thompson and Trexler 1971). We should therefore think of our gut microbiota as completely necessary for the development of healthy gut morphology and function therein.

1.1.2 The human gut microbiome in disease

Many studies have highlighted the associated perturbation of the host gut microbiome following antibiotic therapy in both mice (Antonopoulos *et al.* 2009; Sekirov *et al.* 2008) and in humans (Dethlefsen *et al.* 2008; Jernberg *et al.* 2007). These perturbations can give rise to complications such as antibiotic associated diarrhoea (AAD) (Wiström *et al.* 2001) and pseudomembranous colitis due to overgrowth of *Clostridium difficile*. Both arise as a result of decreased competition (Bartlett *et al.* 1978; Wiström *et al.* 2001). Perturbations of the gut microbiome have been linked to an increased susceptibility of enteric infections in mice (Sekirov *et al.* 2008). The gut microbiota in many instances has been implicated in the progression of complex disorders within the human body including type II diabetes (Larsen *et al.* 2010; Qin *et al.* 2012) and controversially in both obesity (Ley *et al.* 2006b; Schwartz *et al.* 2009) and autism (Finegold 2008; Sandler *et al.* 2000; Song *et al.* 2004). With regards to obesity, it has been suggested that the gut microbiota of some individuals is more adept at extracting energy from food sources (Bäckhed *et al.* 2004) and this contributes to the progression of the disease. Furthermore, it has been shown that the gut bacterial community of obese mice is different to those who are not, with a reduction in the ratio of *Bacteroidetes* to *Firmicutes*, the two major bacteria phyla present in the human gut (Ley *et al.*

2005). However, more recent research, in humans, challenges this observation. Duncan and colleagues (Duncan *et al.* 2008) have shown no significant difference in the percentage of *Bacteroidetes* in obese and non-obese individuals (27.2% vs. 21.9% respectively, $p=0.084$). It must be noted however, that the measure used, body mass index (BMI), does not solely take into account body fat percentage. Another study has shown that GF mice also developed obesity while on a high fat diet (Fleissner *et al.* 2010). This confusion highlights the need for further research into the effect of the human microbiome in health and disease.

1.2 Methods implemented in order to analyse the gut microbiota composition and functions

In order to analyse the gut microbiota and its functions a whole suite of techniques and methods are needed. Initially many of the techniques used to analyse any ecosystem were culture dependent. Researchers became aware of the caveats associated with using these techniques with the coining of the "great plate count anomaly" (Staley and Konopka 1985). With regards to the gut microbiome itself, there was already concern around the issue of biased culture dependent methods prior to Staley and Konopka. There was a particular focus on the effect of the culturing methods implemented at the time on the recovery of anaerobic bacteria from the human gut (Hughes 1972). There have been significant advances in the molecular field of biology. These advances include the advent of the polymerase chain reaction (PCR) (Saiki *et al.* 1985) and Sanger sequencing (Sanger *et al.* 1977). This was followed by next generation sequencing (NGS) through pyrosequencing (Margulies *et al.* 2005) and now through the use of protons (Rothberg *et al.* 2011). There has also been an increase in the understanding of the underlying biology of microorganisms. An example of this is the use of ribosomal RNA (*rRNA*) genes as molecular chronographs (Woese *et al.* 1990), that is to say the use of these genes to provide an account of the evolutionary history of bacteria. The advancement in technical expertise and knowledge has led to a culture independent revolution in biology.

1.2.1 The use of culture dependent methods in order to analyse the gut microbiota and functions

While culture dependent methods have their drawbacks, they still provide a useful set of tools in order to interrogate the gut microbiota and the functions provided therein. It is sometimes advantageous or of interest to select for specific groups of bacteria. This can be achieved through

the use of selective media such as the use of de Man, Rogosa and Sharpe (MRS) agar for the selection of *Lactobacilli* (De Man *et al.* 1960). MRS agar has been used in order to recover *Lactobacillus rhamnosus* from human colonic biopsy samples (Alander *et al.* 1997). Specific media formulations also allow for functional screening of the gut microbiota such as cholesterol degraders and bile salt hydrolase (BSH) tolerant organisms (Pereira and Gibson 2002). Culture dependent methods can also be used in order to test for the genotoxicity of faecal water, that is to say the ability of faecal water to induce DNA damage. The genotoxicity of faecal water can be measured through the incubation of faecal water with a test organism (Venitt and Bosworth 1986).

1.2.2 The use of culture independent methods in order to analyse the gut microbiota and functions

1.2.2.1 The use of 16S *rRNA* as a target in bacterial communities

The use of the 16S *rRNA* as a target for phylogenetic analysis was pioneered by Carl Woese and was used, amongst other *rRNA* targets, in his construction of the three domains of life, namely the Bacteria, Archaea and Eucarya (Woese *et al.* 1990). The 16S *rRNA* genes are still a target for many molecular microbiologists today. The reasons for their use have been previously discussed (Janda and Abbott 2007). 16S *rRNA* genes are essential housekeeping genes and are therefore ubiquitous amongst bacteria. The genes according to Janda and colleagues are also big enough for informatics use. Following the elucidation of its secondary structure in 1980 (Woese *et al.* 1980) it was determined that there are conserved and hypervariable regions, V1-V9. Through designing various PCR primers these variable regions, with flanking conserved regions can be targeted and phylogenetic information can be gleaned (Chakravorty *et al.* 2007). Due to the variable nature of these regions it can be difficult to design primers which can target all bacteria equally. This problem has been recently discussed (Pinto and Raskin 2012) where biases in multi-template 16S *rRNA* PCR affected the relative numbers of bacteria detected through pyrosequencing. This is of particular concern as many researchers focus on the relative abundance of specific bacterial taxa whilst analysing their datasets.

1.2.2.2 Community profiling of faecal DNA

Community profiling of the gut microbiota can be carried out using denaturing gradient gel electrophoresis (DGGE) of the 16S *rRNA* genes obtained from faecal DNA (Simpson *et al.* 2000), terminal fragment length polymorphism (t-RFLP) analysis of faecal DNA (Dicksved *et al.* 2007) or length heterogeneity PCR (LHPCR) of 16S *rRNA* genes obtained from faecal DNA (Bjerketorp *et al.*

2008). These methods are relatively high-throughput and inexpensive to carry out and are appropriate for use in the PROHEMI study, due to their previous published use with faecal DNA. With regards to DGGE and LHPCR the primers used in order to amplify the 16S *rRNA* and the products generated are different and this arises from how the products are treated. In their analysis Simpson and colleagues (Simpson *et al.* 2000) used ~200 bp products to run on denaturing gradient gels for their DGGE analysis. In contrast, LHPCR utilises a labelled forward primer and the use of a capillary sequencer in order to determine the length of fragments generated. The primers used are also varied with some researchers generating ~350 bp (Bjerketorp *et al.* 2008) LHPCR products and others generating ~530 bp products (Eusebio *et al.* 2011).

1.2.2.3 NGS and metagenomics in order to determine bacterial community structure and functions

However, recently more emphasis is being placed upon characterisation of complex communities, such as the gut microbiome, through NGS. This is due to its high throughput nature, vast reduction in cost and volume of information generated. Technologies, such as 454 pyrosequencing, have been applied to the gut microbiome. NGS of amplified 16S *rRNA* genes from faecal DNA were used in order to assess the composition of the core gut microbiome in obese and lean twins (Turnbaugh *et al.* 2009). NGS technologies are constantly evolving. The use of new sequencing platforms such as the Illumina HiSeq and MiSeq have been successfully applied to sequence 16S *rRNA* genes obtained from DNA from a range of sources. These sources include the soil, mouth, skin and more importantly human faecal material (Caporaso *et al.* 2012). It is also possible to predict the functions, such as butyrate synthesis or methanogenesis, present in a community from sequenced 16S *rRNA* genes. This information, obtained from the community in question, can be analysed using software called PICRUSt (Langille *et al.* 2013). The software utilises databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database to assign functions for a given 16S *rRNA* gene sequence. Through this the functions present within the community as a whole may be inferred. While this software infers functions within communities it is possible to interrogate the functions present within a community through metagenomics and metatranscriptomics. Metagenomic approaches have been successfully applied to the human gut through the NGS of a metagenomic library. Libraries are constructed from human faecal DNA (Qin *et al.* 2010) and provide a wealth of information on the genes present within the community. However, metagenomic libraries generated from DNA do not tell us whether the genes are actually expressed within the community under investigation. It

is also possible to combine culture independent and dependent approaches in order to answer questions about the community we are analysing. This can be achieved through functional metagenomics. Metagenomic libraries are created in a host organism such as *Escherichia coli* or *Bacillus subtilis*. Then screening for a function of interest, for example the screening of protease activity (Morris *et al.* 2012), from a metagenomic library can be carried out. However, metagenomic screening does have its drawbacks, such as problems with expression of the gene of interest (de Lorenzo 2005).

1.3 Probiotic supplementation

The definition of a probiotic has long been disputed with Schrezenmeir and de Vrese arguing for the adoption of the following definition: "a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonisation) in a compartment of the host and by that exert beneficial health effects in this host" (Schrezenmeir and de Vrese 2001). This wholly encompassing definition has since been simplified by the World Health Organisation (WHO) and is generally regarded as the definition of a probiotic where probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (WHO 2002). The probiotic market itself is rather lucrative with an estimated market share of \$24.23 billion in 2011 (Markets and Markets 2013). Much of the revenue generated is due to the health claims which surround these products. These health claims are now coming under scrutiny by bodies such as the European Food Safety Authority (EFSA). In order to ensure that consumers were not falling prey to smart marketing, where there was little or no evidence of a health benefit from a specific probiotic preparation, health claims were submitted to the EFSA. These health claims were then reviewed by a panel. Of 143 health claims submitted for probiotic products none were found to be substantiated by the EFSA (Guarner *et al.* 2011). While protecting consumers from unsubstantiated health claims the authors expressed concern that products which did have scientific proof of efficacy were being rejected due to too stringent guidelines. In Japan the Labelling Regulation for Foods for Specified Health Use (FOSHU) decree has been passed. Industrial producers of probiotic products can voluntarily submit evidence of health benefits in order to advertise their claims on the products' labels (Rousseaux *et al.* 2007).

Probiotic supplementation has been and is being researched in healthy people and in the infirm. Although logic would drive us to assume that research is being directed at their effects solely within the gastro-intestinal tract (GIT) this is not the case. It is becoming increasingly apparent that the gut microbiota extends an influence on the host far beyond its niche; positively and negatively affecting host health and well-being (Sekirov 2010) (**Fig. 1.1**). All probiotic organisms destined for human consumption have been isolated from humans and re-administered. The majority of probiotic organisms used are bacteria, including members of the lactic acid bacteria (LAB) family such as *Lactobacillus acidophilus*, *Actinobacteria* such as *Bifidobacterium breve*, even the archetypical gut commensal *E. coli*. However, the yeast *Saccharomyces boulardii* has also been used. The choice of probiotic organism used is often driven by cultural and geographical reasons. For example, the use of a *Weisella* strain after isolation from fermented foods in Nigeria. LAB strains have also been used due to their isolation from fermented milk in Africa and Mongolia and after isolation from the fermented yak milk derived Kurut in China (Fontana *et al.* 2013). The safety of a probiotic organism is of utmost concern as these are usually fed at high numbers and in many instances to those who are unwell. There has been some concern surrounding the use of *Lactobacillus rhamnosus* GG especially in the infirm, as cases of bacteraemia have been reported. In an infant that received heart surgery, broad spectrum antibiotics were administered. Upon development of diarrhoea supplementation with *L. rhamnosus* GG (1×10^{10} cells per day) was carried out. This patient developed fever, an increase in leukocytes and was blood culture positive for *Lactobacillus*. Probiotic treatment was halted and the patient showed improvement in the 48 to 72 hours following cessation of probiotic treatment (Land *et al.* 2005). The authors also highlighted another case where a 6 year old presenting with a urinary tract infection was treated with many courses of antibiotics and received *L. rhamnosus* GG (1×10^{10} cells per day). This was in an effort to combat her perceived antibiotic associated diarrhoea. The girl showed symptoms of fever and was blood culture positive for *Lactobacillus*; probiotic treatment was halted and intravenous administration of ampicillin was introduced for 10 days. The patient showed no symptoms of fever after 4 days of antibiotic treatment and was culture negative for *Lactobacillus*. Bacteraemia was also seen in two patients with short gut syndrome. Blood cultures from these patients tested positive for *Lactobacillus* following treatment with *L. rhamnosus* GG (Kunz *et al.* 2004) *Lactobacillus* bacteraemia was also observed in a paediatric ulcerative colitis (UC) patient (Vahabnezhad *et al.* 2013).

The probiotic yeast *Saccharomyces boulardii* has also been implicated in fungemia. Four cases of this were observed in vascular catheterised patients. It was thought that the opening of packets containing the probiotic contaminated the catheters, which in turn acted as a source for the fungemia (Hennequin *et al.* 2000). Fungemia caused by *S. boulardii* supplementation was also seen in seven critically ill patients, 3 of whom were immunocompromised. All patients had central venous catheters and all patients were undergoing broad spectrum antibiotic therapy (Lherm *et al.* 2002). The probiotic was administered in an effort to reduce antibiotic associated diarrhoea, but instead was linked with fungemia with the exact mechanism unknown. However, the authors suggest that central venous catheter contamination may have occurred, in the same manner as previously described by Hennequin and colleagues. It has also been suggested that overgrowth due to broad spectrum antibiotic therapy, especially in the immunocompromised, was another mechanism for the development of the fungemia. In all of the aforementioned cases the patients had underlying health problems. It is therefore imperative that the safety testing of a specific probiotic strain is carried out prior to mass administration in these ill patients.

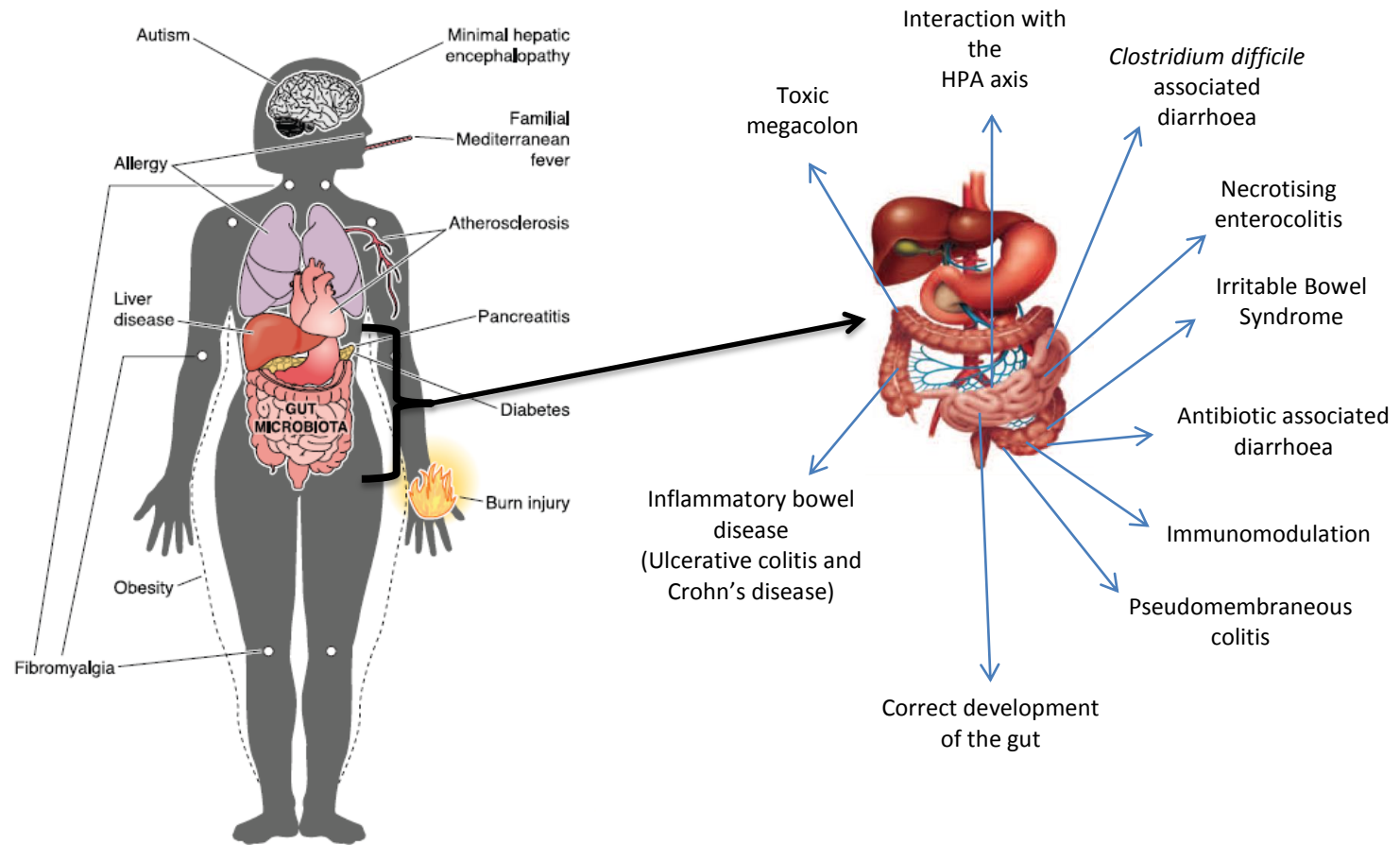


Figure 1.1| The impact of the gut microbiota on the gut and beyond – the figure has been adapted from (Sekirov 2010) and (Culligan *et al.* 2012).

1.3.1 The use of probiotic supplements in unhealthy individuals

The literature on the use of probiotic supplementation is growing. There are studies providing evidence for symptom and disease management in the infirm. Equally studies show little to no effect in some instances.

Many studies focus on the use of probiotic supplements as treatments or adjuvants to current therapies for disorders of the GIT, which is unsurprising due to its microbial load. The positive effects of some probiotic species are shown (**Table 1.1-1.3**) and have been discussed.

There is an abundance of research showing the positive effects that probiotic supplementation can exhibit in the infirm. However, there are also studies which show that supplementation exerts no benefits, and in extreme cases can cause worsening of the disease (**Table 1.4 and 1.5**).

Table 1.1| The positive effects of probiotic supplementation in the infirm

Ailment	Probiotic preparation	Dose	Period of intervention	Effect	Reference
IBS	<i>B. infantis</i> 35624	1 x 10 ¹⁰ /day	8 weeks	Normalisation of IL-10 levels	{O'Mahony, 2005 #540 }
	<i>B. breve</i> Bb99, <i>L. rhamnosus</i> GG, <i>L. rhamnosus</i> LC705 and <i>P. freudenreichii</i> ssp. <i>Shermanii</i> JS	1 x 10 ⁸ CFU/mL	4 weeks	Reduction in pain and scores for bloating, bowel dysfunction, incomplete evacuation, straining and passage of gas	(Whorwell <i>et al.</i> 2006)
		8-9 CFU/day	6 months	Reduction in symptom score*	(Kajander <i>et al.</i> 2005)
IBD	<i>B. longum</i> + FOS + Inulin	2 x 10 ¹¹ viable cells + 6 g twice daily	4 weeks	Reduction in TNF- α and IL-1 α *	(Furrie <i>et al.</i> 2005)
	<i>S. boulardii</i>	250 mg probiotic capsule alongside 1 g mesalazine treatment	4 weeks	Clinical remission Reduction in clinical score index*	(Guslandi <i>et al.</i> 2003)
	<i>E. coli</i> Nissle 1917	three times daily 200 mg capsule	12 months	Probiotic maintained remission as effective as mesalazine treatment	(Kruis <i>et al.</i> 2004)

*marks a significant effect ($p < 0.05$) when compared with the placebo group

Table 1.2| Continued from Table 1. 1

Ailment	Probiotic preparation	Dose	Period of intervention	Effect	Reference
Pouchitis	VSL#3	9 x 10 ¹¹ CFU/day	1 year	Lower occurrence *	(Gionchetti <i>et al.</i> 2003)
				Maintenance of antibiotic induced remission**	(Mimura <i>et al.</i> 2004)
AAD	<i>L. casei</i> , <i>L. bulgaricus</i> and <i>S. thermophilus</i>	1 x 10 ⁸ , 1 x 10 ⁷ and 1 x 10 ⁸ CFU/ml respectively twice daily	During one week of antibiotic therapy and one week after	Reduced incidence*	(Hickson <i>et al.</i> 2007)
	VSL#3	90 x 10 ¹⁰ /day	During the course of antibiotic therapy and one week after	Reduced incidence*	(Selinger <i>et al.</i> 2013)
CDAD	<i>L. casei</i> , <i>L. bulgaricus</i> and <i>S. thermophilus</i>	1 x 10 ⁸ , 1 x 10 ⁷ and 1 x 10 ⁸ CFU/ml respectively twice daily	During one week of antibiotic therapy and one week after	Reduced incidence*	(Hickson <i>et al.</i> 2007)
Liver transplant	<i>P. pentosaceus</i> , <i>Leu. paracasei</i> , <i>L. paracasei</i> and <i>L. plantarum</i> , betaglucan, inulin, pectin and resistant starch	1 x 10 ¹⁰ viable cells with 2.5 g each of betaglucan, inulin , pectin and resistant starch twice daily	Started on the day of liver transplant and continued for 14 days after	Shorter period of antibiotic therapy required Reduced incidence of bacterial infections*	(Rayes <i>et al.</i> 2005)

*marks a significant effect (p<0.05) and ** marks a highly significant effect when compared with the placebo group

Table 1.3| Continued from Table 1. 1

Ailment	Probiotic preparation	Dose	Period of intervention	Effect	Reference
NEC	<i>L. acidophilus</i> and <i>B. infantis</i>	25 mg/kg dose twice daily	Until discharged	Reduced incidence*	(Lin <i>et al.</i> 2005)
	<i>B. infantis</i> , <i>S. thermophilus</i> and <i>B. bifidus</i>	0.35 x 10 ⁹ CFU/day	Continued until VLBW infants reached 36 weeks post-conceptual age	Reduced incidence* Reduced incidence of clinically significant NEC	(Bin-Nun <i>et al.</i> 2005)
AE	<i>L. rhamnosus</i> and <i>L. reuteri</i>	1 x 10 ¹⁰ CFU twice daily	6 weeks	Reduced severity*	(Rosenfeldt <i>et al.</i> 2003)
	<i>L. rhamnosus</i> GG, <i>L. rhamnosus</i> LC705, <i>B. breve</i> Bb99 and <i>P. freudenreichii</i> ssp, <i>shermanii</i> JS + galacto-oligosaccharides in new-borns	5 x 10 ⁹ , 5 x 10 ⁹ , 2 x 10 ⁸ and 2 x 10 ⁹ CFU respectively twice daily. New-borns also received 0.8 g galacto-oligosaccharides	Given to mother's at 35 weeks of gestational age and continued in new-borns for 6 months	Reduced incidence	(Kukkonen <i>et al.</i> 2007)

*marks a significant effect ($p < 0.05$) when compared with the placebo group

Table 1.4| The negative and no effects of probiotic supplementation in the infirm

Ailment	Probiotic preparation	Dose	Period of intervention	Effect	Reference
Pancreatitis	<i>L. casei</i> , <i>L. salivarius</i> , <i>L. lactis</i> , <i>B. bifidum</i> , <i>B. lactis</i>	1 x 10 ¹⁰ bacteria/day	Maximum of 28 days	No significant difference in primary end-point†. More deaths*. Increase incidence of bowel ischemia*.	(Besselink <i>et al.</i> 2008)
IBS	VSL#3	9 x 10 ¹¹ bacteria/day	8 weeks	No significant difference in GIT transit time, relief of symptoms, stool frequency, consistency or ease of passage†	(Kim <i>et al.</i> 2003)
IBD	<i>L. rhamnosus</i> GG	1 x 10 ⁹ CFU twice daily	6 months following antibiotic induced remission	No significant difference in antibiotic induced CD remission†	(Schultz <i>et al.</i> 2004)
	<i>L. johnsonii</i> LA1	1 x 10 ⁹ CFU twice daily	6 months	No significant difference in recurrence rates†	(Marteau <i>et al.</i> 2006)

†when compared with the placebo group

* marks a significant effect (p<0.05) when compared with the placebo group

Table 1.5| Continued from Table 2. 1

Ailment	Probiotic preparation	Dose	Period of intervention	Effect	Reference
Pouchitis	<i>L. rhamnosus</i> GG	0.5-1.0 x 10 ¹⁰ CFU four times daily	3 months	No significant difference in PDAI scores†	(Kuisma <i>et al.</i> 2003)
AE	<i>L. acidophilus</i> LAVRI-A1	3 x 10 ⁹ bacteria/day	6 months	No significant difference in rate of AE†. Increased rate of sensitisation to allergens*.	(Taylor <i>et al.</i> 2007)
	<i>L. rhamnosus</i> GG	5 x 10 ⁹ CFU twice daily	4-6 weeks before expected birth and 6 months following birth	No significant difference in episodes of fever, airway infections, IgE levels nor sensitisation to inhalant allergen†. Increased frequency of recurrent wheezing bronchitis*.	(Kopp <i>et al.</i> 2008)

†when compared with the placebo group

* marks a significant effect (p<0.05) when compared with the placebo group

1.3.1.1 The positive effects of probiotic supplementation in irritable bowel syndrome

Irritable bowel syndrome (IBS), "comprises a group of functional bowel disorders in which abdominal discomfort or pain is associated with defecation or a change in bowel habit, and with features of disordered defecation," (Thompson *et al.* 1999). IBS is thought to affect around 10-20% of adults and adolescents worldwide (Longstreth *et al.* 2006). The exact cause of IBS is unknown and is thought to be multi-factorial with genetic factors, motor dysfunction of the GIT, visceral hypersensitivity, infection, inflammation, immunity as well as psychopathological factors playing roles (Quigley 2003). While most therapies are aimed at reducing patient discomfort, studies are now researching the impact of probiotic supplementation as treatment for the syndrome. The rationales behind their use are the potential of probiotic organisms to produce antibacterial compounds such as bacteriocins (Corr *et al.* 2007), exertion of immunomodulatory effects, improvement of epithelial function and reduction of gut leakiness through tight junction strengthening (Corr *et al.* 2009). The efficacy of *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC4331 in the treatment of IBS have been compared (O'Mahony *et al.* 2005). IBS sufferers consumed a daily malted drink containing either 1×10^{10} viable cells of *B. infantis* or *L. salivarius* for an 8 week period; the malted milk drink alone served as a placebo. Symptom scores were taken for each patient throughout the study with *B. infantis* showing the greatest therapeutic response. Measurements of cytokine release from peripheral blood mononuclear cells were also taken. The authors studied the ratio of Interleukin (IL)-10 to IL-12 in IBS sufferers ($n=69 \pm 15$) and healthy volunteers ($n=176 \pm 31$); the ratio of IL-10 to IL-12 significantly differed between the two groups ($p=0.003$). IBS sufferers had lower levels of IL-10 which, according to the authors, suggests a T helper cell type 1 (Th1) proinflammatory state. However, supplementation with *B. infantis* normalised IL-10 levels with pre-feeding and feeding IL-10 levels differing significantly ($p=0.001$). This suggests that *B. infantis* 35624 can exert anti-inflammatory effects through increasing IL-10 levels in IBS patients. Daily supplementation of *B. infantis* 35624 at 1×10^8 colony forming units (CFU)/mL also alleviated IBS symptoms in women when compared to a placebo taking group (Whorwell *et al.* 2006). Single strains, such as *B. infantis* 35624 are not the only probiotic preparations which have been studied for their effects on IBS sufferers. The mixed preparation containing $8-9 \times 10^9$ CFU/day of *B. breve* Bb99, *L. rhamnosus* GG, *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS in equal amounts was taken daily by IBS sufferers ($n=52$). Sufferers were compared to a placebo group ($n=51$) in a 6 month trial (Kajander *et al.* 2005). The probiotic supplemented group saw a significant total symptom score reduction ($p=0.037$) when compared to the placebo group. It is difficult to assign specific reasons as to why

probiotic organisms alleviate IBS symptoms; we may be seeing a multifactorial response in a multifactorial syndrome.

1.3.1.2 The positive effects of probiotic supplementation inflammatory bowel disease

Inflammatory bowel disease (IBD) is caused by the ongoing activation of mucosal immune responses by our normal commensal gut microbiota at an inappropriate level. It is driven by impaired barrier function at the intestinal mucosal surface and over-reaction of its associated immune system (Podolsky 2002). It has been argued that two disease states exist: UC and Crohn's disease (CD). However, Podolsky highlights that confusion remains in the literature, are these two separate disease states or do these diseases exist on a continuum (Podolsky 2002)? It has been argued that genetic factors play a crucial role in the manifestation and progression of IBD e.g. IL-33 (part of the IL-1 family) and Interleukin receptor like 1 (ILRL1) polymorphism (Latiano *et al.* 2013). Environmental factors such as diet and hygiene have also been implicated (Ng *et al.* 2013). Treatment of these diseases has historically centred on the use of corticosteroids. More recently inhibitors of specific pro-inflammatory factors within the human body, such as the use of infliximab a monoclonal antibody which binds tumour necrosis factor α (TNF- α), have been used (Rutgeerts *et al.* 2004). Surgical intervention is sometimes required in extreme cases. Due to the proposed commensal driven dysregulation of immune responses at the gut epithelial surface, it follows that modulation of this community could provide an alternative therapy for IBD. Probiotics have once again been used for this purpose with studies determining their effect in IBD patients alongside prebiotic supplementation. Prebiotics, as defined by Gibson and colleagues (Gibson and Roberfroid 1995), are “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon”. The synbiotic (probiotic and prebiotic) mixture of *B. longum*, at a concentration of 2×10^{11} viable cells, and a 6 g sachet of the prebiotics fructo-oligosaccharides (FOS) and inulin were fed to active UC patients twice daily for four weeks and compared to a placebo group (Furrie *et al.* 2005). The synbiotic group saw a significant reduction in the levels of pro-inflammatory TNF- α ($p=0.0177$) and IL-1 α ($p=0.0051$). This shows the possible anti-inflammatory effects exerted by the probiotic/prebiotic synergistic mixture.

The probiotic yeast *S. boulardii* has also been assessed as a treatment for UC. In one study a 250 mg capsule of the organism was fed 3 times daily alongside standard anti-inflammatory mesalazine treatment (1 g, 3 times daily) for 4 weeks. The results showed clinical remission in all cases, as confirmed through sigmoidoscopy, a surgical procedure involving inspecting the bowels with a sigmoidoscope. The disease, as with many other diseases, is measured through a clinical index score, whereby symptoms are measured in terms of severity and are scored. Combinatorial UC therapy with mesalazine and *S. boulardii* also gave rise to a significant reduction in clinical index score ($p < 0.05$) and a deemed successful clinical index score of 5 or less in 68% of cases (Guslandi *et al.* 2003). The study used a probiotic organism in conjunction with traditional therapy. However, it has been shown that once UC remission has been achieved *E. coli* Nissle 1917 alone is as effective as mesalazine in maintaining remission (Kruis *et al.* 2004). The probiotic group ($n=110$) and mesalazine placebo group ($n=112$) showed significant equivalence ($p=0.003$) in patient relapse, 36.4% and 33.9% respectively. Probiotic supplementation has also been used in order to treat CD. Active CD patients who failed to reach remission through standard treatment were fed a synbiotic mixture of *B. breve* (3×10^{10} CFU), *Lactobacillus casei* (3×10^{10} CFU), *B. longum* (1.5×10^{10} CFU) daily in conjunction with 3 daily 3.3 g doses of the prebiotic plant psyllium. Crohn's disease activity index (CDAI) scores for these patients were significantly lower after therapy ($p=0.009$) (Fujimori *et al.* 2007).

The aetiology and development of IBD makes researching how probiotic supplementation alleviates this condition difficult. It has been suggested that probiotics exert anti-inflammatory effects, and through this drives alleviation of symptoms. Furthermore, a mechanism for how these anti-inflammatory effects arise has been suggested. Fernandez and colleagues propose that the cell-wall components of probiotic bacteria exert anti-inflammatory effects. Specifically muropeptides, arising during the degradation of peptidoglycan, interact with nucleotide-binding oligomerisation domain 2 (NOD2) in the cytosol of the host through a specific NOD2 ligand. This is associated with a significant down-regulation of pro-inflammatory genes (Macho Fernandez *et al.* 2011). The authors also suggest that probiotic bacteria and cell-wall components can be internalised through phagocytosis. Internalised cell wall components interact with toll-like receptors (TLR) and recruit myeloid differentiation primary response 88 (MyD88) giving rise to anti-inflammatory responses. This interaction was highlighted in MyD88 deficient mice where peptidoglycan lost the ability to protect against colitis.

1.3.1.3 The positive effects of probiotic supplementation in pouchitis

UC cases can become severe, requiring surgical intervention in the form of a colectomy. Since 1977 postcolectomy ileal pouch-anal anastomosis has been routinely carried out, in an attempt to restore function lost through colectomy (McGuire *et al.* 2007). Inflammation events can occur within the ileal pouch reservoir giving rise to pouchitis. Traditional therapy makes use of anti-inflammatory compounds. However, there has been much focus on the use of probiotic organisms as an alternative treatment and preventative of pouchitis. The multi-species (*B. breve*, *B. longum*, *B. infantis*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, *L. delbrueckii ssp. bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* at 9×10^{11} viable cells/day) probiotic VSL#3 has been researched in this respect. Following ileal pouch-anal anastomosis patients either received the probiotic mixture (n=20) or a placebo (n=20) for one year. The number of acute episodes of pouchitis was lower in the probiotic group than the placebo group, 10% and 40% respectively. There was also an observable difference in the number of relapses with 90% of the probiotic group still in remission one year later compared to 60% of patients in the placebo group (Gionchetti *et al.* 2003). In a follow-up study the number of patients in antibiotic induced remission, after one year, was significantly higher in the VSL#3 group than the placebo group (85% vs. 6% respectively, $p < 0.0001$) (Mimura *et al.* 2004).

1.3.1.4 The positive effects of probiotic supplementation in antibiotic associated diarrhoea, *Clostridium difficile* associated diarrhoea and bacterial infections following operative procedures

Treatment of bacterial infections often leads to the unwanted side-effect of antibiotic associated diarrhoea (AAD). This arises from the perturbation caused by consumption of a broad range antibiotic. In turn this leads to diarrhoea, as the commensal gut bacteria are affected. The incidence of antibiotic diarrhoea is estimated at 5-39%; lengthening hospital stays and therefore, increases costs (Vidlock and Cremonini 2012). AAD should not be confused with *Clostridium difficile* associated diarrhoea (CDAD). In this instance administration of a broad spectrum antibiotic allows the overgrowth of *C. difficile* due to reduction of competing gut commensal bacteria. CDAD cases have increased each year since 2000 in North American and European hospitals (Balassiano *et al.* 2012). *C. difficile* infection can give rise to a range of disease states from CDAD through to pseudomembranous colitis and toxic megacolon (Balassiano *et al.* 2012). The two latter diseases have the potential to be life threatening. The twice daily consumption of a probiotic drink containing *L. casei* (1×10^8 CFU/mL), *L. bulgaricus* (1×10^7 CFU/mL) and *S.*

thermophilus (1×10^8 CFU/mL) throughout a week's course of antibiotics, and for one week after course completion, has been shown to decrease the incidence of AAD and CDAD (Hickson *et al.* 2007). The probiotic group (n=57) showed significantly less cases of AAD than the placebo group (n=56), 12% vs. 34% respectively ($p=0.007$). There was an absolute risk reduction of 22% by taking the probiotic mixture. This study also showed a significant reduction in the incidence of CDAD ($p=0.001$) with no cases in the probiotic group and 9 cases in the placebo group. AAD incidence has also been shown to decrease upon twice daily administration of the previously described probiotic mixture VSL#3 during antibiotic therapy, and a week thereafter (Selinger *et al.* 2013). Per-protocol analysis showed a significant reduction of AAD incidence in the probiotic taking group, 0 cases vs. 7 cases in the placebo group ($p=0.006$). Meta-analysis of the literature has also shown that the feeding of probiotics can reduce the relative risk of AAD. McFarland has shown that out of 25 clinical trials probiotic supplementation significantly reduced the relative risk (RR) of AAD (RR = 0.43, $p<0.001$). The research highlighted a significant reduction in the incidence of AAD in 13 trials (52%) when probiotic supplements were administered (McFarland 2006). However, the remaining 12 trials (48%) showed no difference between the probiotic and placebo groups. The author suggests that the ambiguity surrounding the efficacy of probiotic supplementation in reducing AAD incidence could be due to many factors. These include the range of demographics enrolled in different studies, the probiotic used (species, mixture of two or a synbiotic mixture) and also the dose used. It is interesting to note that a high dose of probiotic ($\geq 10^{10}$ cells/day) exhibited significant efficacy against AAD development in 67% of the positive trials. In a more recent meta-analysis, 20 of the aforementioned 25 studies were analysed with an additional 14 new studies (Vidlock and Cremonini 2012). This study showed a reduced relative risk (RR=0.53) in the probiotic supplemented group. Synbiotic supplementation has also been shown to decrease the length of post-operative antibiotic administration in liver transplant patients, as well as the rate of bacterial infection (Rayes *et al.* 2005). These patients received either the synbiotic mixture (*Pediococcus pentosaceus*, *Leuconostoc paracasei*, *L. paracasei* and *L. plantarum* at 10^{10} viable cells with the 4 prebiotics betaglucan, inulin, pectin and resistant starch at 2.5 g each) twice daily, or a twice daily dose of just the 4 prebiotics (control). The synbiotic group (n=33) required a significantly shorter period of antibiotic therapy than the control group (n=33), 0.1 ± 0.1 vs. 3.8 ± 0.9 days respectively ($p<0.05$) and had significantly less bacterial infections, 3% vs. 16% respectively ($p<0.05$).

1.3.1.5 The positive effects of probiotic supplementation in necrotising enterocolitis

Necrotising enterocolitis (NEC) is a poorly understood disease which primarily affects premature very low birth weight (VLBW) infants. The disease is associated with high mortality rates ranging from 10-50% (Henry and Moss 2009). The exact mechanisms of the disease remain unclear. However, it has been suggested that the disease is multifactorial with inflammation and bacterial overgrowth playing roles (Ballance *et al.* 1990). Often surgical intervention is used as treatment for the disease. It has been argued that the gut bacterial make up of NEC infants is different to that of healthy infants. Using sequencing approaches it has been shown that NEC infants have a significantly lower number of operational taxonomic units (OTU)s than healthy infant controls (10.4±6.1 vs. 19±6.7 respectively, $p=0.008$), indicating a lower level of diversity (Wang *et al.* 2009). This study also showed that the relative abundance of *Proteobacteria* compared to other phyla was significantly higher in the NEC infant group ($p<0.01$). This suggests that the gut bacterial composition of NEC infants is different from healthy infants.

Conversely however, in a more recent study using 16S *rRNA* gene 454 pyrosequencing of faecal samples, NEC infants ($n=10$) were compared to control infants ($n=10$) over 7 weeks. No significant difference in the gut bacterial community of NEC infants was observed (Normann *et al.* 2013). While there were no differences between the two groups overall there were observable, albeit non-significant, differences between the two groups during the first week. The samples of NEC infants during the 1st week were dominated by *Enterobacteriaceae* and *Bacillales*, while *Enterococcus* was more abundant in the samples from healthy individuals. While these differences were non-significant, the authors suggest that differences in the gut bacterial community of preterm babies could play a pivotal role in the onset and progression of this disease. It therefore follows that modulation of the gut bacterial community in VLBW infants could provide a normalising effect. To this end the efficacy of probiotic supplementation as a prophylactic treatment for NEC development is now being researched. Supplementation of the probiotic organisms *L. acidophilus* and *B. infantis* in VLBW infants has been carried out (Lin *et al.* 2005). The probiotic group ($n=180$) showed a significantly lower NEC incidence than the control group ($n=187$), 1.1% vs. 5.3% ($p=0.04$). When data for the incidences of death, NEC or sepsis were combined there was a significant difference between the probiotic treatment group and control (17.2% vs. 32.1% respectively, $p<0.009$). The authors suggest that feeding probiotic organisms to VLBW infants may provide competition against pathogens at the epithelial surface. This could also give rise to an increased barrier to translocation of bacteria and their products across the

epithelial surface. In another study the feeding of a different set of probiotic organisms (*B. infantis*, *S. thermophilus* and *Bifidobacterium bifidus* all at 0.35×10^9 CFU/day) alongside either mother's or formula milk also showed a significantly reduced NEC incidence, at 4% in the active group vs. 16.4% in the control group ($p=0.03$) (Bin-Nun *et al.* 2005). It was shown that only 1% of NEC cases were clinically significant in the probiotic group compared to 14% of cases in the placebo group. This was a statistically significant difference ($p=0.013$), suggesting that if probiotic administration fails to protect against the onset of NEC it can decrease the severity. This study is in concurrence with the previously mentioned study by Lin and co-workers. The authors of this study suggest that probiotics exert their protective effects through strengthening intestinal epithelial barrier function. The authors also suggest that bacteriocin production could play a crucial role in bacterial exclusion at the mucosal surface.

Meta-analysis of the literature on the effect of probiotic administration on NEC in in preterm VLBW infants has been carried out by Deshpande and colleagues (Deshpande *et al.* 2010). Probiotic organisms administered in the 11 studies used for meta-analysis included LAB, *Bifidobacteria*, and *S. boulardii* with the majority being administered at 10^9 CFU/day. The authors show that a higher proportion of VLBW infants developed NEC in the placebo group ($n=1082$) than the probiotic group ($n=1094$), 6.56% vs. 2.37% respectively. In addition there was a significantly lower risk of developing NEC in the probiotic group, with a relative risk of 0.35 ($p<0.0001$). Probiotic supplementation showed a reduced risk of mortality versus the control group with a relative risk of 0.42 ($p<0.00001$). In their conclusions Deshpande and colleagues highlight that parents, in light of the favourable evidence, would be unlikely to take a chance with their infant's health and would opt for probiotic treatment instead of taking part in a clinical trial.

1.3.1.6 The positive effects of probiotic supplementation in atopic eczema

Interactions within the gut can have implications far beyond its locality. Atopic eczema (AE), an inflammatory skin condition, was shown to affect 20% of children under 12 months in England in Wales during 2006 (Schofield *et al.* 2011). The ailment cost an estimated £465 million to treat in 1996 (Ellis *et al.* 2002). In a review of the literature by Williams and Grindlay the causes of AE were suggested as being method of birth (vaginal vs. caesarean) and mutations in the gene filaggrin (Williams and Grindlay 2010). The influence of the gut and its resident microbiota were overlooked in this instance. Characterisation of the gut microbiota of AE sufferers has been carried out in a recent study (Abrahamsson *et al.* 2012). Next generation sequencing of the 16S *rRNA* gene amplified from faecal sample DNA from infants with atopic eczema and healthy controls has shown differences in their associated microbiota. Shannon diversity indices show that AE infants have a significantly lower total diversity ($p=0.04$) at one month. Furthermore, there is significantly lower diversity of *Bacteroidetes* and specifically *Bacteroides* spp. within the phylum ($p=0.02$ and $p=0.01$ respectively) at one month. The study also highlighted lower diversity of *Proteobacteria* and *Bacteroidetes* at 12 months in the AE group ($p=0.02$ and $p=0.08$ respectively). The results suggest that AE sufferers have lower bacterial diversity than healthy controls. The authors have also shown a lower abundance of *Proteobacteria* in infants presenting with AE and highlight that the cell walls of the bacteria in this phylum contain lipopolysaccharide (LPS) which has the ability to elicit an immune response, in particular the response of Th1. The authors highlight the findings of another study (Gehring *et al.* 2001) in which low exposure to LPS in infancy was linked with a higher risk of AE. The lower level of LPS coupled with low bacterial diversity could possibly play a role in the development of the disease through poor stimulation of the immune system. However, it must be noted that the effect of the gut microbiota on the incidence and progression of AE is difficult to elucidate and indeed is likely to be multifactorial.

Considering that there is a link between our gut microbiota and the incidence of AE, it follows that the efficacy of probiotic supplementation as a preventative measure and also as a treatment for AE has been researched. In a cross over study it has been shown that twice daily administration of *L. rhamnosus* and *Lactobacillus reuteri* significantly ($p=0.001$) reduced the severity of the eczema in the patients' opinion. Following treatment 56% of active patients ($n=39$) believed that their eczema severity had decreased vs. 15% in the placebo group ($n=39$) (Rosenfeldt *et al.* 2003). In another study, expectant mothers of children at high-risk of developing immunoglobulin E (IgE) associated atopic diseases were randomised to a placebo

group or active group at 35 gestational weeks (Kukkonen *et al.* 2007). The active probiotic group received a twice daily capsule containing *L. rhamnosus* GG (5×10^9 CFU), *L. rhamnosus* LC705 (5×10^9 CFU), *B. breve* Bb99 (2×10^8 CFU) and *P. freudenreichii* ssp. *shermanii* JS (2×10^9 CFU) up until delivery. Supplementation was continued after birth where a single capsule was opened and mixed with 20 drops of sugar syrup and 0.8g of galacto-oligosaccharides (prebiotic), and fed daily to the new-borns for 6 months after birth. The placebo group (expectant mothers) received twice daily capsules containing micro-crystalline cellulose while the new-borns received a single open placebo capsule mixed with 20 drops of sugar but no prebiotic. Probiotic treatment reduced the incidence of atopic eczema by 34% when compared to the placebo group. In a meta-analysis of the literature it was shown that probiotics given in a pre and/or post-natal manner reduced the risk of prolonged AE as much as 61% (Lee *et al.* 2008). The analysis showed that probiotic supplementation is effective as a preventative measure, but showed no statistical significance as a treatment.

1.3.1.7 The negative effects of probiotic supplementation in pancreatitis

Inflammation of the pancreas, pancreatitis, is characterised by 3 phases which include: activation of trypsin, induction of inflammatory pathways giving rise to inflammation of the pancreas and inflammation that is not localised to the pancreas (Banks and Freeman 2006). It is estimated that there are 210,000 admissions of acute pancreatitis each year in the US (Banks and Freeman 2006). In this disease pancreatic tissue can become necrotised, due to translocation of intestinal bacteria as a result of the pro-inflammatory response arising from this disease. This in turn gives rise to infectious complications within this tissue (Besselink *et al.* 2008). Probiotic supplementation has been investigated as a prophylactic treatment for patients who were predicted to develop severe acute pancreatitis (Besselink *et al.* 2008). Patients admitted with a first episode of acute pancreatitis were enrolled in the multi-centre, randomised placebo-controlled study. The probiotic group (n=153) received a mixture of *L. acidophilus*, *L. casei*, *L. salivarius*, *Lactococcus lactis*, *B. bifidum*, *Bifidobacterium lactis* at a total dose of 10^{10} bacteria with cornstarch and maltodextrins daily. However the placebo group received cornstarch and maltodextrins only. Both groups received treatment for a maximum of 28 days. There was no significant difference between the two groups in terms of primary endpoint (any infectious complication, infected necrosis, bacteraemia, pneumonia, urosepsis and infected ascites). Alarmingly however, there were significantly more deaths in the probiotic group than the placebo group (16% vs. 6% respectively, $p=0.01$). Bowel ischaemia occurred at a significant

number in the probiotic group (9 patients vs. 0 patients in the placebo group, $p=0.004$), 8/9 patients passed away. It has therefore been advised that probiotic supplementation should not be given to patients likely to develop severe acute pancreatitis

1.3.1.8 The little to no effects of probiotic supplementation in irritable bowel syndrome

The aetiology and symptoms of IBS have previously been discussed, as have the positive effects that probiotic supplementation can have on sufferers. However, probiotics can sometimes have no effect upon the sufferer. The probiotic mixture VSL#3 was fed twice daily (4.5×10^{11} bacteria per day) to sufferers who exhibited diarrhoea predominant IBS ($n=12$). Whereas the placebo group ($n=13$) received a powder which contained starch. Both groups received treatment for 8 weeks. There were no significant differences between GIT transit times, relief of symptoms, stool frequency, consistency and ease of passage between the two groups. The only positive metric from this study was a significant reduction of bloating ($p=0.05$) (Kim *et al.* 2003). In a follow-up study using the same treatment regime (active group $n=24$, placebo $n=24$) in IBS sufferers with significant abdominal bloating, VSL#3 did not exert positive effects once again (Kim *et al.* 2005). Treatment with this mixture showed no significant effect on urgency, pain, ease of passage and stool form. Furthermore, in contradiction to the previous study bloating was not significantly reduced in the active group. In this study the only symptom which showed a significant difference was the score for flatulence ($p=0.01$).

1.3.1.9 No effect of probiotic supplementation in inflammatory bowel disease

Whilst there is an abundance of evidence for the positive effects of probiotic supplementation in IBD sufferers in general, there are studies which show that probiotic supplementation has no effect upon CD sufferers. It has been shown that feeding *L. rhamnosus* GG to CD sufferers at 2×10^9 CFU/day made no significant difference to time in antibiotic induced remission. The median time to relapse in this instance was 16 ± 4 weeks in the probiotic group and 12 ± 4.3 weeks in the placebo group ($p=0.5$) (Schultz *et al.* 2004). The use of another probiotic organism, *L. johnsonii* LA1, has been assessed as a prophylactic treatment for CD (Marteau *et al.* 2006). In this study the active group ($n=48$) received two daily doses of *L. johnsonii* (2×10^9 CFU per dose), and the placebo group ($n=50$) received maltodextrin. Both treatments were given for a 6 month period. Although the recurrence rate was lower in the probiotic group than the placebo group (49% and 64% respectively) the effect failed to reach significance ($p=0.15$). Recurrence differences, as measured through endoscopy, also failed to reach significance. Recurrence was observed in 63%

of the placebo group compared to 49% of the active group ($p=0.21$). Severe endoscopic recurrence occurred in 26% of the placebo group and 21% of active patients ($p=0.61$). The authors therefore concluded that *L. jonsonii* LA1 at the dose given did not provide a statistically significant benefit.

The treatment of ileal pouch-anal anastomosis following postcolectomy in UC patients has previously been discussed, as have the benefits of probiotic supplementation. The use of *L. rhamnosus* GG has also been researched in the treatment of pouchitis (Kuisma *et al.* 2003). Participants were enrolled if they had been treated for pouchitis at least once. Gelatine capsules of *L. rhamnosus* ($0.5-1.0 \times 10^{10}$ CFU/capsule) were fed 4 times daily for 3 months to participants in an active group ($n=10$). Microcrystalline cellulose was fed to the placebo group ($n=10$). There was no significant difference in pouchitis disease activity index (PDAI) scores between the two groups; with a pre-treatment PDAI mean score of 8.4 ± 0.7 in the placebo group and 8.0 ± 0.8 in the active group ($p=0.44$). This trend was not altered by probiotic administration as the mean PDAI scores were highly similar in both post-treatment groups (8.0 ± 0.7 in the placebo group and 8.0 ± 1.1 in the active group, $p=0.97$).

1.3.1.10 No effect and the negative effect of probiotic administration in atopic eczema

Studies have suggested that probiotic administration is beneficial for the prevention of IgE associated AE. However, ambiguity still remains as there are studies which show that probiotic supplementation has no effect upon this ailment. High-risk new-borns, that is to say new-borns from mothers with atopic disease, were recruited for a feeding study (Taylor *et al.* 2007). Treatments were administered for 6 months with the active group ($n=89$) receiving a daily dose of 3×10^9 *L. acidophilus* LAVRI-A1 and the placebo group ($n=89$) receiving maltodextrin. The rates for AE were similar between the active and placebo group after 6 months of feeding, 25.8% and 22.7% respectively ($p=0.629$). When patients were reassessed at 12 months there were no significant differences in AE rate nor severity between treatment groups ($p=0.581$ and $p=0.995$ respectively). It was in fact shown that probiotic supplementation in this instance was linked to a higher rate of sensitisation to common allergens ($p=0.03$). This suggests that supplementation with this organism in this instance can potentially have negative effects as opposed to positive ones.

Twice daily prenatal (4-6 weeks before delivery) and postnatal feeding (for 6 months) with *L. rhamnosus* GG at 5×10^9 CFU/dose showed no significant difference in outcome when compared to a microcrystalline cellulose placebo (Kopp *et al.* 2008). For this study 105 pregnant women were recruited from families with ≥ 1 member suffering from an atopic disease. The criteria included mother, father or any other children in the family. The probiotic group (n=50), at the age of 2 years, showed no significant difference in episodes of fever, airway infections, total IgE nor sensitisation to an inhalant allergen (p=0.22, p=0.23, p=0.38 and p=0.61 respectively). Although there were no beneficial significant differences, there was a detrimental significant difference. Recurrent episodes of wheezing bronchitis (≥ 5 episodes) occurred significantly more frequently in the probiotic group than the placebo group (26% vs. 9.1%, p=0.03). Once again there is evidence that probiotic supplementation can have no effect and furthermore can exacerbate the condition.

1.3.2 The use of probiotic supplements in healthy individuals

While many researchers focus on the effects of probiotics in the infirm, others research the effects of probiotic supplementation in healthy individuals. Many healthy people purchase probiotic supplements therefore it is imperative that scientific research into their effects is carried out to benefit the individual and the producing company (due to EFSA guidelines).

1.3.2.1 Gut bacterial modulation and survivability of probiotic strain in the GIT

Much attention has been focused on whether probiotic consumption can modulate the gut bacterial community in healthy individuals. It has been shown in numerous instances that the organism which is fed can be recovered after passage through the GIT, and the organism increases the levels of its own genus. Feeding of *B. lactis* HN019 at 1.9×10^7 CFU/day with 2.4 g/day of prebiotic galacto-saccharide in healthy children showed a significant increase in *B. lactis* in faecal material when compared to a placebo group (p<0.001) (Prasad *et al.* 2013). *L. casei* has the ability to survive GIT transit with a study showing that *L. casei* Shirota levels were 7.1 ± 0.4 Log₁₀ CFU/gram of faeces after 7 days of supplementation. This level was maintained with no significant difference for 21 days during the feeding study and persisted for a further 7 days after the study. (Tuohy *et al.* 2007). There was also a significant increase in the total numbers of lactobacilli recovered in faecal material throughout the study.

Another strain of *L. casei*, DN-114001, was delivered to healthy individuals (n=12) three times daily for 10 days in milk which this organism had fermented along with *S. thermophilus* and *L.*

bulgaricus at a total of 10^8 CFU/mL (Rochet *et al.* 2006). Faecal samples were taken at day 0 (before supplementation), day 10 (end of supplementation) and at day 20 (10 days after supplementation ceased). There was a significant ($p < 0.01$) increase in the number of *L. paracasei* group specific quantitative PCR (qPCR) products (of which *L. casei* is part) per gram of faeces at day 10. Research has shown that recovery of *B. lactis* BB12 occurs in a dose-dependent manner (Larsen *et al.* 2006). BB12 was fed with *L. paracasei* CRL-431 at 10^8 , 10^9 , 10^{10} or 10^{11} CFU/day and compared to a placebo group ($n=15$ per group). The results showed a dose dependent response in faecal recovery when the dose was treated as a linear and continuous variable. *L. paracasei* CRL-431 however, was not detected in any of the faecal samples.

While these studies have shown that recovery of the fed organism occurs, there is no significant impact on the gut microbiota as a whole from feeding these probiotic organisms. Tuohy and colleagues show no significant changes in *Bacteroides* spp., *Eubacterium rectale* group and *Atopobium* group numbers (Tuohy *et al.* 2007). Rochet and co-workers showed no significant changes in mean proportions of *Atopobium*, *Bacteroides-Prevotella*, *Bifidobacterium*, *Clostridium coccoides*, *Faecalibacterium prausnitzii*, *Enterobacteria* and *Lactobacillus-Enterococcus* groups (Rochet *et al.* 2006). Similarly Larsen and associates saw no significant change in major gut bacterial groups including *Bacteroides* and *Clostridia* (Larsen *et al.* 2006). The administration of the probiotic yeast *S. boulardii* also did not alter the global gut bacterial community of healthy individuals. The probiotic yeast was fed at a daily concentration of 2.5×10^9 viable cells for 4 weeks (Vanhoutte *et al.* 2006). However the daily feeding of lactulose, a prebiotic, at 10 g showed a bifidogenic response with a significant increase in total bifidobacteria observed during feeding than levels at baseline ($p=0.007$). The aforementioned studies suggest that probiotics are unable to alter the global community of the gut microbiota. However, it has been shown that probiotic consumption can modify the gut bacterial community. Healthy but over-weight individuals ($n=28$) received either a control yoghurt, yoghurt containing 1.39×10^9 CFU *L. amylovorus* or yoghurt containing 1.08×10^9 CFU *L. fermentum* in a cross-over trial. All participants received each treatment for 43 days separated by a 6 week washout period (Omar *et al.* 2013). Both *L. amylovorus* and *L. fermentum* significantly ($p=0.008$) increased its own genus *Lactobacillus* spp., as measured through qPCR of faecal DNA, mirroring the previously mentioned studies. However in contradiction to the previously mentioned studies, *L. amylovorus* supplementation gave rise to a significant ($p < 0.038$) 2-log fold reduction in *Clostridium* cluster IV as measured by qPCR of faecal DNA. These data suggest that modulation of gut bacterial

community can be achieved, in this instance, through the consumption of this probiotic organism.

1.3.2.2 Immunomodulatory effects

Probiotic organisms are often regarded as beneficial due to the immunomodulatory effects which they reportedly exert. Researchers are keen to answer whether or not probiotic strains exert these effects in healthy individuals. In order to assess this question Olivares and colleagues recruited 30 healthy individuals. These individuals were randomly assigned to receive control yoghurt (yoghurt made with the conventional starters *L. bulgaricus* and *S. thermophilus*) or probiotic yoghurt. The probiotic yoghurt was made using *S. thermophilus*, at the same concentration as the placebo. However, *L. bulgaricus* was replaced with *Lactobacillus coryniformis* CECT5711 and *L. gasseri* CEC5714 at 2×10^9 CFU each as starter cultures (Olivares *et al.* 2006). Participants received either the control or probiotic yoghurt daily for 4 weeks and provided a weekly faecal sample. Leukocyte subset proportions were analysed, and immunoglobulin and cytokine measurements were taken. Comparisons were carried out against week 0. After two weeks of consumption the probiotic group showed a significant ($p < 0.05$) increase in proportion of monocytes and T lymphocytes. A significant increase was also observed in the proportion of neutrophils and a significant ($p < 0.05$) decrease of lymphocytes at week 2 in the control group. Whilst there was a significant difference in neutrophil and lymphocyte proportions in the control group, highly significant differences were observed in the probiotic group. These included a highly significant ($p < 0.01$) increase in neutrophil proportion and a highly significant ($p < 0.01$) decrease in lymphocyte proportion. Comparisons between week 0 and week 4 showed a highly significant ($p < 0.01$) decrease in B lymphocytes in the control group; no other leukocyte subsets were significantly different. However, comparisons of leukocyte subset proportions between week 0 and week 4 in the probiotic group showed a highly significant ($p < 0.01$) increase in the proportion of neutrophils. A highly significant ($p < 0.01$) decrease in T lymphocytes and significant ($p < 0.05$) decreases in the proportion of lymphocytes, T memory cells and B lymphocytes was also observed. Furthermore, at week 2 there was a significant ($p < 0.05$) increase in the proportion of natural killer (NK) cells in the probiotic group when compared to week 0. This trend returned to a non-significant difference after 4 weeks. The immunomodulatory effect at two weeks was also shown through a significant ($p < 0.05$) increase in IL-4, IL-10 and a significant ($p < 0.05$) decrease in IgE. This study suggests that probiotics may exert immunomodulatory effects in the short-term.

Immunomodulatory effects may well be strain specific as while the previous study shows that this probiotic mixture exerts these effects, the same cannot be said for other probiotic species. *B. lactis* BB12 and *L. paracasei* ssp. *paracasei* CRL-431 were fed to healthy adults who were randomised to receive the probiotic mixture at either 10^8 , 10^9 , 10^{10} , 10^{11} CFU/day or a placebo (n=15 for each group) daily for 3 weeks (Christensen *et al.* 2006). There was no significant difference between the probiotic groups and placebo group at any concentration in terms of phagocytosis difference (difference between activity before intervention and immediately after). No significant change in faecal immunoglobulin A (IgA) and no significant difference in stimulated blood sample production of interferon- γ (IFN- γ) and IL-10 was also observed. The authors also measured the recovery of *B. lactis* BB12 and *L. paracasei* CRL-431 from faecal material. *L. paracasei* CRL-431 could not be recovered while *B. lactis* BB12 could. When the authors plotted IFN- γ difference (difference in levels before intervention and immediately after) against recovery of *B. lactis* BB12 ($\log \text{CFU/g}^{-1}$) a significant negative ($p=0.011$) correlation was observed. Increased *B. infantis* BB12 recovery correlated with a decrease in IFN- γ difference. This work furthers the idea that different probiotic strains possess different immunomodulatory capabilities. Furthermore, this evokes the notion that survivability of the chosen probiotic organism is a key factor for exertion of any positive effects.

1.3.2.3 Effects on bowel habit and incidence of diarrhoea

Probiotic supplementation in healthy individuals has also been shown to exert other benefits. One such benefit is the improvement in bowel habits with multiple species showing a positive effect. It has been shown that supplementation with *B. lactis* BB12 at an increasing dose has a significant ($p=0.018$) linear effect on faecal consistency whereby the stool became looser as the probiotic dose increased from 10^8 CFU/day through to 10^{11} CFU/day (Larsen *et al.* 2006). Improvements in bowel habits were also seen through the consumption of *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 in food products (both species were at 1×10^9 CFU per serving) (Verdenelli *et al.* 2011). Active participants (n=25) were told to consume at least one portion of these functional foods per day for 12 weeks, while placebo participants (n=25) consumed the same foods with no probiotic organisms added. Participants recorded their bowel habits using a zero to ten scale (0 = worse, 5 = no change, 10 = best). There was a significant ($p<0.05$) increase in intestinal regularity and stool volume observed in the probiotic group when compared to the placebo group. These two studies suggest a role for probiotics in improving the well-being of

healthy individuals. Participants did not have bowel complaints, but probiotic supplementation still improved these two facets of bowel function.

Closely linked to intestinal well-being, the effect of probiotic supplementation on acute diarrhoea in healthy infants has been researched (Thibault *et al.* 2004). Formula fed infants were randomised to receive either formula fermented by *B. breve* C50 and *S. thermophilus* 065 (n=464) or standard formula (n=449). The incidence, number of episodes, duration and severity of diarrhoea were recorded. There was no significant difference in the incidence, number of episodes, duration of diarrhoea or number of hospitalisations between the two groups. However, there was a significant reduction in signs of dehydration ($p=0.01$) and prescriptions of oral rehydration salts ($p=0.003$) in the probiotic group. The data suggest that in those infants who require hospitalisation due to the severity of diarrhoea (0.9% in the probiotic group and 0.5% in the placebo group), probiotics may exert a positive effect through alleviating the level of dehydration. In another study young and healthy male soldiers were recruited to assess the effect of *L. casei* DN-114 on diarrhoea. The incidence of diarrhoea was non-significantly reduced (12.2% in the probiotic group and 16.1% in the placebo group, $p=0.207$) (Pereg *et al.* 2005). The probiotic was provided in the form of a commercially available fermented yogurt which contained 10^8 CFU/mL of the organism which was taken daily for 6 days per week for 8 weeks. There was also no significant difference in mean duration of diarrhoea, fever, vomiting or abdominal pain ($p=0.276$, $p=0.12$, $p=0.082$) between the two treatment groups. We can therefore surmise that strains exert specific effects and that the age of the participants and length of treatment can all play a role in the end result.

1.3.2.4 Effects in other illnesses

It is thought that probiotics exert immunomodulatory effects and through these effects can prime the immune system. It is therefore logical to study the effect of probiotics on the prevention of some infectious illnesses such as the common cold. Healthy Taiwanese school children (age 8-13) were randomised to receive either a twice daily capsule containing *L. acidophilus* and *B. bifidum* (both at 1×10^9 per capsule) or a placebo for 3 months (Rerksuppaphol and Rerksuppaphol 2012). The probiotic group (n=40) had significantly fewer children developing fever and cough ($p=0.025$ and $p=0.01$ respectively) and significantly fewer secondary outcomes of school absence and cold-related school absence ($p<0.001$ and $p=0.001$ respectively). However, probiotic supplementation did not decrease the number of children developing vomiting, diarrhoea or decrease antibiotic usage ($p=0.600$, $p=0.630$ and $p=0.432$ respectively). The data suggest a role for probiotic

supplementation in the prevention of common school acquired ailments and could decrease absenteeism due to illness. The age at which intervention is administered seems to have an impact on the efficacy of probiotic supplementation in the prevention of infectious illnesses. Formula fed infants (4 to 10 months old) from child care centres were recruited to assess the effect of probiotic supplementation on acquired infections (Weizman *et al.* 2005). Participants were randomised to receive either a control milk formula (n=60) or the control milk formula enriched with either *B. lactis* BB12 (n=73) or *L. reuteri* (n=68) each at 1×10^7 CFU/g of formula powder for 12 weeks. Species specific effects were once again observed with *L. reuteri* significantly reducing the number of days with fever, clinic visits, absences from child care centres and prescriptions of antibiotics ($p < 0.001$, $p = 0.002$, $p = 0.015$ and $p = 0.037$ respectively). Whereas both *L. reuteri* and *B. lactis* showed a significant decrease in the number of fever episodes, days with diarrhoea and episodes of diarrhoea ($p < 0.001$) in each instance. Probiotic supplementation did not have a complete protective effect however. The number of days and episodes of respiratory illness did not differ significantly between the placebo group and either of the probiotic groups.

1.4 The Probiotics in Healthy Gut Microbiota (PROHEMI) study

There is an apparent discrepancy in the number of studies which look at the effect of probiotic supplementation in healthy individuals. Furthermore, in these recent studies the active period is relatively short and therefore only addresses effects seen in the short term. In an older study the effect of 6 months of probiotic supplementation in healthy individuals was carried out, which constitutes a far more long-term study than the more recent literature (Tannock *et al.* 2000). The aim of the study was to determine whether the gut microbiota and functions therein could be modulated in healthy individuals through long-term probiotic supplementation. The probiotics in healthy gut microbiota (PROHEMI) study was the vehicle for data generation in order to answer our global questions. This pilot study aimed to determine whether daily supplementation with a probiotic supplement had an effect upon healthy individuals. I aimed to achieve this through the use of both culture dependent and independent approaches. The PROHEMI probiotic mixture has previously been researched with regards to its efficacy in the infirm; where a difference in the number of elderly patients who were *C. difficile* toxin positive (46% in the probiotic group vs. 78% in the placebo group) was observed (Plummer *et al.* 2004). The same probiotic mixture has been shown to reduce the symptom severity scores in IBS sufferers (Williams *et al.* 2009). It must be noted however, that in this study significant reductions in symptom score were also apparent in the placebo group; this highlights a rather marked placebo effect in this instance. Even though symptom score reduction was also seen in the placebo group, the biggest reduction in symptom score was seen in the probiotic group. Strain type tracking through random amplified polymorphic DNA (RAPD) of *L. acidophilus* Cul21 has also been carried out. Recovery of this strain was observed in the faecal material of 10/12 healthy participants following daily feeding of this strain for 14 days (Mahenthiralingam *et al.* 2009). This study shows that this strain can survive transit through the GIT.

1.5 Project aims

This PhD studentship was funded by a Knowledge Economy Skills Scholarship (KESS) with funding from European Social Funds (ESF). KESS aims to promote knowledge exchange between higher education institutions and small and medium enterprises (SMEs). The overall aim of the project was to determine the efficacy of a probiotic product produced by Cultech (Cultech Ltd., Port Talbot, UK) in healthy human males. The specific hypotheses of the project were as follows:

1. Probiotic administration affects the distal gut bacterial community of healthy male individuals (Chapters 3 and 5)

Through the use of community fingerprinting and next generation sequencing techniques we aim to determine whether long-term probiotic consumption modulates the distal gut bacterial community of healthy male individuals or not

2. Probiotic administration affects functions provided by the commensal distal gut microbiota of healthy male individuals (Chapter 4)

Culture dependent techniques will be utilised in order to screen faecal samples from healthy male individuals for a range of functions provided by the commensal gut microbiota. We aim to determine whether long-term probiotic consumption affects the expression of these functions in healthy male individuals or not.

3. Probiotic administration affects the genotoxicity of faecal waters from healthy male individuals (Chapter 4)

Using a bacterial genotoxicity testing strain, the effect of long-term probiotic supplementation on the genotoxicity of healthy male faecal waters will be tested.

4. Probiotic administration affects the metabonomic profiles of faecal waters from healthy male individuals (Chapter 5)

¹H Nuclear Magnetic Resonance spectroscopic analysis will be carried out in partnership with Dr. Jia Li (Imperial College London) in order to determine whether long-term probiotic supplementation affects the metabonomic profiles of healthy male individuals.

5. There are no geographical differences in the gross distal gut bacterial community, functions, and metabonomic profiles of faecal waters of healthy male individuals in the UK (Chapters 3, 4 and 5)

The gross bacterial community, functions and metabonomic profiles of faecal waters of healthy male individuals from the UK will be compared in order to determine whether there are geographical differences.

6. Freezing faecal material affects its bacterial composition

The effect of storage at -20°C on the bacterial composition of faecal material will be determined in a 6 month study.

2. GENERAL METHODS AND MATERIALS

2.1 The PROHEMI study design

2.1.2 The probiotic mixture

The probiotic supplement used in this pilot study was a mixture of *Bifidobacterium bifidum* (Cul20, NCIMB 30153), *Bifidobacterium lactis* (Cul34, NCIMB 30172) and two strains of *Lactobacillus acidophilus* (Cul21, NCIMB 30156) and (Cul60, NCIMB 30157) at 2.5×10^{10} viable cells and was produced by Cultech.

2.1.2 Ethical approval

Ethical approval for the PROHEMI study was granted by Cardiff School of Biosciences, Cardiff University (Cardiff University's Research Ethics Committee. Ref: 1010-3) and the University of Sheffield Research Ethics committee (Ref: SMBRER168).

2.1.3 Study design

Healthy male adults (n=36), with an age range of 21-48 years old were enrolled for this placebo controlled, double blinded, multi-centre (Cardiff/Port Talbot and Sheffield) pilot study. The Cardiff/Port Talbot arm of the study comprised of a Pre-feeding period with no supplementation, an Active period of 6 months where supplementation took place and a Washout period. Participants from the Cardiff/Port Talbot (n=18) arm of the study were anonymised through random number assignment and randomly assigned to an Active group or placebo group. The Active group were instructed to consume a probiotic capsule daily with a meal, while the placebo group received a daily dummy capsule containing maltodextrin (300 mg). Both types of capsule looked identical and were provided in identical bottles labelled with the participant's unique number (**Fig 2.1**). The Sheffield arm (n=18) of the study was carried out in the same manner where all participants were anonymised. However, all received the Active probiotic treatment for 6 months, this was due to ethical reasons and the initial Pre-feeding period was slightly longer (**Fig 2.2**). During the course of the study there were withdrawals with participant PH33 from the Cardiff/Port Talbot arm giving no samples, and participants PH3 and PH10 from the Sheffield arm withdrawing during the Pre-feeding period. Other withdrawals occurred during the course of the study (**Fig 2.3**). All participants were instructed not to consume other foods which contained probiotic organisms. Power and sample size calculations were not carried out for the PROHEMI study as it was a small-scale pilot study. Following completion of the Active

period, participants were asked to return any capsules which were not taken in order to calculate compliance. Participants who remained in the study throughout had a compliance percentage of >80% (data not shown), this was in line with other probiotic feeding trials

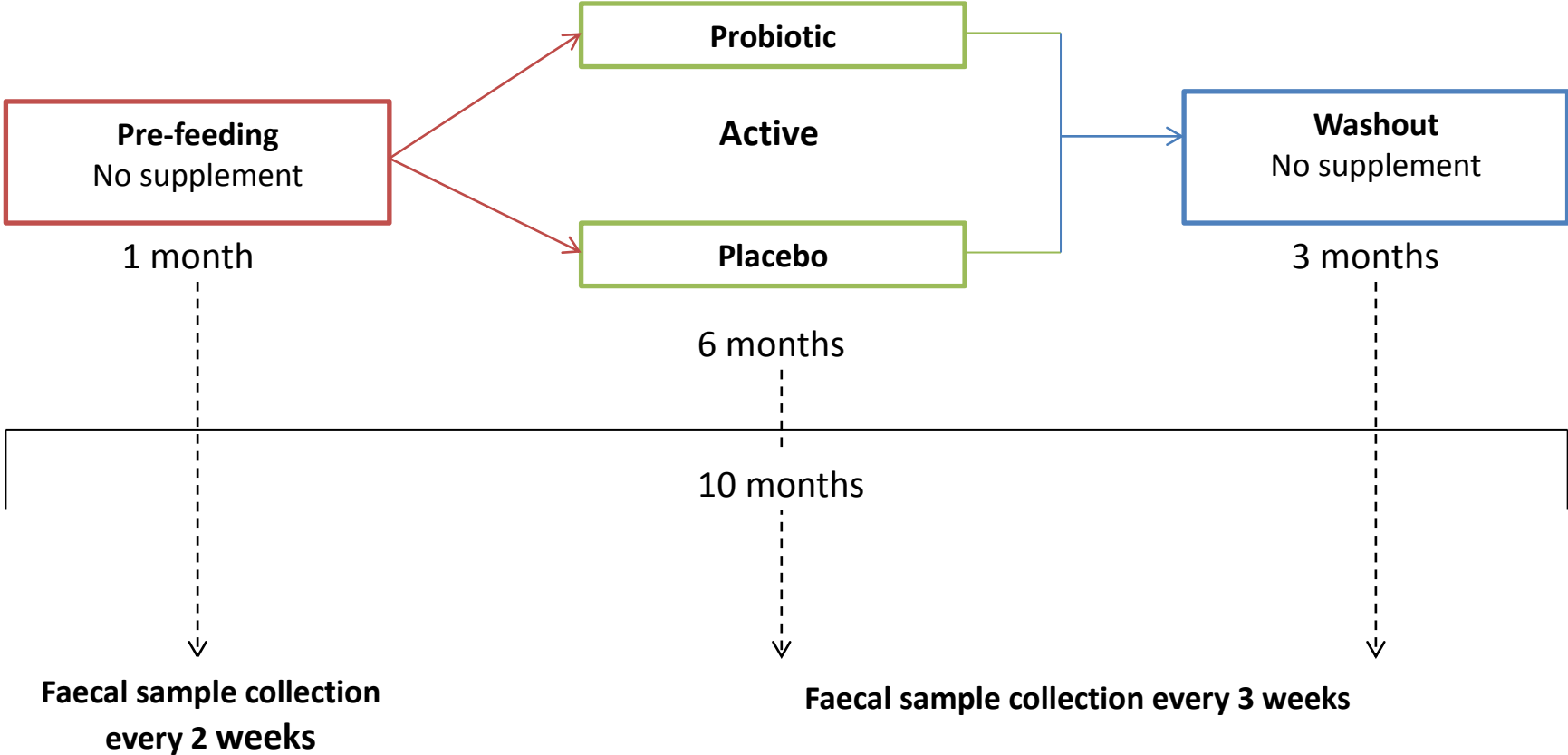


Figure 2.1 | Schematic of the Cardiff arm of the PROHEMI study

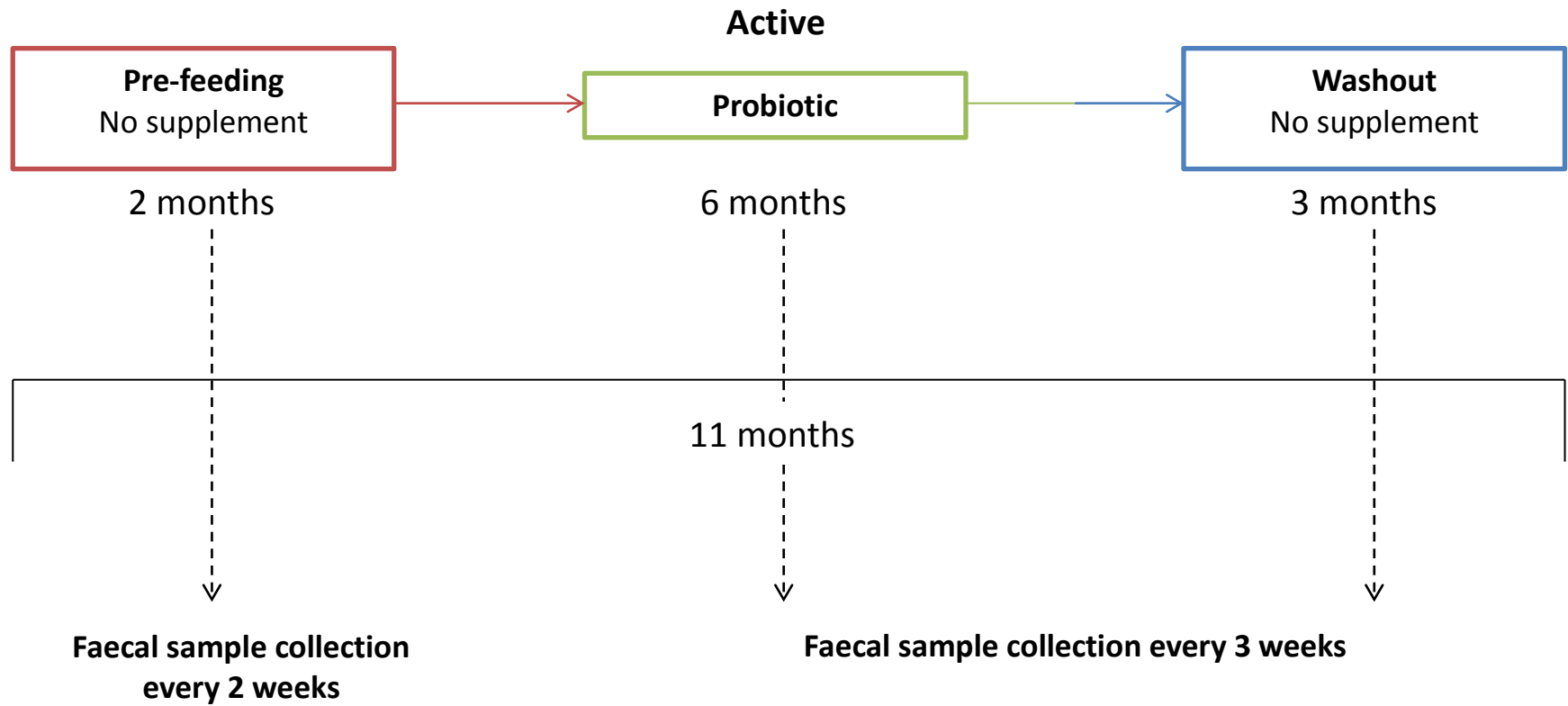


Figure 2.2 | Schematic of the Sheffield arm of the PROHEMI study

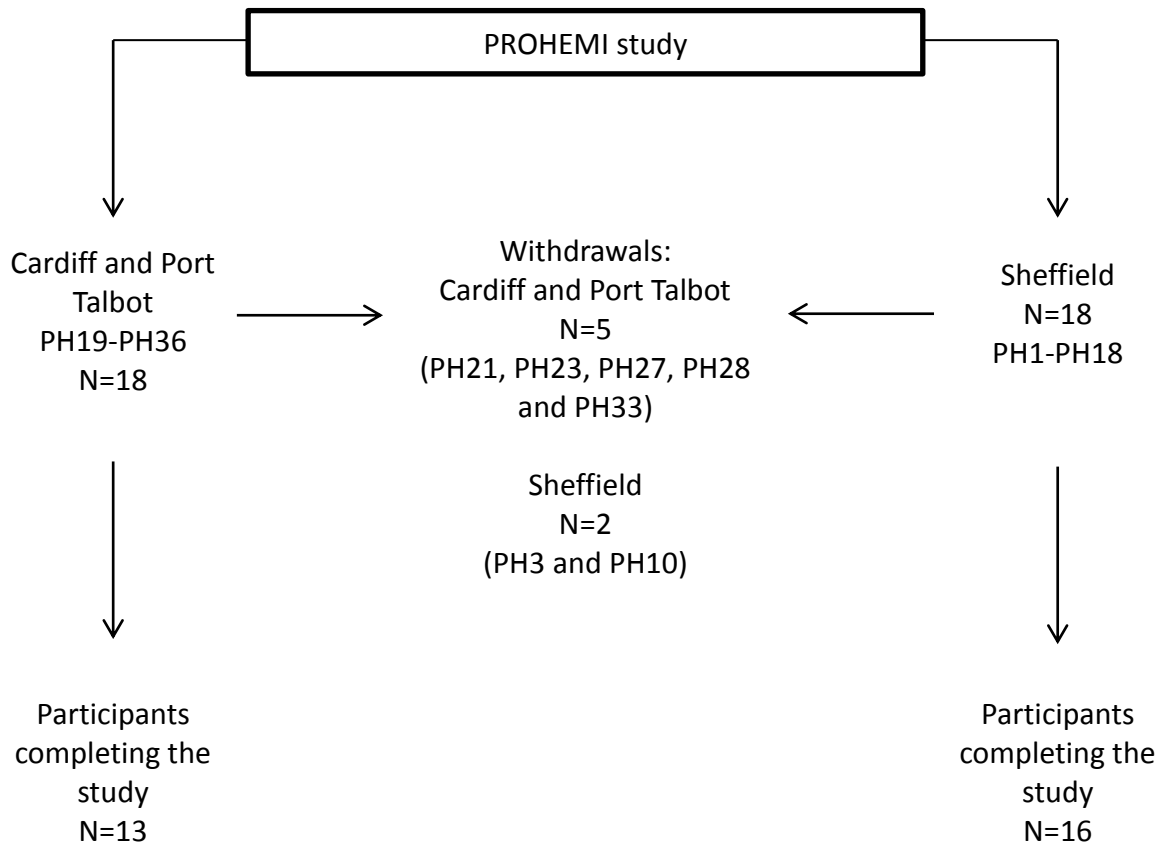


Figure 2.3| The PROHEMI study enrolment – the numbers of participants enrolled in each arm of the study are shown. Withdrawals have been shown along with their respective participant numbers.

2.2 Processing of faecal samples

Both study sites followed the same protocols for faecal sample processing whereby fresh faecal samples were weighed and scored using the Bristol Stool form Scale (Heaton 1999). Samples were homogenised with a spatula. Homogenised faecal samples were separated into relevant tubes for storage.

2.2.1 Storage of faecal material for DNA extraction and for preparation of faecal water

The homogenised faecal material (5 g) was stored at -20°C in preparation for DNA extraction and faecal water preparation within one week of freezing.

2.2.2 Storage of faecal material in order to preserve bacterial cells

Homogenised faecal material (1 g) was placed in 9 mL of maximum recovery diluent (Oxoid Ltd, Basingstoke, England) which contained 20% w/v glycerol (Fisher Chemical, Loughborough, Leicestershire, UK) and 0.5% w/v L-cysteine hydrochloride (Sigma Aldrich company Ltd. Dorest, UK). The faecal material and cryo-protective liquid was mixed by vortex until a uniform suspension was achieved. The faecal suspension was stored at -80°C for future work.

2.3 DNA extraction from bacterial cells

2.3.1 SOP for DNA extraction from faecal material

DNA extractions were carried out at both study centres from their respective participant samples using the same equipment, reagents, consumables and no sample transportation. DNA was obtained from human faecal material by using the QIAamp® DNA Stool Mini Kit (QIAGEN LTD. West Sussex, UK) according to manufacturer's guidelines. However, an additional bead beating step was used, whereby 0.5 g of 0.1 mm zirconia/silica beads (BioSpec products Inc. Bartlesville, OK 74005, USA) were added to each sample and the cells lysed by 3 one min bursts at 5 m/s using a Fastprep®-24 machine (MP Biomedicals, Solon, OH 44139, USA). This bead beating step was carried out following the addition and mixing of the faecal sample with the first buffer.

2.3.2 DNA extraction from single species

Over-night culture (1 mL) was dispensed into a 1.5 mL microcentrifuge tube and centrifuged at a relative centrifugal force of 4,000 g for 2 min. The supernatant was removed and the

pellet was resuspended in 100 μL of 10 mM TRIS/EDTA (10 mM TRIS/HCl pH8 and 10 mM EDTA pH8) (MP Biomedicals, Aurora, Ohio). The cell suspension was transferred to a 2 mL tube which contained 0.5 mL of 0.1 mm zirconia/silica beads (BioSpec products Inc. Bartlesville, OK 74005, USA). To this 500 μL of lysis buffer (50 mM TRIS/HCl pH8, 70 mM EDTA pH8, 1% sodium dodecyl sulphate (SDS)) (Fisher Scientific, Loughborough, Leicestershire, UK) and 20 μL proteinase K (20 mg/mL) (QIAGEN LTD. West Sussex, UK) was added. The bacterial cells were lysed at 5 m/s for 30 s using a Fastprep[®]-24 machine (MP Biomedicals, Ohio, USA). The cells were incubated at 37°C for 30 min before 200 μL of saturated ammonium acetate was added and vigorously mixed by vortex and tube inversion. To this suspension 600 μL of chloroform (Fisher Scientific, Loughborough, Leicestershire, UK) was added and vigorously mixed by vortex and tube inversion. The suspension was centrifuged at a relative centrifugal force of 14,000 g for 5 min before 700 μL of the clear supernatant was taken and transferred into a sterile 1.5 mL microcentrifuge tube. To this 700 μL of propan-2-ol (Fisher Scientific, Loughborough, Leicestershire, UK) was added and mixed vigorously by tube inversion. The suspension was incubated at -20°C for 30 min before centrifugation at 14,000 g for 10 min. The supernatant was removed, leaving a white pellet of DNA which was washed with 100 μL of ethanol. Excess ethanol was removed and the remaining ethanol and DNA were centrifuged for 15 s at 14,000 g to form a pellet. The ethanol was removed and the sample was allowed to air dry before resuspension of the pellet in 200 μL of TRIS/HCl (10 mM, pH8).

2.4 Ethanol precipitation of DNA samples

NaCl (1 M) was added as a ratio of 0.3 to the volume of the DNA sample. To the combined DNA and NaCl, 2 volumes of cold 100% ethanol was added and mixed by inversion before incubation at -20°C over-night. The following morning, the mixture was centrifuged at 16,000 g at 4°C for 15 min. The supernatant was poured off and 600 μL of cold ethanol (70%) was added before centrifugation at 16,000 g at 4°C for 15 min. The supernatant was poured off and the sample was allowed to air dry until all remaining ethanol had evaporated. The remaining pellet was resuspended in 30 μL of 10 mM TRIS/EDTA (10 mM TRIS/HCl pH8 and 10 mM EDTA pH8).

2.5 SOP for faecal water generation from PROHEMI faecal samples

Faecal water generation was carried out at both study centres from their respective participant samples using the same equipment, consumables and no sample transportation as previously described (Marchesi *et al.* 2007). Briefly, thawed faecal material was weighed and 2 volumes (v/w) of sterile phosphate buffered saline (1.9 mM Na₂HPO₄, 8.1 mM NaH₂PO₄, 150 mM NaCl, pH 7.4) was added. Homogenisation of this mixture was achieved through vortex mixing for 60 s in order to generate faecal slurries. The slurry was then centrifuged at 3,000 g for 15 min. Following this, the supernatant was removed and filtered through a 30 µm filter (Whatman 113V, GE Healthcare Life Sciences, Buckinghamshire, UK). The filtrate was further filtered through a 0.2 µm centrifugal filter (VWR International Ltd, Leighton Buzzard, UK) at 14,000 g for 15 min.

3. COMMUNITY PROFILING OF FAECAL DNA FROM THE PROHEMI STUDY

3.1 Introduction

Culture independent analysis of microbial communities has provided a wealth of knowledge and has the main advantage of negating “the great plate count anomaly” (Staley and Konopka 1985). The method, DGGE, was initially developed in order to discern differences in DNA mutation (Fischer and Lerman 1983). However, the technique has also been used as a community fingerprint method through the use of 16S *rRNA* genes (Muyzer *et al.* 1993). The method has been used in order to discern the bacterial community of complex ecosystems. These ecosystems include the soil (Ellis *et al.* 2003) and more importantly the human distal gut (Schwiertz *et al.* 2003). DGGE has the advantage of the user being able to sequence excised bands in order to determine which organism/organisms are present within the sample. The advantages and disadvantages of this method have previously been discussed (Kirk *et al.* 2004). Although highly reproducible, this method relies on good laboratory techniques in order to compare gels. These techniques include, but are not limited to, good pipetting technique when setting up the PCR mixture and the ability to pour gels of a consistent volume. The difficulty in comparing different gels becomes apparent when band intensity is measured as a proxy for the abundance of a particular organism or organisms in the sample.

Fingerprinting methods such as LHPCR, ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA) provide a snapshot of the community as a whole. These methods however, do not easily provide information on the specific bacterial make up of a community. It must be noted that DGGE, ARISA and LHPCR suffer with the 16S *rRNA* gene bias. Organisms of potential importance with a low copy number of 16S *rRNA* genes within a community may be overlooked, while those with a high copy number are not (Crosby and Criddle 2003). Due to the fact that these techniques rely on PCR prior to analysis, many biases can be introduced. These biases and problems have previously been discussed (V. Wintzingerode *et al.* 1997). This then produces a biased community fingerprint. These techniques however, can be used in order to assess gross changes within the community under investigation. LHPCR has been shown to be reproducible and has been successfully applied to complex communities, such as the soil (Ritchie *et al.* 2000) and more importantly the gut (Bjerketorp *et al.* 2008). LHPCR has also been used as a screening method for interesting samples which can be put forward for next

generation sequencing (Gillevet *et al.* 2010). ARISA has also been applied to assess complex communities such as freshwater (Fisher and Triplett 1999) and RISA to assess the microbiota associated with plant biomass in the herbivore gut (Larue *et al.* 2005). Both LHPCR and ARISA have the benefits of being high-throughput and the abundance of a peak can be easily calculated using dedicated software packages.

It is difficult to ascertain which bacterial species are responsible for generating a fragment of a given length when using LHPCR and ARISA. However, it is not impossible to estimate which bacterial species will give rise to a fragment of a given length. In the instance of ARISA there is a dedicated database for this purpose. The ADAPT system takes ARISA data and uses multiple databases to assign taxonomic information to the submitted data (Schmieder 2008). To my knowledge, there is no such database for LHPCR data. Therefore, an *in silico* LHPCR experiment using 16S *rRNA* gene information obtained from the ribosomal database project (RDP) (Maidak, 2000) was carried out. This information can be combined with LHPCR data for rough taxonomic identity assignment for a given fragment length.

Research into the effects of probiotic supplementation focuses on the potential of these organisms to exert immunomodulatory effects and to protect against disease. Some research has also focused on the ability of probiotics to modulate the gut bacterial community of individuals. Much of the research makes use of *in vitro* models, such as the anaerobic faecal batch culture system (Saulnier *et al.* 2008), in order to assess the ability of probiotics to modulate bacterial community. Due to the increasing evidence that the gut bacterial community of individuals may impact upon diseases such as obesity, it follows that modulation of our commensal gut bacterial community could be used as a therapy. There is a scarcity of research on the effect of probiotic supplementation in healthy individuals. Research into the effect of probiotic supplementation on the gut bacterial community of healthy individuals is scarcer. Therefore, in this study a mixture of community fingerprint profiling techniques were tested and applied in order to answer whether the long-term consumption of a probiotic, in healthy individuals, modulates the gut bacterial community.

3.1.1 Chapter Aims

The aims of this chapter are as follows:

1. to develop and apply published community profiling techniques on extracted faecal DNA from PROHEMI participants;
2. to determine whether probiotic administration affects the gross bacterial community of healthy male individuals;
3. to determine whether there is a difference in the gross bacterial community of individuals from the two study centres.

3.2 Materials and methods

3.2.1 Length heterogeneity PCR (LHPCR)

The universal 27F (5'-AGAGTTTGATCMTGGCTCAG-3') primer was labelled with the fluorescein molecule 6-FAM at the 5' end (Sigma Aldrich, United Kingdom) and used in conjunction with 3 different reverse primers: 338R (5'-GCTGCCTCCCGTAGGAGT-3') (Rogers *et al.* 2003), 358R (5'-ACTGCTGCCTCCCGTAGGAGT-3') (Bjerketorp *et al.* 2008) and 534R (5'-ATTACCGCGGCTGCTGG-3') (Eusebio *et al.* 2011). Reactions (20 μ L) were set-up as previously described. The thermal cycler (C1000™; BIO-RAD, Hertfordshire, UK) was programmed with an initial denaturing step of 95°C for 1 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min with a final extension step of 72°C for 10 min.

All products were visualised by gel electrophoresis using the 2-log ladder DNA standard (New England Biolabs® Inc. Ipswich, MA 01938-2723) as a reference. Products were concentrated using the Eppendorf concentrator 5301 (Eppendorf UK Limited, Cambridge, UK). Hi-Di™ Formamide (10 μ L) (Applied Biosystems, Cheshire, UK) and 0.25 μ L of MRK 1000 ROX ladder (Gel Company, San Fransisco, CA 94107, US) were added to each concentrated product. Fragment analysis of products was carried out using the ABI 3130xl Genetic Analyzer (Applied Biosystems, Life Technologies Corporation, CA 92008, US).

3.2.1.2 Duplex target length heterogeneity PCR (DT-LHPCR)

The use of a dual reverse primer combination for LHPCR was trialled. DT-LHPCR was carried out by setting-up 20 μ L reactions as previously described. However; both 338R and 534R primers were added at a concentration of 0.04 μ M each with 0.08 μ M 6-FAM labelled 27F to give a final primer concentration of 0.16 μ M. The thermal cycler programme used was the same as previously described for LHPCR. Products were visualised and treated as previously described for LHPCR.

3.2.1.3 Length heterogeneity PCR (*in silico*)

Sequences for gut bacterial *rRNA* genes were obtained from the Ribosomal Database Project (available at: <http://rdp.cme.msu.edu>). The sequences were aligned using the ClustalW (Larkin *et al.* 2007) programme in BioEdit v7.0.5.3 (Hall 1999). Forward and reverse primers (27F and 358R respectively) were aligned to the sequences with excess bases and gaps subsequently removed. Sequences were uploaded as FASTA files to the compute sequence length function in Galaxy tools (available at: <https://main.g2.bx.psu.edu/>). The sequence length for a given bacterial species was used to assign an approximate bacterial species to a given peak in the electropherograms generated through LHPCR. The approximate fragment sizes generated are shown in **Appendix I**.

3.2.1.4 LHPCR analysis of the probiotic organisms used in the study

DNA from over-night cultures of *B. bifidum* (Cul 20), *L. acidophilus* (Cul 21) and *L. acidophilus* (Cul 60) was extracted as previously described (see **Chapter 2.3.2**). DNA from *B. lactis* (Cul 34) was not extracted due to problems culturing the organism from freezer stocks. The extracted DNA was subjected to LHPCR analysis using the 358R primer as previously described.

3.2.1.5 Bioinformatic analysis of LHPCR products

Fragment analysis files generated from LHPCR products were imported into SoftGenetics GeneMarker Version 1.91 (SoftGenetics LLC, PA 16803, USA). Electropherograms were generated from fragment analysis files in the programme. These were inspected in order to ensure that the file was not corrupted or missing data. The areas for given peaks within a sample were exported and normalised. This was achieved by calculating the area ratio for each peak through dividing the area for a given peak by the total peak area within a sample.

Normalised proportional peak area ratios (NPPARs) for given samples were imported into R Statistical software (R-Core-Team 2012). Heatmaps of data were generated using the heatmap2 function in the package gplots (Warnes 2010), or through the aheatmap function of the NMF package (Gaujoux and Seoighe 2010). Heatmaps were coloured using the colour palettes available in the RColorBrewer package (Neuwirth 2007) (**Appendix II**). Samples within the heatmap were clustered together by hierarchical clustering using calculated Euclidean distances of NPPARs through Ward's method. Principal coordinate analysis (PCoA), using calculated Euclidean distances of NPPARs, was carried out in R statistical software. This was achieved through the use of a custom script which utilised both vegan (Oksanen *et al.*

2011) and *labdsv* (Roberts 2010) packages (**Appendix II**). Agglomerative nested clustering (AGNES), using Ward's method, of the calculated Euclidean distances of NPPARs and export in Newick format was carried out in R statistical software. In order to carry AGNES a custom script was used. The script utilised *ape* (Paradis *et al.* 2004), *BiodiversityR* (Kindt and Coe 2005) and *cluster* (Maechler *et al.* 2005) packages (**Appendix II**). Once exported in Newick format, the tree was manipulated using *interActive tree of life (iTol)* software online (Letunic and Bork 2011).

Percentage changes between samples from the same individual were calculated as previously described (Marzorati *et al.* 2008). Pearson's *r* correlation coefficients were calculated between samples using SPSS (IBM, Portsmouth, UK) and subtracted from 100 in order to give the percentage change. The mean percentage change was calculated.

Normality testing of data followed by Kruskal – Wallis H tests were carried out using IBM SPSS statistics 20 (IBM, Portsmouth, UK).

3.2.2 Ribosomal Intergenic Spacer Analysis (RISA)

RISA of faecal sample DNA was carried out by amplifying the intergenic region between the 16S *rRNA* genes and 23S *rRNA* genes. Amplification of the ribosomal intergenic spacer region was achieved through the primer combination of 1406F (5'-TGYACACACCGCCCGT-3') and 23SR (5'-GGGTTBCCCCATTCRG-3') (Fisher and Triplett 1999). PCR reactions (20 µL) were set-up as previously described however; each reaction contained 40 ng of template DNA. The thermal cycler was programmed with an initial denaturing step of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s with a final extension step of 72°C for 5 min.

Products were initially visualised by gel electrophoresis before being loaded into either an Agilent 1000 or 7500 DNA chip (Agilent Technologies, Edinburgh, UK). Samples in the chip were visualised using the Agilent BioAnalyser 2100 (Agilent Technologies, Edinburgh, UK) micro-fluidics platform following manufacturer's guidelines.

3.2.2.1 Bioinformatic analysis of RISA products

RISA data was exported from the Agilent Bioanalyser 2100 and was imported into GelCompar®II Version 4.5 (Applied Maths NV, Sint-Martens-Latem, Belgium) using a custom script. The script took the comma separated data from the Bioanalyser and converted the

data to GelCompar®II format Cluster analysis of percentage similarities between samples, as generated through Pearson's correlation coefficient, was carried out using Ward's method. Calculated Pearson's correlation coefficients for each sample were exported from the software.

3.2.2.2 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The same primers were used for ARISA as those in RISA. However, the primer 1406F was labelled with the fluorescein molecule 6-FAM at the 5' end. ARISA products were treated as previously described for LHPCR.

3.2.2.3 Bioinformatic analysis of ARISA products

ARISA fragment analysis files were imported into SoftGenetics GeneMarker Version 1.91 and were processed as previously described for LHPCR.

Raw AB1 ARISA fragment analysis files, generated through fragment analysis, were also uploaded to the ADAPT (Schmieder 2008) database for analysis.

3.3 Results

3.3.1 Length heterogeneity PCR (LHPCR) testing

3.3.1.1 Primer testing

The single 6-FAM labelled 27F primer was tested with 3 different reverse primers (338R, 358R and 534R) and a combination of 2 reverse primers (338R and 534R). For this experiment primer combination reproducibility was tested through the amplification of a single DNA sample in quadruplicate reactions using each primer combination. Through PCoA it was found that all primer combinations were highly reproducible (**Fig. 3. 1**). Both 534R and the dual target combination of 338R and 534R, clustered closely together. In order to inspect the reproducibility further, a heatmap (**Fig. 3. 2**) was generated which consolidated all the information from the electropherograms (**Fig. 3. 3**) of all the quadruplicate primer reactions. Samples NO9-NO16 appear to be clustered closely together, with no differentiation between the 534R primer alone and when it is combined with 338R as a dual target. Further inspection of the heatmap shows that the peaks generated by the 338R primer in the dual reaction (NO13-NO16) are low in their proportional abundance. Therefore, separate clustering of these 4 replicates from the 534R primer replicates (NO9-NO12) was not achieved. For further LHPCR experiments 358R will be used as the primer of choice, as this primer has previously been applied to LHPCR analysis of human faecal samples (Bjerketorp *et al.* 2008).

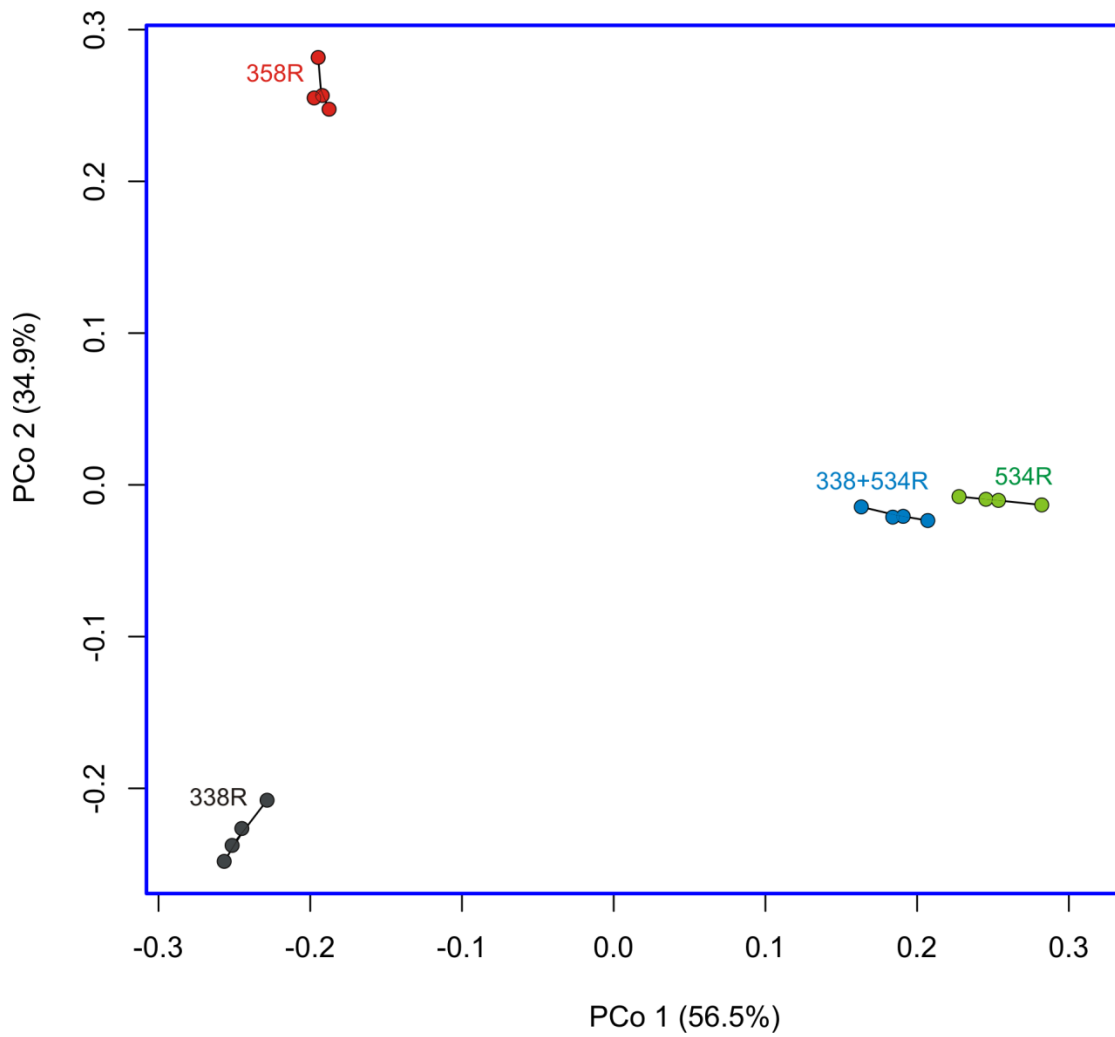


Figure 3.1| PCoA of LHPCR primer combinations– the first and second principal coordinates have been plotted, the variance explained is shown in brackets. The reverse primer used in conjunction with 6-FAM 27F have been colour coded, 338R, 358R, 534R and 338+534R are black, red, green and blue respectively.

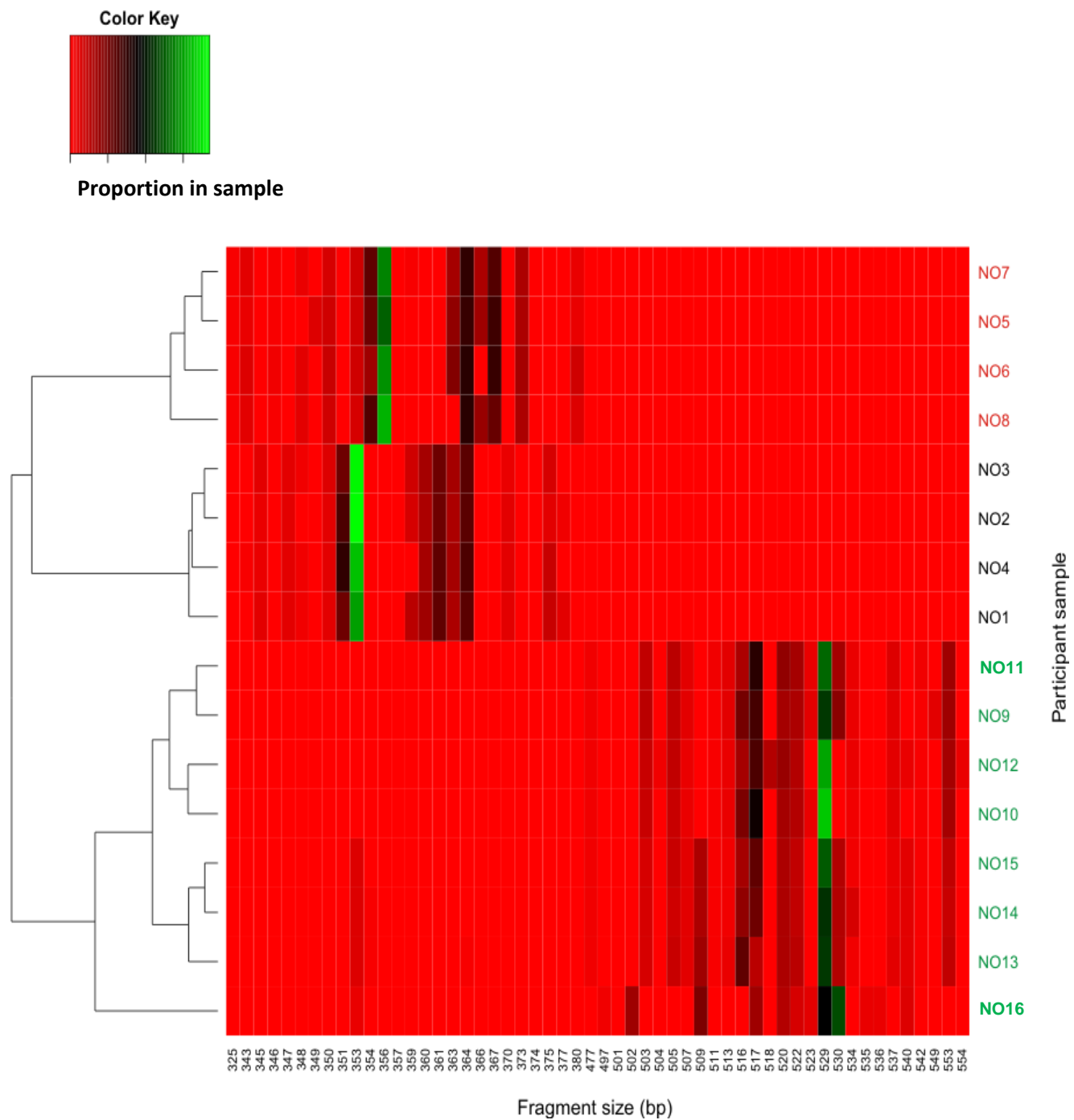


Figure 3.2| Heatmap of LHPCR primer reproducibility– samples are shown on the right of the heatmap, NO1-NO4 = 338R, NO5-NO8 = 358R, NO9 -NO12 = 534R and NO13-NO16 = 338/534R. Fragment sizes are shown in base pairs (bp) on the bottom of the heatmap. The proportion of each peak shown within a given sample is represented by a colour, which is shown in the Key. The software clusters the most similar samples next to one and other.

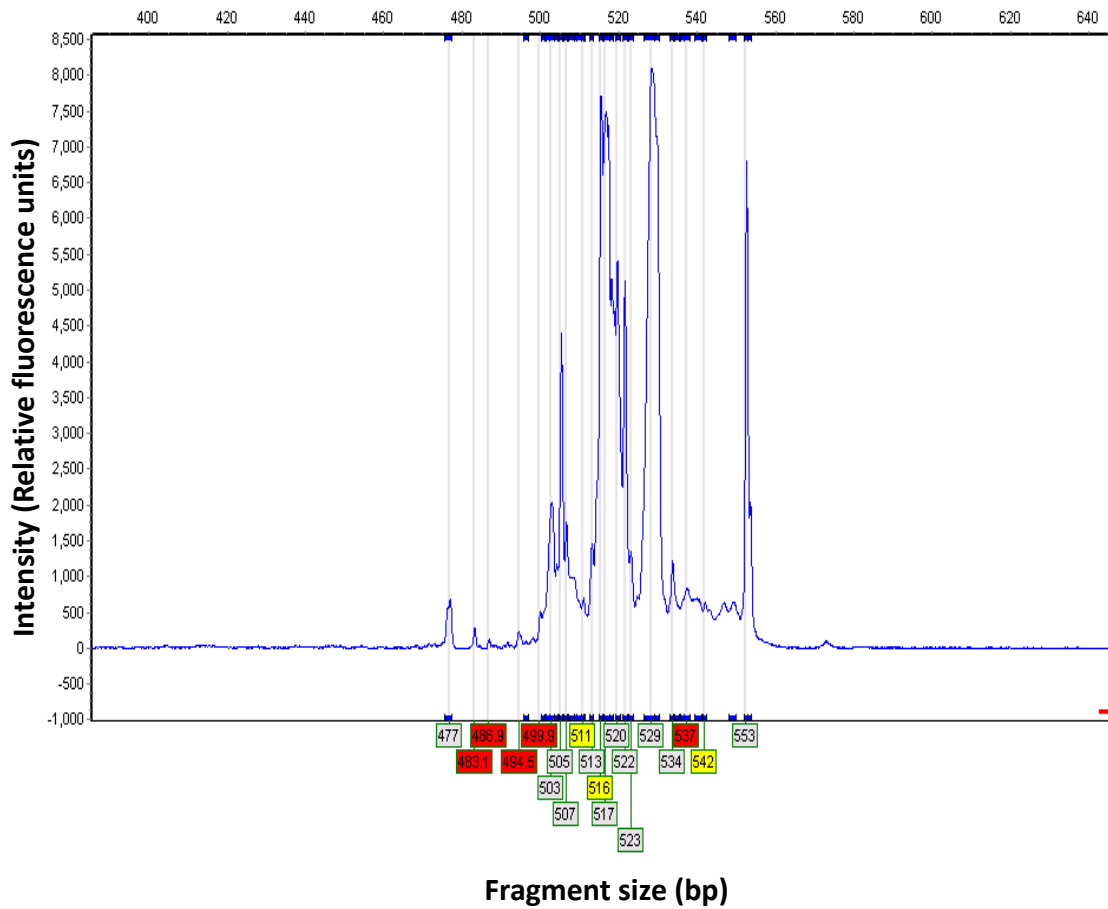


Figure 3.3| Electropherogram of a 534R LHPCR sample – Fragment sizes are shown on the bottom of the electropherogram and the fragment abundance, measured by fluorescence intensity, is shown by the peak height (which is measured on the left of the electropherogram).

3.3.1.2 Stability of the 6-FAM label on LHPCR products

DNA was extracted from the 1st set of Cardiff faecal samples and subjected to LHPCR analysis. Products were subjected to immediate fragment analysis and also stored at -20°C for 2 weeks and 1 month before fragment analysis, to give 3 replicates for a given sample. The heatmap (**Fig. 3. 4**) shows that most replicates of a given sample clustered together (e.g. PH35 and PH30), while some replicates in a given sample did not (e.g. PH21 and PH25). Percentage change of samples, after storage at -20°C for 2 weeks and 1 month, was also calculated. This was achieved by comparing peak area ratios of stored LHPCR samples to their respective samples where fragment analysis was carried out immediately. The mean percentage change after storage for 2 weeks and 1 month was 15.71% (standard deviation from the mean (SD) = \pm 16.9%) and 16.69% (SD = 18.8%) respectively (**Fig. 3. 5**). Therefore, it was decided that samples will be sent for fragment analysis without delay after LHPCR product generation.

3.3.1.3 Reproducibility of LHPCR using the 358R primer and the reproducibility of faecal DNA extraction

LHPCR was carried out twice on a single DNA sample obtained from three participants. The two LHPCR results for a given individual cluster together. Importantly individuals S1, S2 and S3 cluster separately from one and other (**Fig. 3. 6**). Through Pearson's correlation coefficient it is possible to see that S1 A and S1 B share 100% similarity, S2 A and S2 B share 99.9% similarity and S3 A and S3 B share 99.5% similarity (**Table 1**). Therefore it is possible to conclude that intra-sample similarity is higher than the inter-sample similarity. For example S3 A and B share 89.2% and 88.9% similarity with S1 A respectively. This suggests that the technique is highly reproducible and can distinguish between DNA samples from different individuals.

Five DNA extractions were performed on a single faecal sample and LHPCR analysis carried out on the DNA. After analysis and export of NPPARs the data was subjected to Pearson's correlation coefficient analysis (**Table 2**). It is possible to see that the majority of the samples are highly similar. However, Extraction 3 shows a lower percentage similarity to the other extractions. This highlights the importance of the extraction method in a fingerprinting technique.

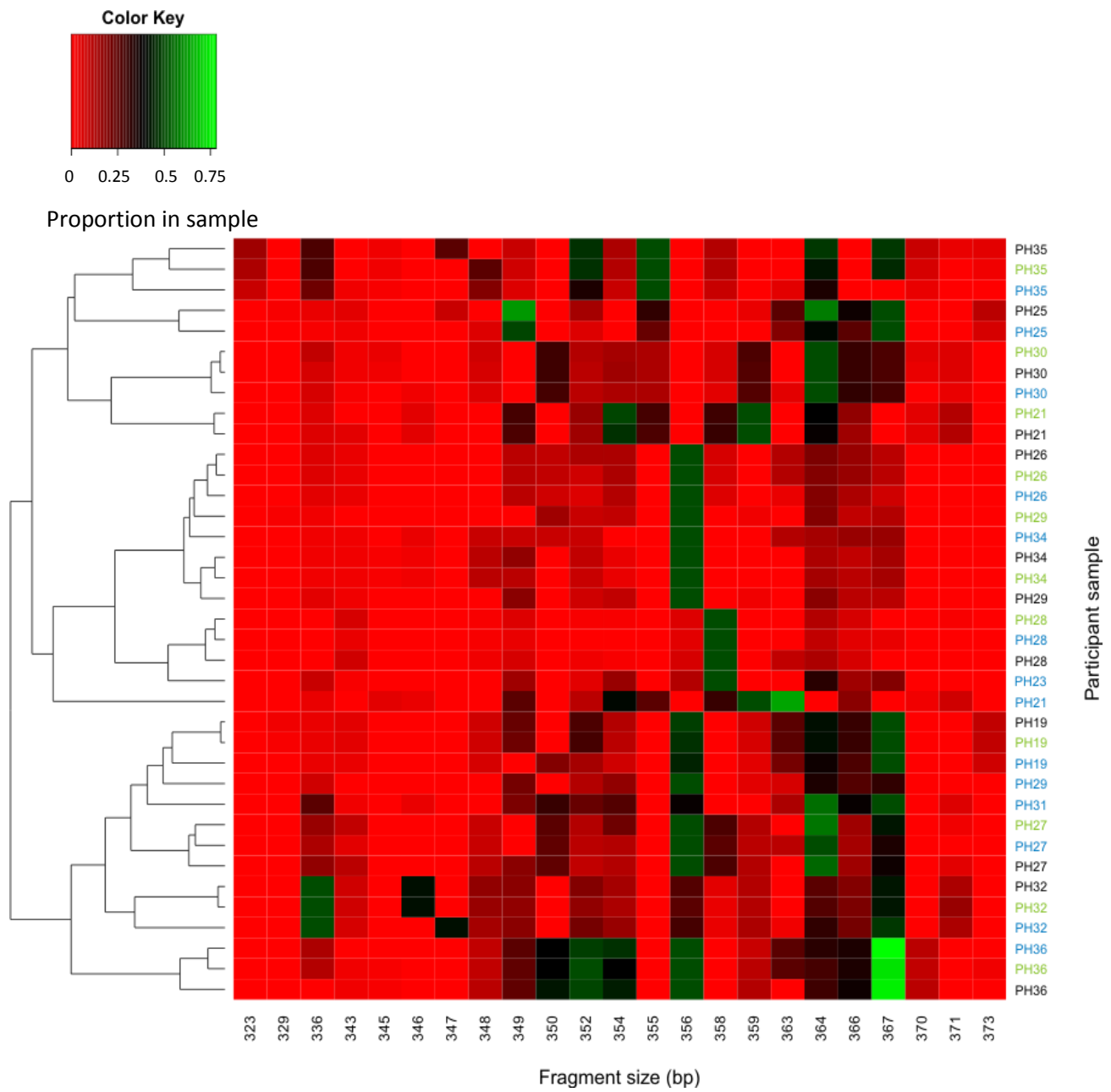


Figure 3.4| Heatmap to show 6-FAM stability – samples which were sent for fragment analysis immediately are shown in **black**, after 2 weeks of storage at -20°C in **green** and after 4 weeks of storage at -20°C in **blue**. The proportional abundance of a fragment is represented by a colour, as shown in the key.

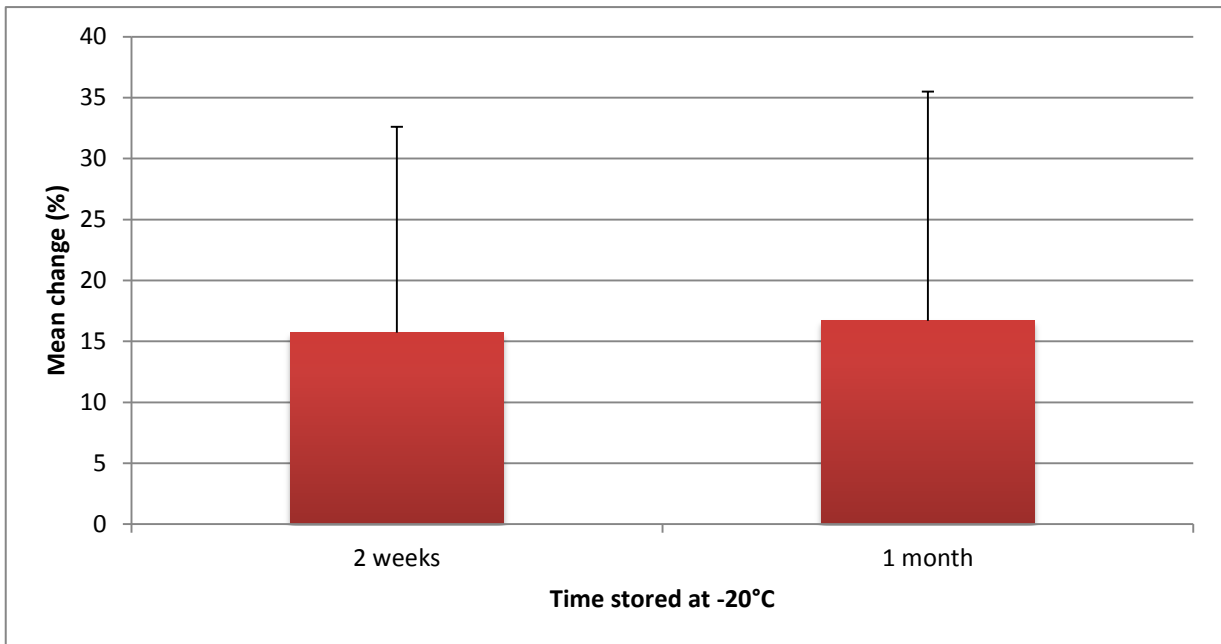


Figure 3.5| Mean percentage change in normalised peak area ratios of samples after storage at -20°C – normalised peak area ratios of stored samples were compared to normalised peak area ratios of the same samples following immediate fragment analysis; error bars represent standard deviation from the mean.

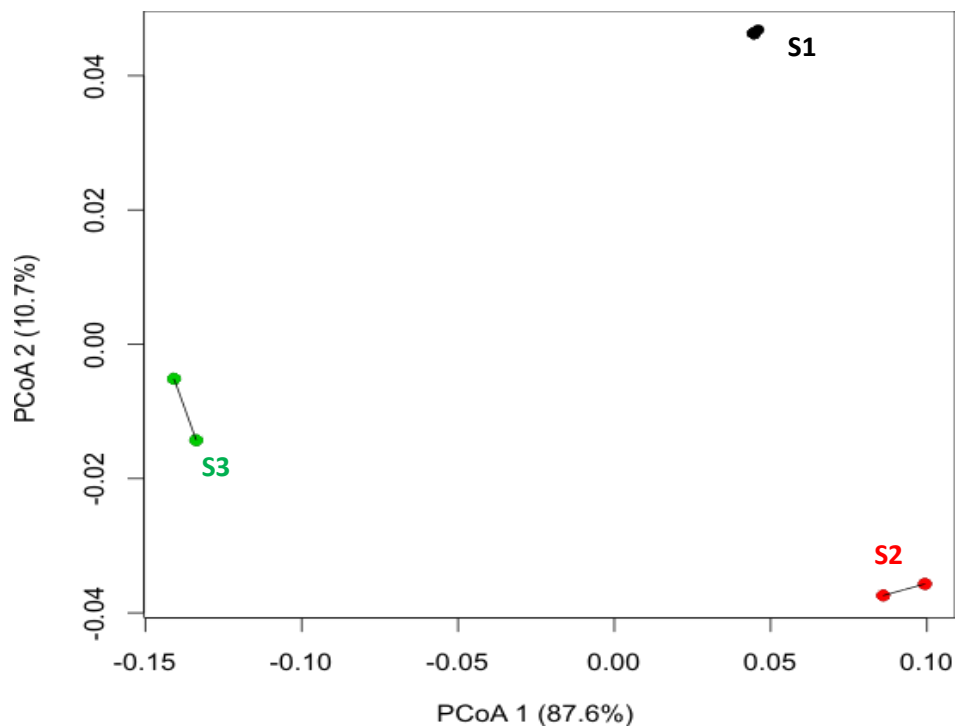


Figure 3.6| PCoA of two LHPCR analyses of a single DNA sample from 3 individuals – The first and second principal coordinates have been plotted, the variance explained is shown in brackets.

Table 3.1| Pearson's correlation of two LHPCR runs from 3 individuals

	S1 A	S1 B	S2 A	S2 B	S3 A	S3 B
S1 A	1.000					
S1 B	1.000	1.000				
S2 A	.987	.987	1.000			
S2 B	.988	.988	.999	1.000		
S3 A	.892	.892	.893	.884	1.000	
S3 B	.889	.889	.892	.884	.995	1.000

Single DNA extractions from 3 individuals (S1, S2 and S3) were subjected to LHPCR analysis twice; A = 1st LHPCR analysis and B= 2nd LHPCR analysis.

Table 3.2| Pearson's correlation of 5 DNA extractions using a single faecal sample

	Extraction 1	Extraction 2	Extraction 3	Extraction 4	Extraction 5
Extraction 1	1.00				
Extraction 2	0.958	1.00			
Extraction 3	0.693	0.802	1.00		
Extraction 4	0.95	0.987	0.839	1.00	
Extraction 5	0.848	0.901	0.84	0.938	1.00

5 DNA extractions were performed in a single faecal samples and were subjected to LHPCR analysis followed by Pearson's correlation.

3.3.1.4 LHPCR analysis of probiotic strains used in study

When subjected to LHPCR analysis the two *L. acidophilus* strains (Cul 21 and 60) showed a peak (fragment) at 372 bp while *B. bifidum* (Cul 20) showed a peak (fragment) at 351 bp. This information will be used while analysing the LHPCR profiles generated from study samples.

3.3.2 LHPCR analysis of PROHEMI faecal DNA samples

3.3.2.1 LHPCR analysis of all PROHEMI samples from Cardiff/Port Talbot and Sheffield based upon study period

All Cardiff/Port Talbot and Sheffield faecal DNA samples were subjected to LHPCR analysis. PCoA of NPPARs based upon study period shows no effect of probiotic supplementation on the gross community profiles. Pre-feeding, Active and Washout samples clustered together (**Fig. 3. 7**). In order to interrogate the dataset further, the study centres were analysed separately. Probiotic supplementation showed no effect upon gross community profiles as shown by PCoA of Sheffield samples as Pre-feeding, Active and Washout samples clustered together (**Fig. 3. 8**). The same was observed in the Cardiff/Port Talbot samples where the Active and placebo groups were separated from one another and blindly assigned either Active group 1 (A1) or Active group 2 (A2). These two Active groups clustered together with Pre-feeding and Washout (**Fig. 3. 9**) following PCoA. It must be noted that outliers were observed in all groups, however, the majority of samples from each group clustered together.

3.3.2.2 LHPCR analysis of all PROHEMI samples from Cardiff/Port Talbot and Sheffield based upon study centre

PCoA of NPPARs, generated through LHPCR analysis, shows clustering based upon the study centre. Cardiff/Port Talbot and Sheffield samples clustered separately from one another (**Fig. 3. 10**). Although there is an observable overlap between the two study centre clusters due to a few samples, it is clear that the two study centres cluster separately.

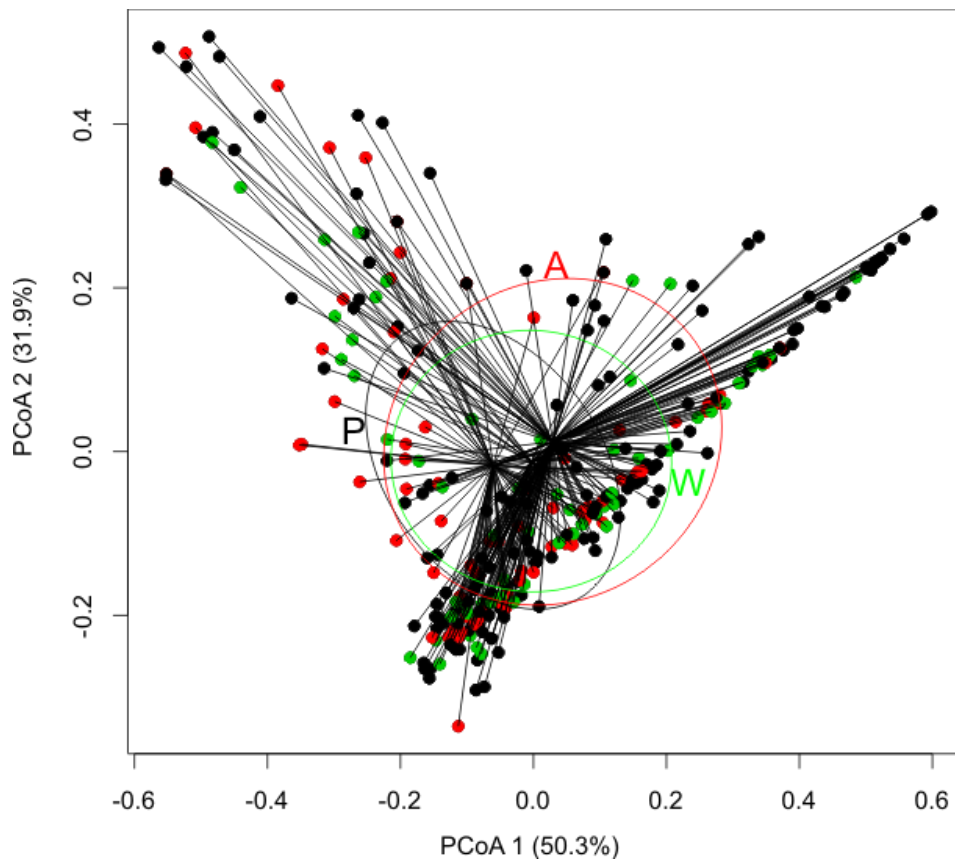


Figure 3.7| PCoA of all PROHEMI LHPCR samples with study period shown – study periods are shown on the plot (P = Pre-feeding, A = Active, W = Washout) and variation explained shown for each axis. PCoA was carried out using calculated Euclidean distances of NPPARs.

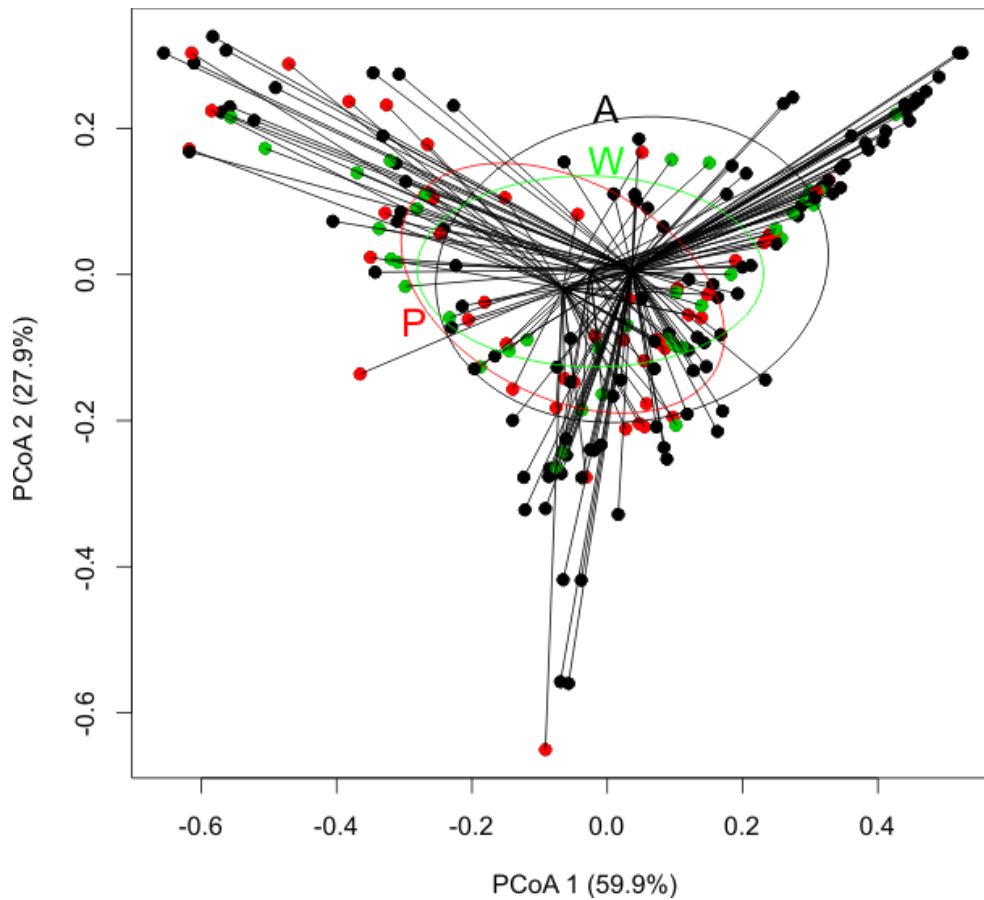


Figure 3.8| PCoA of Sheffield PROHEMI LHPCR samples with study period shown – study periods are shown on the plot (**P** = Pre-feeding, **A** = Active, **W** = Washout) and variation explained shown for each axis. PCoA was carried out using calculated Euclidean distances of NPPARs.

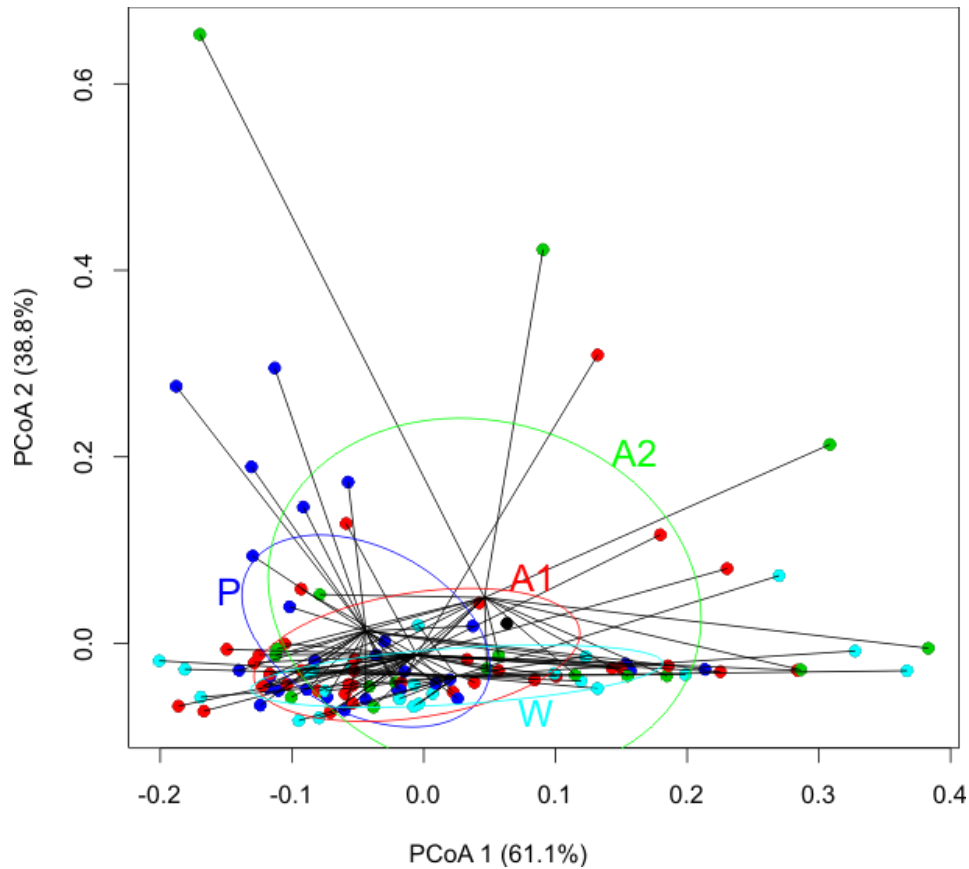


Figure 3.9| PCoA of Cardiff/Port Talbot PROHEMI LHPCR samples with study period shown – study periods are shown on the plot (**P** = Pre-feeding, **A1** = Active group 1, **A2** = Active group 2, **W** = Washout) and variation explained shown for each axis. PCoA was carried out using calculated Euclidean distances of NPPARs.

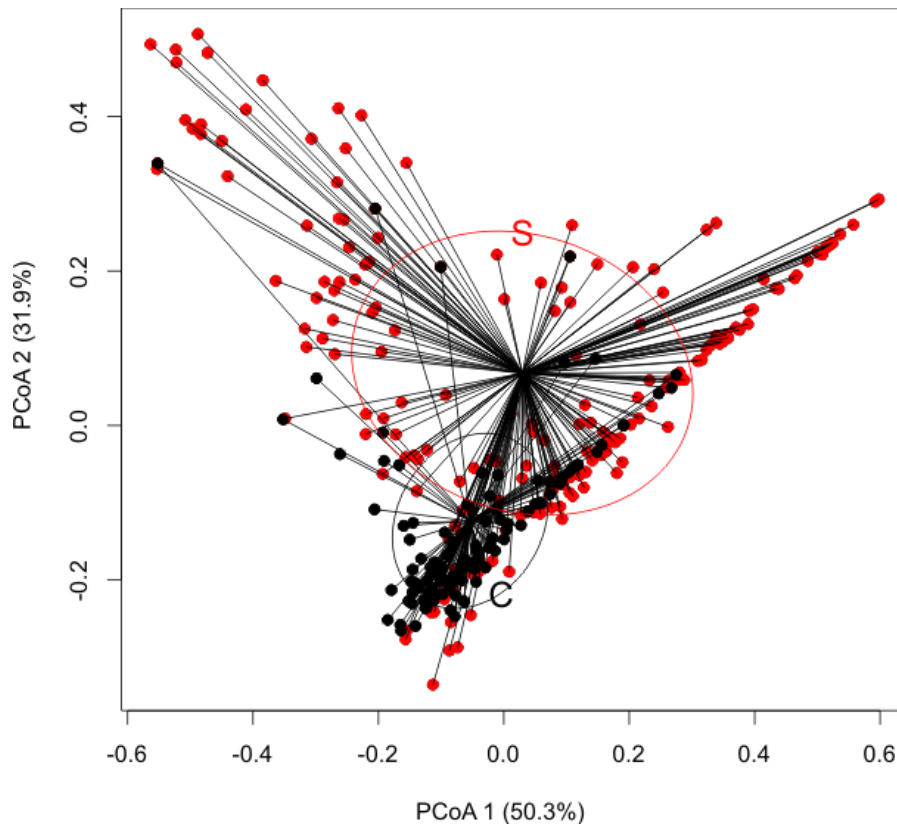


Figure 3.10| PCoA through distance of all PROHEMI LHPCR samples with study centre shown – study centres are shown on the plot (C = Cardiff/Port Talbot, S = Sheffield) and variation explained shown for each axis. PCoA was carried out using calculated Euclidean distances of NPPARs.

3.3.2.3 LHPCR analysis of all PROHEMI samples from Cardiff/Port Talbot and Sheffield based upon study centre and study period

Cluster analysis of all samples from both study centres suggests that samples cluster together due to the study centre and not study period (**Fig. 3. 11**). It is possible to see that Sheffield samples cluster together to the right hand side of the dendrogram, while Cardiff/Port Talbot samples cluster together to the left of the dendrogram. There are a few samples from Sheffield which cluster with the Cardiff/Port Talbot samples and vice versa. This would explain the slight overlap in clusters generated through PCoA (**Fig. 3. 10**). In addition a heatmap, generated through LHPCR analysis, was applied to the cluster analysis. The observed study centre clustering is driven by a difference in the community profiles of participants from the two study centres (**Fig. 3. 12**). Sheffield samples appear to have high proportional abundance of two to three peaks, as represented by the deep red colour. However, Cardiff/Port Talbot samples show a far lower proportional abundance of a range of peaks. This observation was further investigated through the generation of a heatmap from selected samples from different portions of the dendrogram (**Fig. 3. 13**). Sheffield samples cluster together and have a high proportional abundance of a peak at 356 base-pairs (bp). Cardiff/Port Talbot samples have a range of peaks at a lower proportional abundance. Samples from Cardiff/Port Talbot also have peaks at 365-367 bp which are absent to low in the Sheffield samples. Furthermore, it is possible to see that samples which cluster with samples from the other centre, do so due to similar NPPARs e.g. 6PH36 and 11PH7 (**Fig. 3. 13**). This information was combined with an *in silico* LHPCR experiment. Through the combination of these two methods, I hypothesise that the major driver for the separate clustering of Sheffield samples from Cardiff/Port Talbot samples, is the high peak area proportion seen in these samples at 356 bp. According to my *in silico* LHPCR experiment this fragment corresponds to the *Bacteroidetes* group of bacteria. Low and sometimes non-existent proportional peak area abundances at 363-367 bp in the Sheffield samples were also observed. The *in silico* LHPCR experiment suggest that the *Firmicutes* group of bacteria would generate fragments of this size. The high abundance of the 356 bp fragment coupled with the low and sometimes non-existent proportional peak area abundance observed between 365-367 bp, drives the separate clustering of these two study centres (**Appendix I**).

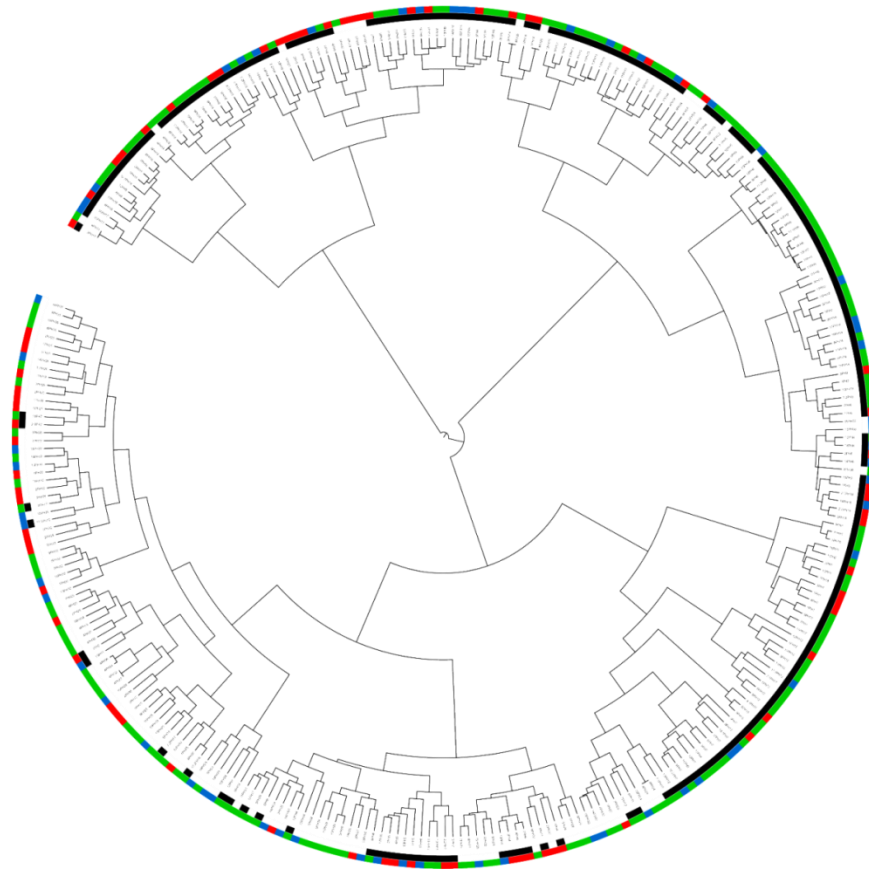


Figure 3.11 | Cluster analysis of all PROHEMI LHPCR samples with study centre and study period shown – study centre is shown on the inner ring (Cardiff/Port Talbot = blank and Sheffield = **black**) while study period is shown on the outermost ring (Pre-feeding = **red**, Active = **green** and Washout = **blue**).

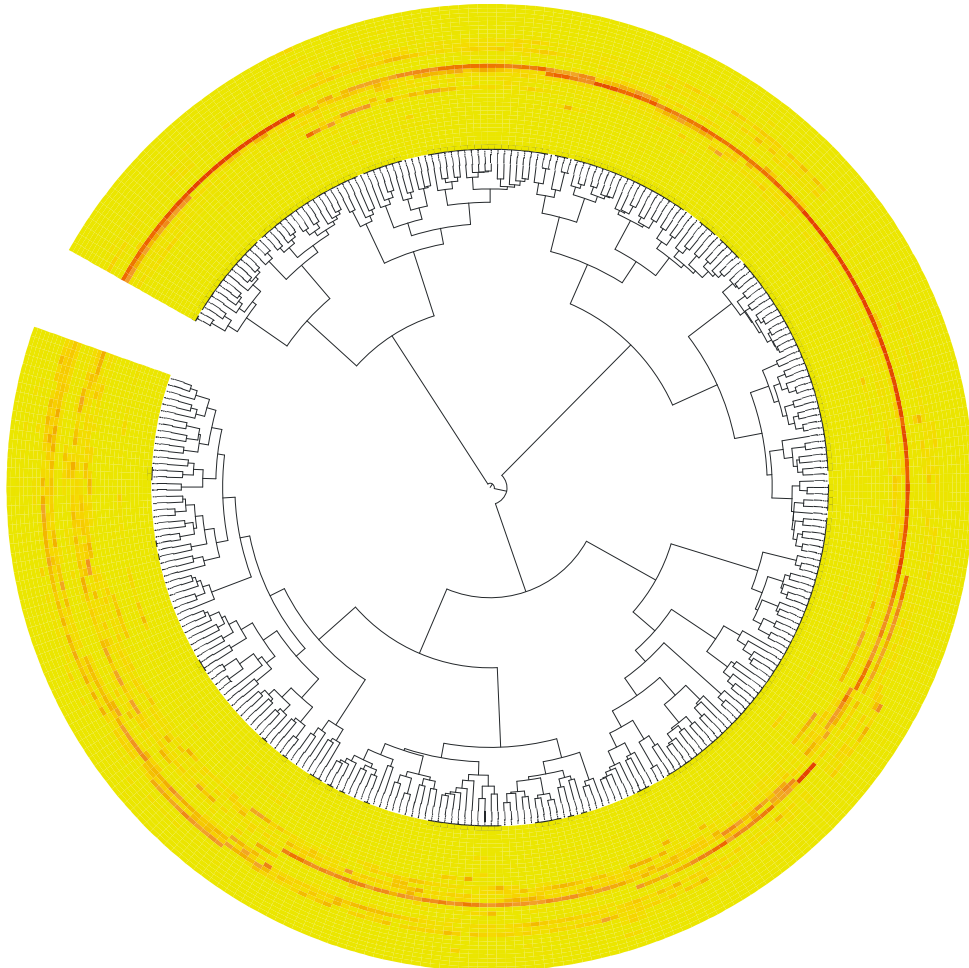


Figure 3.12| Cluster analysis of all PROHEMI LHPCR samples with a heatmap of the community profile, generated through LHPCR, applied – electropherograms, generated through LHPCR, for a given sample were converted into a heatmap and applied to the cluster analysis. Proportional abundance is represented through a colour continuum from yellow (lowest) to orange to red (highest). Sheffield samples are represented by a black line on the inner side of the heatmap while Cardiff samples have been left blank.

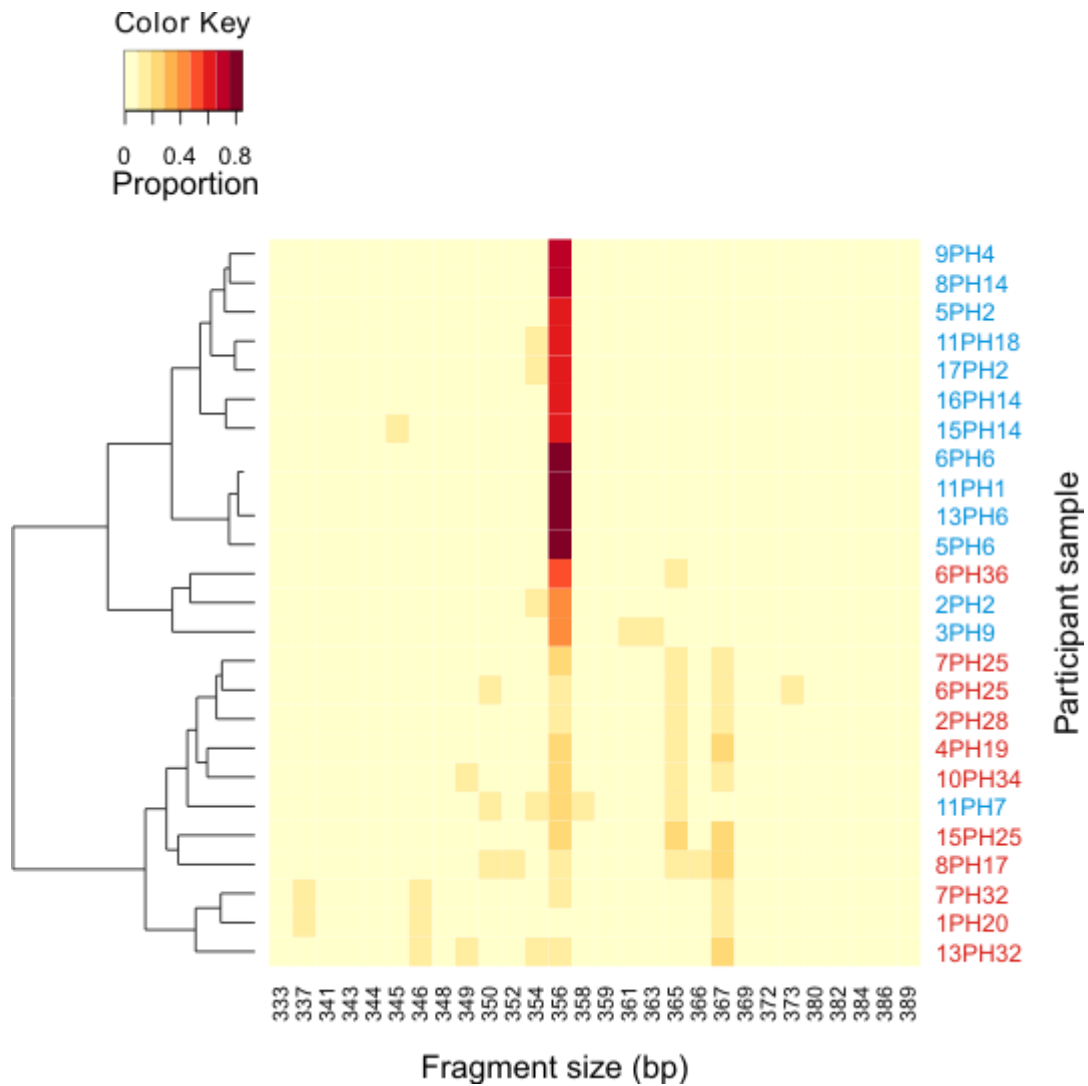


Figure 3.13| Heatmap of selected samples from different study centres – generated through LHPER analysis of PROHEMI samples. Participant sample is shown (Cardiff/Port Talbot = red and Sheffield = blue) with LHPER fragment size and a key for proportional abundance.

It is also possible to see this difference in centre proportional peak abundances in another heatmap, where all the treatment groups and study periods are represented along with the study centre (**Fig. 3. 14**). It is once again possible to see a high proportional abundance of the fragments at 356 bp and also 358 bp in the Sheffield samples with little to no proportional abundance of fragments between 365-367 bp. Cardiff/Port Talbot samples exhibit an increased distribution of fragment sizes at lower abundance levels. The proportional abundances of 356, 363, 365, 366 and 367 fragments were plotted in a box-plot (**Fig. 3. 15**) In order to determine whether there was a significant difference in these proportional abundances the Kruskal-Wallis H test was utilised. The data were not normally distributed therefore, this test was used. The ranked mean of the 356 bp fragment was significantly higher in Sheffield samples than Cardiff samples [$\chi^2 (1) = 42.349, p < 0.001$]. Conversely, the mean rank of fragments, at 365-367 bp, were significantly higher in the Cardiff samples than Sheffield samples ([$\chi^2 (1) = 113.763, p < 0.001$], [$\chi^2 (1) = 64.285, p < 0.001$] and [$\chi^2 (1) = 53.753, p < 0.001$] respectively).

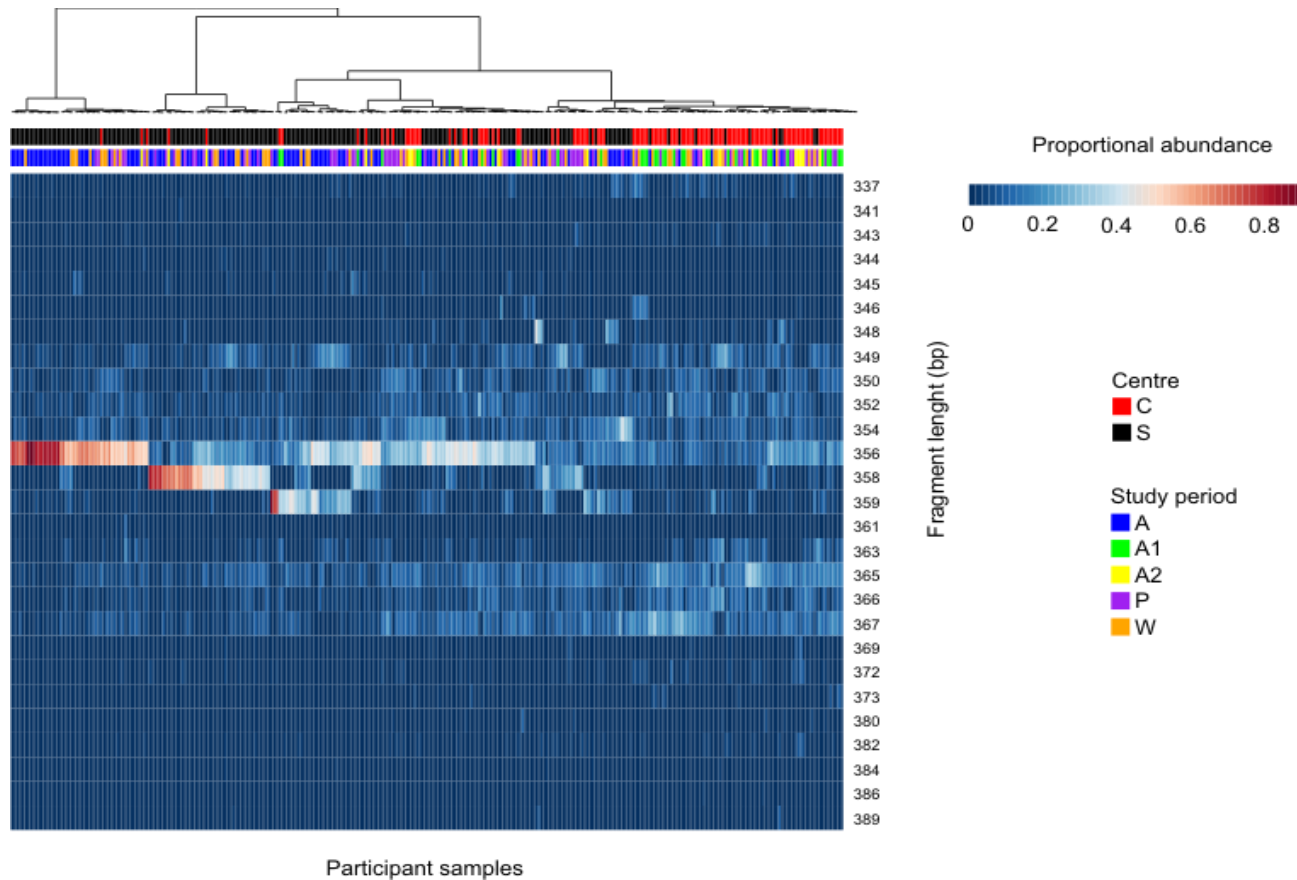


Figure 3.14 | Heatmap of all PROHEMI LHPCR samples – study centre is shown (C = Cardiff/Port Talbot and S = Sheffield) with study period (P = Pre-feeding, A = Active Sheffield, A1 = Active group 1 Cardiff/Port Talbot, A2 = Active group 2 Cardiff/Port Talbot and W = Washout) and proportional abundance key.

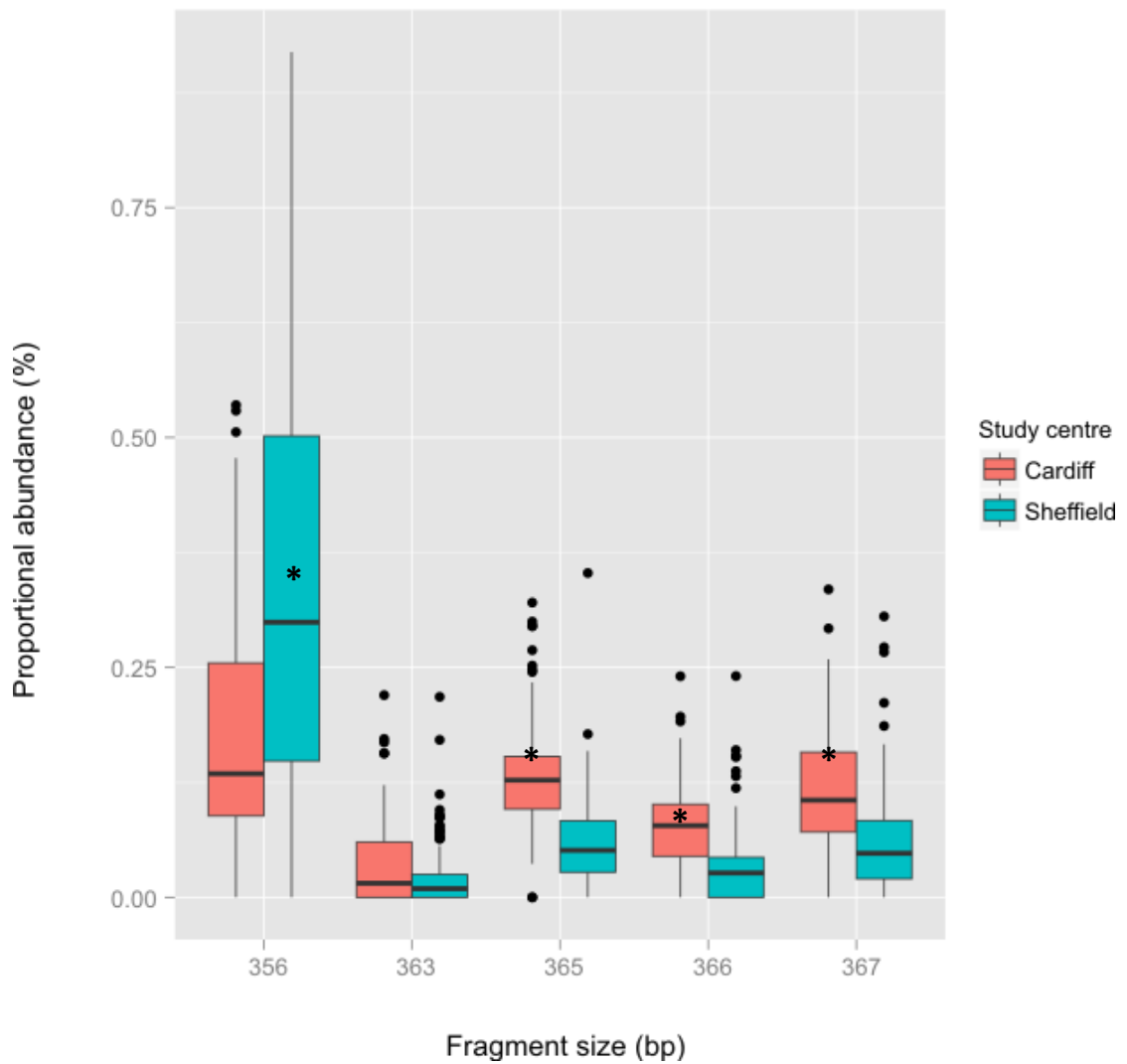


Figure 3.15 | The abundance of NPPARs from PROHEMI LHPCR samples – the fragment size is shown in bp with a key for the centre where the sample came from. Box-plots represent the median, first and third quartiles of a given group of samples while outliers are represented by black dots. *denotes a significantly higher mean rank ($p < 0.05$)

3.3.2.4 LHPCR analysis of Sheffield faecal samples and comparison with pervious LHPCR data

Stored faecal samples from Sheffield participants were obtained and DNA was extracted from these samples at Cardiff University. LHPCR analysis was carried out on the newly extracted DNA. Repeat fragment analysis was also carried out on LHPCR products obtained from the corresponding original Sheffield DNA extractions, which were stored at -20°C. The newly generated LHPCR profiles were compared to the original LHPCR profiles generated from Sheffield DNA extractions, and also the LHPCR profiles generated from stored LHPCR products. Therefore, there are 3 repeats of LHPCR analysis for participants from the same time point. The results show a stark difference between the samples. Freshly extracted DNA clustered separately from the stored LHPCR products and original LHPCR profiles (**Fig. 3. 16**). Further inspection through the generation of a heatmap shows the driver for separate clustering (**Fig. 3. 17**). While the original Sheffield extractions showed a high proportional abundance of the fragments at 365-367 bp, this is not seen in the later LHPCR analysis of DNA extracted from the same samples at Cardiff. In this instance what there appears to be little to no proportional abundance of these fragments. Instead there is a high proportional abundance of the 365 bp fragment. This is a complete reverse to what was originally seen. Fragment analysis of stored LHPCR products from the same original Sheffield samples cluster with the original Sheffield LHPCR profiles. This is likely as a result of the stability of the 6-FAM molecule as previously discussed.

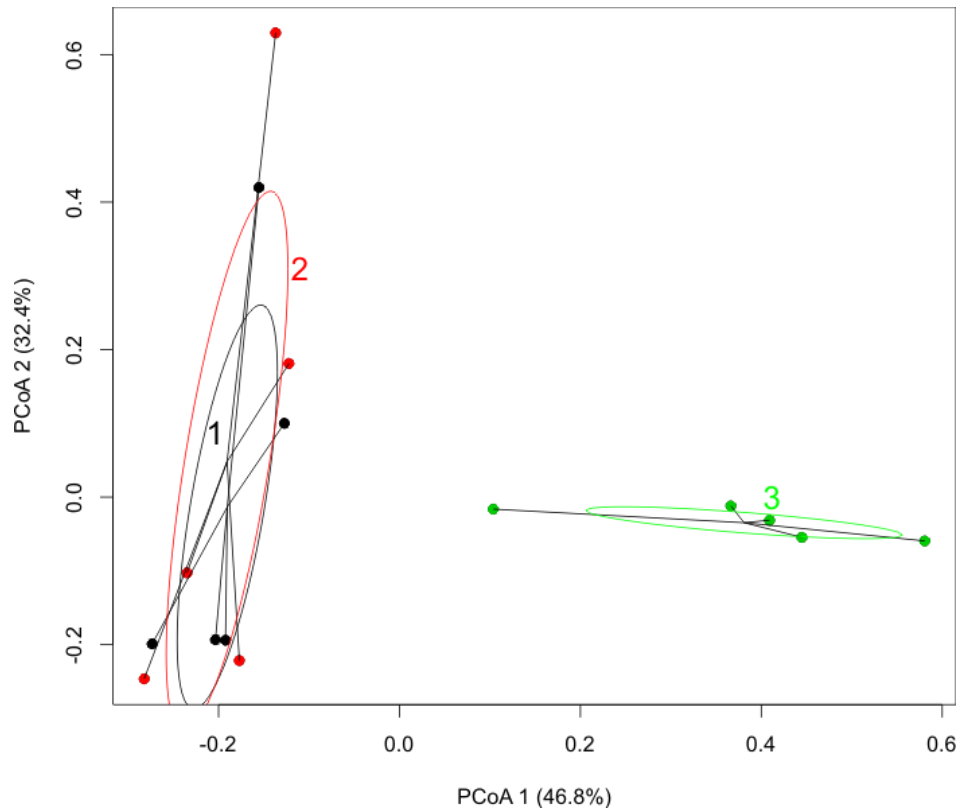


Figure 3.16 | PCoA of multiple LHPER analyses on the same samples after storage – samples are clustered together based on the condition (**1** = original LHPER profiles from DNA extracted at Sheffield, **2** = LHPER products of DNA extracted at Sheffield after storage at -20°C and **3** = LHPER profiles obtained from DNA extracted at Cardiff University after storage of the faecal sample). The variation explained for each axis is shown.

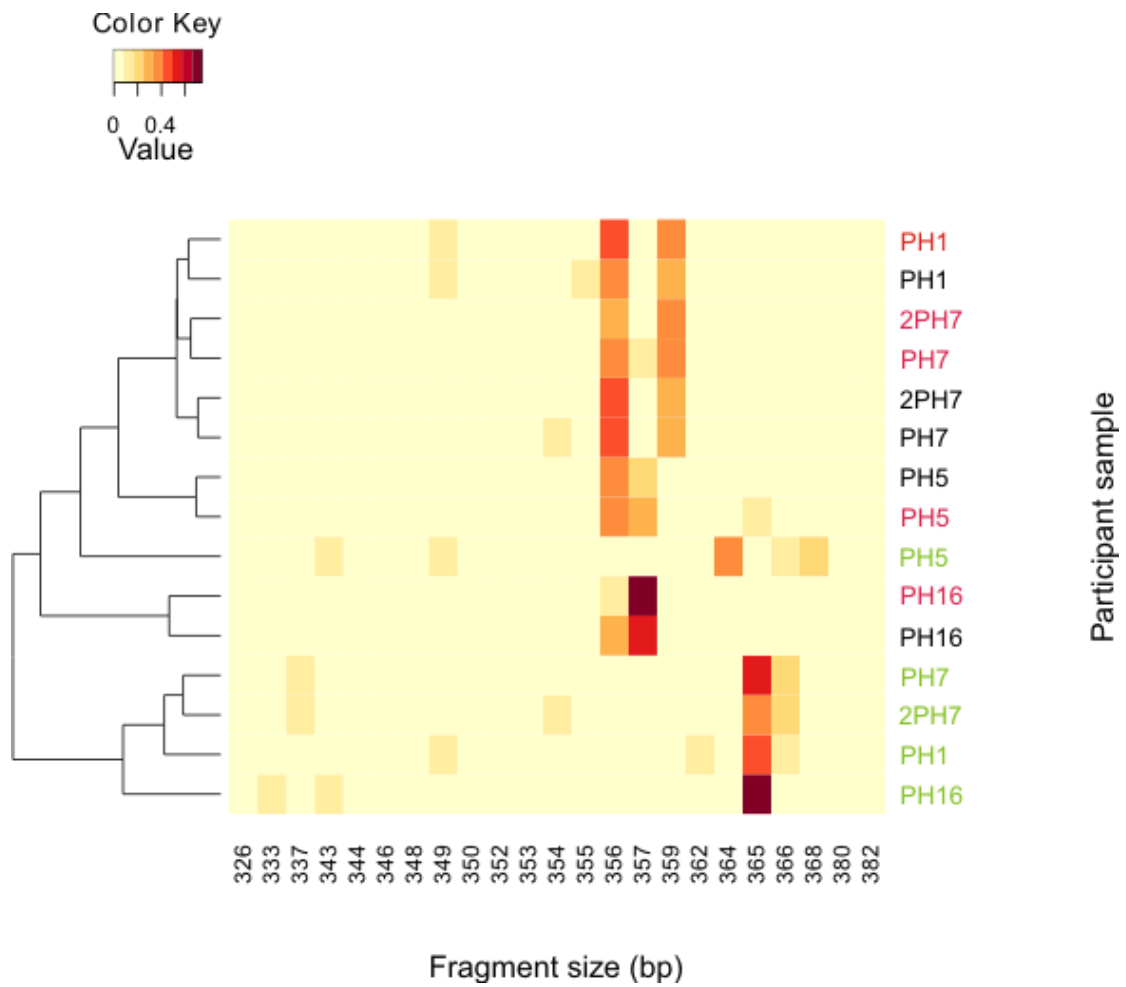


Figure 3.17| Heatmap of multiple LHPCR analyses carried out on the same samples after storage – the heatmap shows the proportional abundance of fragments within a sample. Conditions for each sample are indicated by colour with **black** = original LHPCR profiles from DNA extracted at Sheffield, **red** = LHPCR profiles of LHPCR products from DNA extracted at Sheffield after storage at -20°C and **green** = LHPCR profiles obtained from DNA extracted at Cardiff University after storage of the faecal sample.

3.3.2.5 Percentage changes in LHPCR profiles of all PROHEMI samples from Sheffield based upon study period

The average percentage change between LHPCR profiles within a given study period was calculated. One-way ANOVA showed no significant difference [F (2, 799) = 0.411, p=0.663] between the Pre-feeding, Active and Washout periods was observed (**Fig. 3. 18**). Percentage changes for Pre-feeding, Active and Washout periods were 19.61%, 19.3% and 16.39% respectively. The average percentage change was also calculated on an individual basis for each study period (**Fig. 3. 19**). There were different observed responses to probiotic administration. Participants PH6 and PH9 showed a lower percentage change during the Active period which increased upon cessation of the probiotic. However, participants PH1, PH4 and PH17 showed an increase in percentage change during the Active period which decreased upon cessation of the probiotic. In addition, some participants such as PH5 and PH16, showed little response to probiotic administration with similar percentage changes for all study periods. It was not possible to calculate percentage changes for some participants, such as PH12 and PH18, due to a lack of samples.

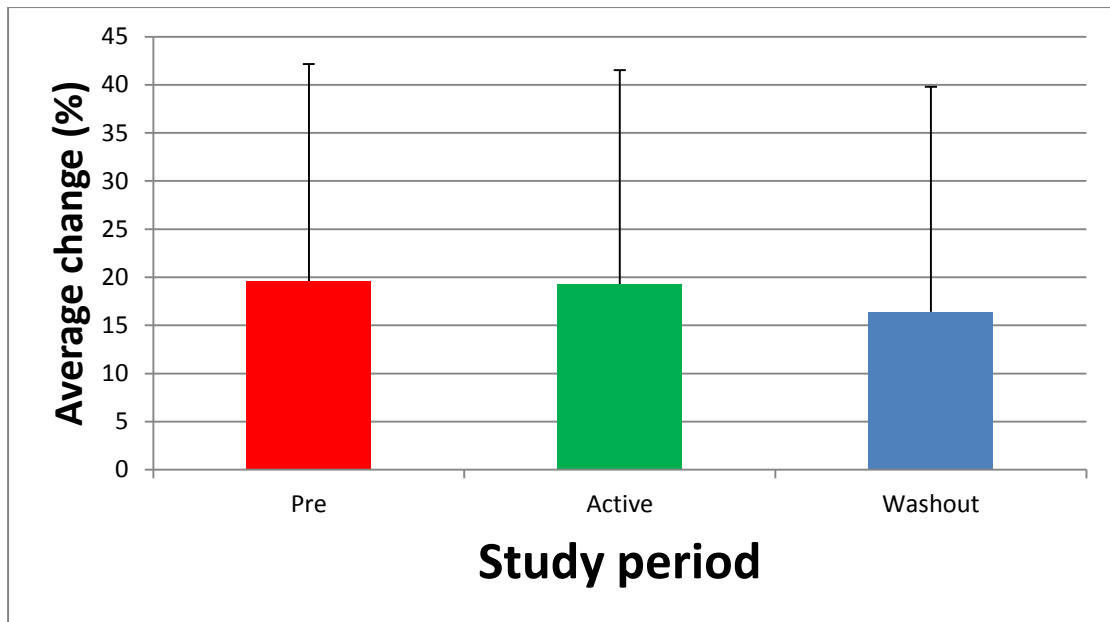


Figure 3.18| Percentage change between Sheffield LHPCR samples within a given study period – the average percentage change between LHPCR profiles from Sheffield within a given study period is shown with error bars representing the standard deviation from the mean.

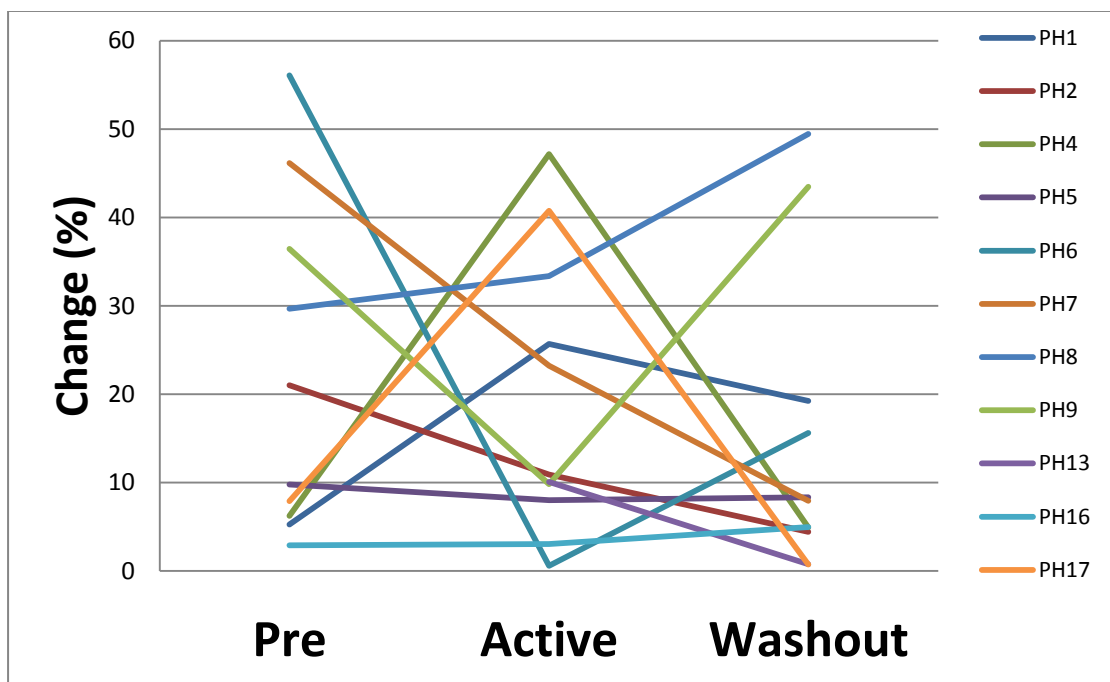


Figure 3.19| Percentage change between Sheffield participants' LHPCR samples within a given study period – the average percentage change in LHPCR profiles of individuals from Sheffield within a given study period is shown.

3.3.3 Ribosomal Intergenic Spacer Analysis (RISA) of faecal DNA

RISA profiles of 3 individuals at two different time points (before the feeding study and one week into the study) were tested along with an extra individual's sample from before the commencement of the study (**Fig. 3. 20**). Three DNA extractions from a single sample (PH27 1, PH27 2 and PH27 3) were also compared. The RISA products obtained were bunched closely at the 700-1000bp region, making it difficult to identify inter and intra individual differences (**Fig. 3. 21**). The RISA products were then loaded onto a 7500 chip and the products were once again bunched (**Fig. 3. 22**). Pearson's correlation coefficient analysis of the two chips shows $\geq 95\%$ similarity with the 1000 chip and $\geq 98\%$ with the 7500 chip. Both inter and intra individual differences are highly similar.

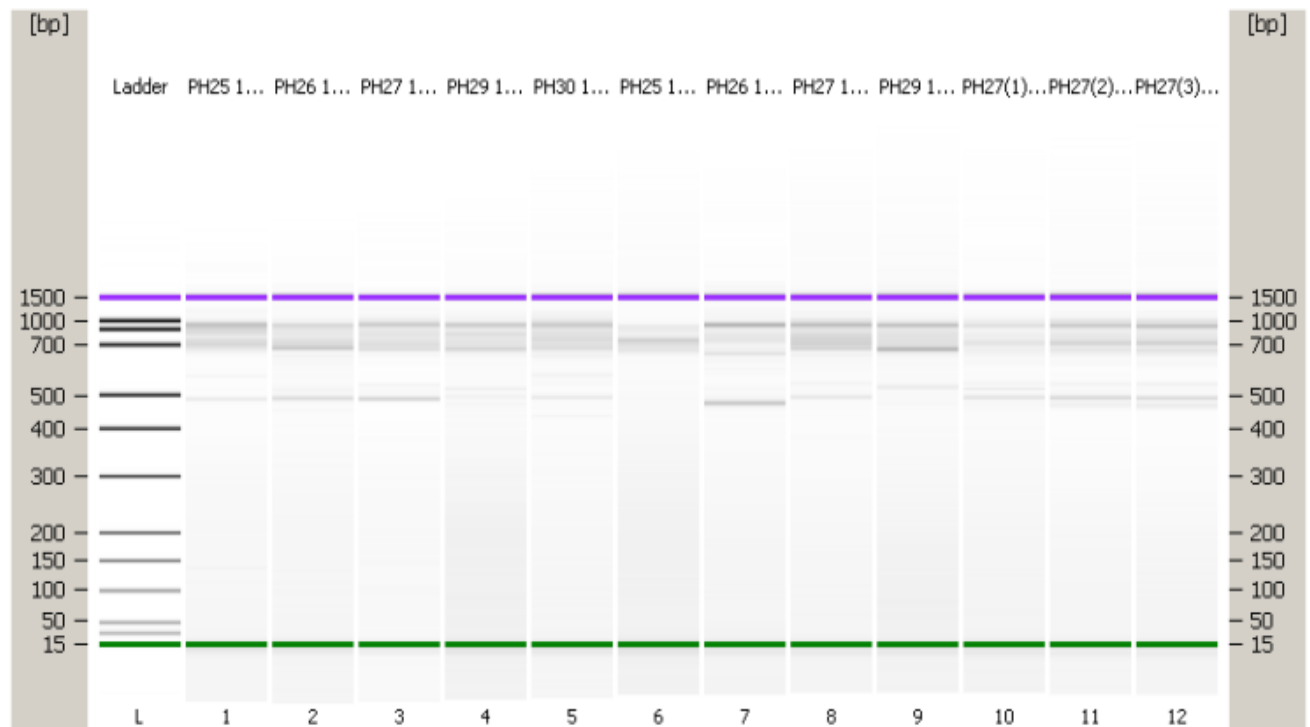


Figure 3.20 | RISA of faecal DNA from the PROHEMI study - lanes 1-5 show the RISA profiles participants PH25, PH26, PH27, PH29 and PH30 respectively at the beginning of the feeding study. Lanes 6-9 show the RISA profiles participants PH25, PH26, PH27 and PH29 respectively following one week of probiotic supplementation. While lanes 10-12 show the RISA profiles of 3 DNA extractions from one homogenised faecal sample.

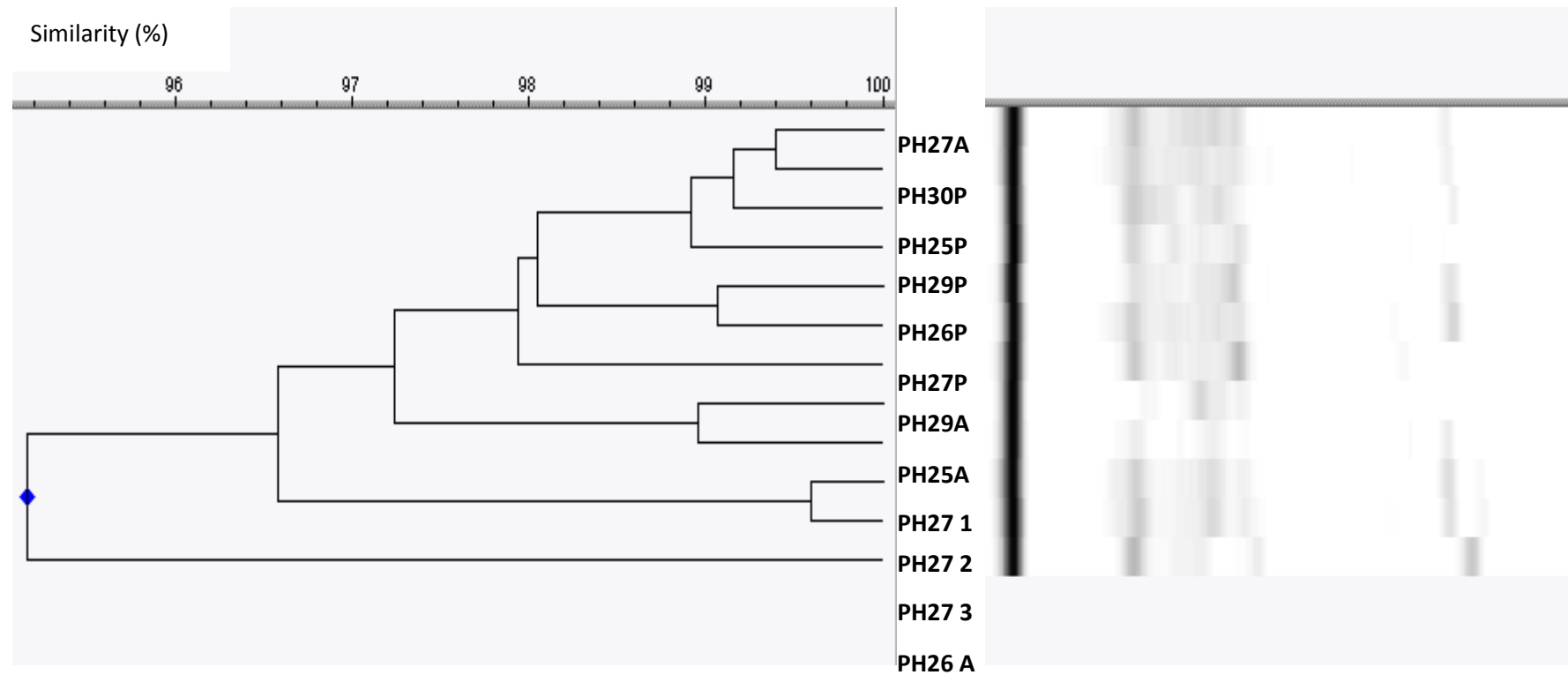


Figure 3.21| Composite dendrogram generated through RISA of PROHEMI samples using the Bioanalyzer 1000 chip – samples are clustered based on percentage similarity where P = Pre-feeding and A= Active. The analysis also contains three sets of RISA products from a single DNA extraction of DNA obtained from participant PH27. The figure shows the RISA profile generated for each sample also.

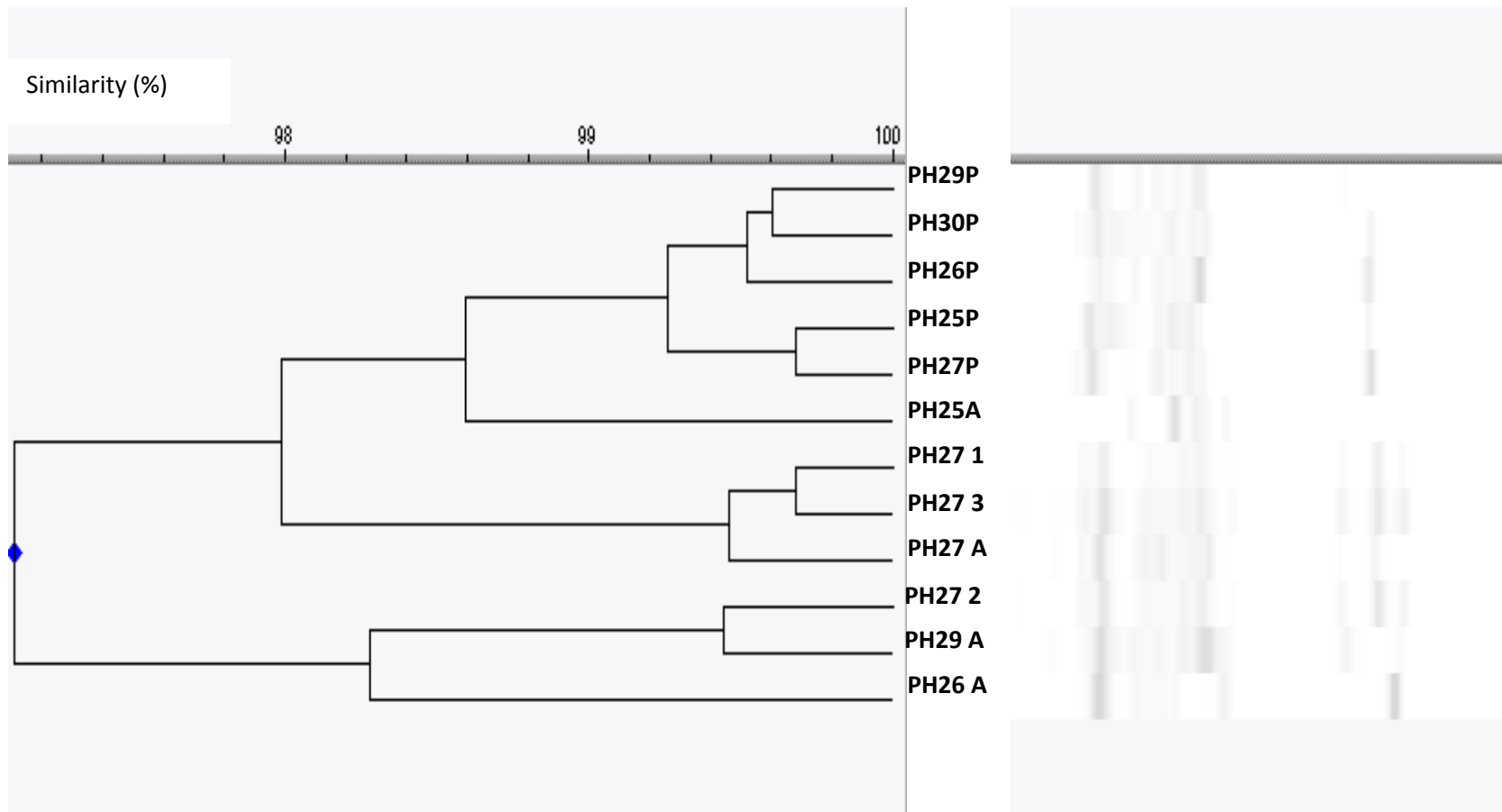


Figure 3.22| Composite dendrogram generated through RISA of PROHEMI samples using the Bioanalyzer 7500 chip – samples are clustered based on percentage similarity where P = Pre-feeding and A= Active. The analysis also contains three sets of RISA products from a single DNA extraction of DNA obtained from participant PH27. The figure shows the RISA profile generated for each sample also.

Table 3.3| Pearson's correlation coefficient analysis of RISA products obtained from a Bioanalyzer 1000 chip

	Sample												
	PH27A	PH30P	PH25P	PH29P	PH26P	PH27P	PH29A	PH25A	PH27 1	PH27 2	PH27 3	PH26A	
PH27A	1.000												
PH30P	0.994	1.000											
PH25P	0.993	0.991	1.000										
PH29P	0.99	0.993	0.988	1.000									
PH26P	0.985	0.989	0.981	0.984	1.000								
PH27P	0.989	0.991	0.988	0.989	0.991	1.000							
PH29A	0.989	0.986	0.98	0.985	0.983	0.98	1.000						
PH25A	0.979	0.987	0.977	0.986	0.984	0.98	0.966	1.000					
PH27 1	0.988	0.993	0.985	0.986	0.987	0.989	0.977	0.99	1.000				
PH27 2	0.99	0.999	0.986	0.977	0.983	0.989	0.977	0.976	0.993	1.000			
PH27 3	0.988	0.985	0.985	0.971	0.976	0.982	0.977	0.967	0.988	0.996	1.000		
PH26A	0.974	0.974	0.974	0.968	0.955	0.964	0.965	0.951	0.966	0.97	0.97	1.000	

The table contains 5 Pre-feeding samples from different participants (P), 4 Active samples (A) from different participants and 3 RISA products from different DNA extractions from PH27 at a single time-point (PH27 1, PH27 2 and PH27 3).

Table 3.4| Pearson's correlation coefficient analysis of RISA products obtained from a Bioanalyzer 7500 chip

	Sample												
	PH29P	PH30P	PH26P	PH25P	PH27P	PH25A	PH27 1	PH27 3	PH27A	PH27 2	PH29A	PH26A	
PH29P	1.000												
PH30P	0.996	1.000											
PH26P	0.996	0.995	1.000										
PH25P	0.996	0.994	0.993	1.000									
PH27P	0.995	0.994	0.995	0.997	1.000								
PH25A	0.99	0.987	0.989	0.993	0.989	1.000							
PH27 1	0.995	0.996	0.992	0.991	0.991	0.986	1.000						
PH27 3	0.992	0.993	0.999	0.993	0.988	0.984	0.997	1.000					
PH27A	0.994	0.993	0.99	0.992	0.991	0.987	0.995	0.996	1.000				
PH27 2	0.999	0.988	0.988	0.991	0.99	0.989	0.99	0.993	0.993	1.000			
PH29A	0.987	0.983	0.985	0.987	0.985	0.986	0.982	0.987	0.993	0.995	1.000		
PH26A	0.986	0.979	0.98	0.984	0.982	0.983	0.981	0.981	0.986	0.986	0.985	1.000	

The table contains 5 Pre-feeding samples from different participants (P), 4 Active samples (A) from different participants and 3 RISA products from different DNA extractions from PH27 at a single time-point (PH27 1, PH27 2 and PH27 3).

3.3.3.1 Automated Ribosomal Intergenic Spacer Analysis (ARISA) of faecal DNA

RISA profiles were successfully generated from faecal DNA using the newly 6-FAM labelled forward primer (Fig. 3. 23). However, analysis using GeneMarker and analysis using the ADAPT system was not possible due to the generation of incomplete fragment analysis files.

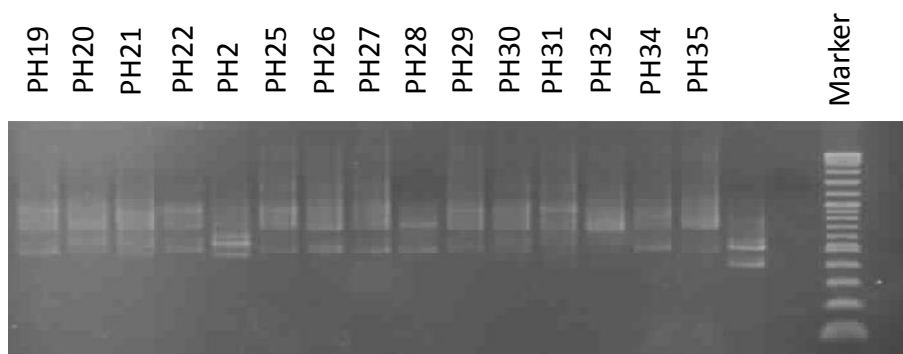


Figure 3.23 |. Gel electrophoresis of ARISA products using a 6-FAM labelled forward primer – successful amplification of 16 faecal DNA samples with a negative control and 2-log ladder.

3.4 Discussion

3.4.1 Length heterogeneity PCR (LHPCR)

LHPCR has previously been used as a community fingerprinting method using faecal DNA as the template material. However, studies such as these are often short-term and often have a limited number of subjects, e.g. one patient and only 3 samples (Bjerketorp *et al.* 2008), or sampling only occurs once (Suzuki *et al.* 1998). The long-term PROHEMI study scrutinised the suitability of LHPCR as a viable fingerprinting method for long-term use and for multiple individuals. While all LHPCR primer combinations showed promise, with the exception of the DT-LHPCR primers, it was decided that the primer combination of 27F and 358R would be used. This primer combination has previously been applied to the human gut (Bjerketorp *et al.* 2008). Pearson's correlation coefficient analysis of multiple DNA extractions from one individual, shows that the extraction method can impact upon the LHPCR profile obtained. However, LHPCR analysis itself appears to be highly reproducible as intra-sample similarity is higher than inter-sample similarity. This is provided that LHPCR samples are subjected to fragment analysis as soon as possible due to stability of the 6-FAM label.

Following primer and reproducibility testing, LHPCR was applied in order to monitor the community fingerprints of individuals' faecal DNA samples during the course of the study. The results show no gross change in the LHPCR profiles when samples are analysed within distinct period groups. Samples from each period and group (Pre-feeding, Active Sheffield, Active group 1, Active group 2 and Washout) cluster together through AGNES clustering and PCoA. Percentage change analysis was also carried out on Sheffield samples alone and showed no significant difference between the Pre-feeding, Active and Washout periods. This suggests that probiotic supplementation does not alter the gross bacterial community of a healthy individual. In order to scrutinise the dataset further, information from my *in silico* LHPCR experiment, fragment sizes of the probiotic organisms and LHPCR data from the PROHEMI samples were combined. There was no increase in fragment abundance at 372 bp or 351 bp, the fragment sizes expected to be generated by *L. acidophilus* (Cul 21 and 60) and *B. infantis* (Cul 20) respectively from my analyses, while probiotic supplementation takes place. It must be noted that the dendrogram generated through AGNES clustering of LHPCR samples shows clustering of Active Sheffield samples. However, I do not believe that this is due to probiotic supplementation. Instead, I believe this is due to geographical differences in the distal gut bacterial community of individuals and this will be discussed later.

Analysis was also carried out on an individual basis using Sheffield samples alone. This was in order to ensure that participants who potentially showed a response to probiotic treatment were not masked by those who did not. Varied responses to the consumption of the probiotic were observed. Some participants, such as PH4, show an increase in percentage change while consuming the probiotic supplement suggesting a bacterial modulating effect. However some participants, such as PH6, show a decrease in percentage change while consuming the probiotic suggesting a potential stabilising effect. This observed duality in responses is further complicated by participants, such as PH16, who show little change in response to probiotic supplementation. It is therefore difficult to draw any conclusions from this particular analysis. It may be that there is a case of responders to supplementation against non-responders. The incidence of responders and non-responders to probiotic treatment is not unheard of (Reid *et al.* 2010). This phenomenon is often seen when the infirm are treated with probiotics. However, these results suggest that this occurs in healthy individuals also. The responders group is further complicated by different responses. This suggests that the response to probiotic supplementation is individual and differs from person to person. While some individual changes were observed during probiotic treatment, there is no clear response across the whole probiotic taking cohort. This makes the role of probiotic supplementation in modulating the gut bacterial community difficult to elucidate.

Analysis of LHPCR data was carried out based upon study period also, with Cardiff/Port Talbot samples being compared to Sheffield samples. The analyses show separate clustering of samples based upon study centre. PCoA shows separate clustering of the two study locations in agreement with the dendrogram generated through AGNES clustering. In order to ascertain the driving force between the observed clustering a heatmap was applied to the dendrogram. There is a large abundance of a few fragments in the Sheffield samples while there is a lower abundance of a larger range of fragments in the Cardiff/Port Talbot samples. Where samples from Cardiff/Port Talbot cluster with Sheffield samples, it is possible to see a similar fingerprint profile in Cardiff/Port Talbot samples to the Sheffield samples and vice versa. Samples from each cluster were selected and a heatmap generated in order to determine the fragment sizes observed. This data was once again combined with my *in silico* LHPCR experiment in order to assign an approximate taxonomic identity to the fragment of a given size. Following the *in silico* LHPCR experiment I believe that the Sheffield samples have a high abundance of *Bacteroidetes* fragments as shown by the significantly high proportional abundance of the 356 bp fragment. The Sheffield samples show a significantly lower proportional abundance of fragments at 365-367 bp than the Cardiff samples. The *in silico*

LHPCR experiment suggests that the *Firmicutes* would generate fragments of this size and are therefore present at low numbers in the samples obtained from Sheffield (**Appendix I**). Investigation of the samples obtained from Cardiff/Port Talbot shows a greater distribution of fragments at lower proportional abundance. In addition, the putative fragment sizes generated by the *Bacteroidetes* and *Firmicutes* are generally evenly represented in samples from Cardiff/Port Talbot.

It has been noted previously that the ratio of *Firmicutes/Bacteroidetes* alters with age (Mariat *et al.* 2009). The authors show that the level of *Firmicutes* is lower in infants than in adults and the elderly. However, *Bacteroidetes* levels remain fairly uniform in infants, adults and the elderly. The participants in the study were all adults and not elderly. Therefore, the results suggest geographical differences between the gut bacterial community of individuals as Cardiff and Port Talbot are in Wales, while Sheffield is in the north of England. It has been shown previously that geographical differences can drive changes in the gut bacterial community of an individual, usually with diet being the major driver. The gut bacterial community of children from the European Union (EU) has been compared to children from Burkina Faso (De Filippo *et al.* 2010). Children from the EU had a significantly higher level of *Firmicutes* than children from Burkina Faso (63.7% vs. 27.3% respectively, $p = 7.89 \times 10^{-5}$). The expected reverse was shown in children from Burkina Faso as a significantly higher level of *Bacteroidetes* was observed compared to children from the EU (57.7% vs. 22.4%, $p = 1.19 \times 10^{-6}$). The believed reasons behind this difference are different diet habits. In another study PCoA of unweighted Unifrac distances derived from 16S *rRNA* datasets, show separate clustering of USA adults from Malawians and Amerindians. The Malawian and Amerindian populations clustered together (Yatsunenکو *et al.* 2012). These studies show differences between very different populations, with different ethnicities and from very different countries.

However, my results suggest a possible difference in the gut bacterial community of individuals based on geographical location in the UK. I believe that the *Firmicutes/Bacteroidetes* ratio is responsible for the clustering of Active Sheffield samples. During the Active period of the study a larger number of samples were provided. Therefore, the *Firmicutes/Bacteroidetes* ratio difference gives the appearance of clustering due to probiotic supplementation. While a geographical difference in gut microbiota composition is an interesting conclusion it would be prudent to meet this conclusion with some scepticism. Due to the nature of this study, DNA extractions were carried out at the two study centres and not at one alone. However, the apparent lack of *Firmicutes* in the Sheffield samples immediately raises the question of whether DNA extraction protocol was followed correctly; particularly the need to bead beat the samples. In order to ensure that there were no lab generated differences, I carried out DNA extractions on faecal samples from Sheffield which had DNA extracted previously. When compared, there was a completely different community fingerprint from the original extracted DNA observed. That is to say that the DNA extraction which I carried out generated different results to the DNA which was originally extracted at Sheffield. When the more recent DNA extractions were compared to their respective original DNA extractions, a difference in the fragments and abundances generated was seen. The recent extractions showed a high proportional abundance of fragments at 365-367 bp, putatively *Firmicutes*, and a low proportional abundance of the 356 bp, putatively *Bacteroidetes*. This observable antithesis between community fingerprints obtained from the same faecal samples is most likely due to the effect of storage on the faecal sample. The effect of storage and the effect of DNA extraction method on the bacterial community acquired has been discussed in **Chapter 6**. It is difficult to say with complete certainty that there is a geographical difference in the gut bacterial community of individuals in this study. Unfortunately, the possibility of a methodological artefact in this instance cannot be ruled out.

LHPCR has been successfully applied to a range of complex ecosystems. The technique has been applied to monitor changes in bacterial composition of industrial processes. Tiirola and colleagues monitored the change in bacterial composition of a thermophilic biofilm process for the treatment of pulp and paper mill white-water lining (Tiirola *et al.* 2003). For this study, a different primer combination was used. The primer fD1 (5'-AGAGTTTGATCCTGGCTCAG-3'), which was designed to amplify the majority of eubacteria (Weisburg *et al.* 1991) was used as a forward primer in combination with 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer *et al.* 1993). In this instance the reverse primer was labelled with the fluorescent molecule IRD-800. Differences in LHPCR profiles were observed between attached and suspended biomass. Furthermore, LHPCR profiles altered following pH shocks in the biofilm. This shows that LHPCR can discern differences in communities and also can detect differences in the bacterial community following perturbations. LHPCR has also been applied in order to monitor the succession of LAB during the ensiling of maize (Brusetti *et al.* 2006). In this study, the universal primer 27F was used in conjunction with 338R primer (Rogers *et al.* 2003). The technique showed changes in the microbial composition as the fermentation process progressed from day 0 to day 30. This study shows that the technique can be used in order to measure the differences in a microbial community temporally.

While LHPCR is a useful technique for comparing microbial communities in terrestrial ecosystems and industrial processes, it has also been applied to assess changes within the human body. Sputum from cystic fibrosis patients was compared using this technique (Rogers *et al.* 2003). The previously mentioned reverse primer 338R was used in conjunction with 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), which was labelled with 700IR. The technique showed differences in the bacterial community of individuals. The technique showed 10 distinct LHPCR profiles for 14 cystic fibrosis individuals. The technique has also been utilised in order to assess whether alcoholism affects the colonic microbiome (Mutlu *et al.* 2012). Faecal material was not the source of DNA in this study instead; biopsies of the colonic tissue were taken. The study used the universal primer 27F labelled with 6-FAM in conjunction with 355R (5'-GCTGCCTCCCGTAGGAGT-3') in order to generate LHPCR profiles. PCoA and plotting of the first, second and third components showed that alcoholics with and without alcoholic liver disease clustered away from the healthy controls. The results show that LHPCR is a suitable technique in order to determine lifestyle driven differences in the composition of the mucosa-associated bacterial community.

Mutlu and colleagues used colonic biopsy material as a source of DNA (Mutlu *et al.* 2012). However, faecal material has also been used as a source of DNA. Bjerketorp and colleagues used faecal samples of a healthy man before, during and after antibiotic intervention as a source of DNA for LHPCR analysis (Bjerketorp *et al.* 2008). The primers used, 27F and 358R, were utilised in this study. The reproducibility of this primer pair was tested and also applied in order to determine differences in the bacterial community composition following antibiotic therapy. The technique shows differences in the LHPCR profiles between the three samples from different times of treatment. Preliminary testing of this primer combination during the PROHEMI study also showed that the technique is reproducible. While the technique itself is highly reproducible, variability in LHPCR profiles was generated through DNA extraction. Researchers should be aware of this before drawing biological conclusions on a low sample dataset. This method can be applied to a large study in order to assess changes in bacterial community composition of the human distal microbiome. The method is relatively high-throughput and inexpensive to carry out. Researchers may use the technique, as Mutlu and colleagues (Mutlu *et al.* 2012) have, in order to select samples of interest for next generation sequencing.

3.4.2 Ribosomal Intergenic Spacer Analysis (RISA) and Automated Ribosomal Intergenic Spacer Analysis (ARISA)

RISA has been successfully applied to monitor changes in complex ecosystems such as the soil (Ranjard *et al.* 2001) and freshwater (Fisher and Triplett 1999). The technique has also been applied to monitor gut bacterial DNA in faecal material, obtained from colorectal cancer patients (n=20) (Scanlan *et al.* 2008). Patients were followed for a period of 3 months, showing the suitability of the technique to monitor bacterial community fingerprints over a period of time. Due to its precedent of use in complex ecosystems, the aim was to use RISA/ARISA as another community fingerprinting method to complement LHPCR. Initially RISA samples were separated using the Bioanalyzer 1000 chip. The chip used in this instance provided poor fragment separation as samples from different individuals showed $\geq 95\%$ similarity. I therefore separated the RISA fragments through the use of a Bioanalyzer 7500 chip. Inspection of the composite dendrogram shows that the 7500 chip also provided poor fragment separation. Further analysis through Pearson's correlation coefficient analysis shows that samples from different individuals show $\geq 98\%$ similarity when RISA products are separated using the 7500 chip. Therefore this chip was poorer than the 1000 chip in terms of fragment separation. Due to the encountered problems with poor

separation of bands using the Bioanalyzer microfluidics platform I experimented with the use of ARISA. The forward RISA primer was labelled with the fluorescein molecule 6-FAM with the aim to carry out fragment analysis in a similar manner to LHPCR. Unfortunately, fragment analysis of these samples generated incomplete files which did not contain the size standard in its entirety and did not contain complete ARISA profiles. Therefore, downstream processing of the samples could not be carried out. I believe that the size of fragments generated from ARISA exceeds the capabilities of the ABI 3130xl machine and through this generates incomplete files.

Owing to the results from RISA and ARISA, it was decided not to apply this technique to the dataset. RISA profiles generated through the use of the Bioanalyser microfluidics platform were too similar, due to poor fragment separation, to discern small differences. LHPCR showed a greater amount of inter-sample variation than RISA and was therefore seen as a more viable method to use in the study. It was hoped that ARISA could be used in conjunction with LHPCR as fragment analysis using the ABI 3130xl machine would have provided greater fragment separation than the Bioanalyser. However, the generation of incomplete fragment analysis files ensured that this technique could not be utilised in this study.

3.5 Conclusions

The main conclusions for this chapter are as follows:

1. LHPCR can be used successfully in order to monitor gross changes in the community fingerprint of faecal DNA;
2. probiotic supplementation does not appear to modify the gross community fingerprint of faecal DNA;
3. there appears to be a geographical difference in the community fingerprint of faecal DNA of PROHEMI participants with a difference in the ratio of *Bacteroidetes* to *Firmicutes* observed;
4. through an *in silico* LHPCR experiment it is possible to assign broad taxonomic identities to peaks in LHPCR profiles.

4. CULTURE DEPENDENT ANALYSIS OF FAECAL MATERIAL AND FAECAL WATERS FROM THE PROHEMI STUDY

4.1 Introduction

In recent years there has been more of a focus on the use of culture independent methods in order to analyse complex ecosystems. These ecosystems include the soil (Janssen 2006), freshwater (Glöckner *et al.* 2000) and humans (The Human Microbiome Consortium 2012). While 16S *rRNA* gene cataloguing of ecosystems has become fairly routine, due to the reduction in sequencing costs, the technique does not directly show the functions expressed within an ecosystem. In order to meet this requirement PICRUSt was developed in order to predict the functions present within a community using only 16S *rRNA* genes as the starting point (Langille *et al.* 2013). However, the technique only infers possible expression and presence of functions. Metagenomic libraries may be screened for functions of a particular interest, through functional metagenomics in a surrogate host. This technique has been applied to identify functional bile salt hydrolases (BSH) in the human gut (Jones *et al.* 2008). Studies such as this hint at the functions which may be expressed within a given community. Alternatively, RNA can be converted into complementary DNA (cDNA) in order to create a metagenomic library of expressed genes. The technique has been applied in order to analyse ocean surface waters (Frias-Lopez *et al.* 2008). Drawbacks with the use of metagenomic interrogation strategies have previously been discussed (de Lorenzo 2005), and include the inability of a host to correctly express the gene of interest.

While the importance of community profiling of 16S *rRNA* gene sequencing cannot be overlooked, it is also important to understand the functions which are being expressed. With regards to our microbiome, it is estimated that 100-fold more unique genes are expressed by this virtual organ (Qin *et al.* 2010). Bacterial encoded enzymes, which may have previously seemed insignificant, are now beginning to attract attention. Polysaccharides, including starch and pectin, have been shown to be broken down by bacterial glycosidases and polysaccharidases (Englyst *et al.* 1987). Recent research has also shown that bacterial encoded β -glucuronidases are also important. These enzymes, expressed in the gut, are responsible for a high level of free catecholamines in the gut lumen (Asano *et al.* 2012). β -glucuronidases in the gut free catecholamines from their conjugated inactivated forms, with dopamine the highest catecholamine produced. Furthermore, bacterial β -glucuronidases affect the body's excretion of toxic compounds, undoing the process of glucuronidation. It

has been shown that the glucuronide form of bisphenol A, an oestrogen which has been shown to damage the reproductive system of animals, is deconjugated in the cecum of rats (Sakamoto *et al.* 2002). It has also been shown that the glucuronidation of the carcinogen 2-amino-3-methylimidazo[4, 5-f] quinioline is reversed by bacterial β -glucuronidase (Humboldt *et al.* 2007). Deconjugation of its inactive form releases the active carcinogen in the gut lumen. Research such as this shows the pivotal role that bacterial β -glucuronidases have upon the body's excretion of toxic compounds.

As well as producing enzymes to break down carbohydrates, gut bacteria also produce proteases. Proteases have been implicated in the progression of IBS and IBD. Research has shown that proteolytic activity was 2-3 and 6-fold higher in IBS and IBD patients respectively, than healthy patients (Cenac *et al.* 2007). Metalloproteinases, including collagenases, have been implicated in the progression of IBD. UC patients have a significantly ($p = 0.0051$) higher level of metalloproteinase activity at inflamed areas of the mucosa than unaffected areas. Furthermore, the unaffected mucosal areas of UC patients had a significantly ($p < 0.001$) higher level of metalloproteinase activity than unaffected areas in control patients. The proteases implicated in the progression of these two inflammatory conditions were thought to be host-derived. However, the importance of bacterial proteases in the progression of these two conditions is now being researched. The bacterial gelatinase enzyme GelE has been shown to contribute to the development of experimental colitis in a murine model (Steck *et al.* 2011). GelE is produced by the abundant gut commensal *Enterococcus faecalis*, suggesting a role for our resident commensal microbiota in the progression of this disease. Bacterially encoded proteases, such as elastase-like enzyme from *Pseudomonas* spp. and mirabilysin of *Proteus mirabilis*, are also capable of degrading immunoglobulin G₁ (IgG₁) (Brezski and Jordan 2010). It is thought that these proteases act as virulence factors and may be an evasion tactic employed by these bacteria.

Cholesterol, a sterol produced by our own bodies and obtained from our diet, is a major risk factor for cardiovascular disease development (British Heart Foundation 2013). It has been shown that bacteria, such as soil dwelling *Streptomyces*, express a cholesterol oxidase enzyme (*choA*) and has been expressed in *E. coli* (Solaiman and Somkuti 1991). Earlier research hinted that the ability to degrade cholesterol is not limited to *Streptomyces*. The feeding of milk fermented with *Streptococcus thermophilus* lowered the plasma levels of cholesterol in male rats and also lowered liver cholesterol levels (Rao *et al.* 1981). In a more recent study, faecal bacteria of healthy individuals were shown to assimilate cholesterol (Pereira and Gibson 2002). Amongst the cholesterol assimilators were bifidobacteria, lactobacilli and enterococci. The lactobacilli and bifidobacteria shown to exhibit cholesterol assimilation were isolated from faecal material and commercial probiotic products. Cholesterol assimilating enterococci were isolated from faecal material. The results highlight the ability of our resident microbiota to degrade and assimilate cholesterol as well as bacteria used in commercial products.

Gastric and pancreatic lipases break down complex lipids into monoglycerides for intestinal absorption (Carriere *et al.* 1993). These enzymes also play a pivotal role in essential mammalian processes such as non-shivering thermogenesis. This is where thermogenin (uncoupling protein), produced in the mitochondrion, produces heat in brown adipose tissue. Within brown adipose tissue the expression of lipoprotein lipase is upregulated, increasing triglyceride turnover and therefore making more energy available for heat production (Cannon and Nedergaard 2004). Bacteria also produce lipases; this has been previously discussed in detail (Jaeger *et al.* 1994). These enzymes are used as colonisation factors by *Propionibacterium acnes* and *Staphylococcus epidermidis* (Jaeger *et al.* 1994) and are thought to act as a virulence factors in some instances. Research has shown that the ExoU cytotoxin, produced by *Pseudomonas aeruginosa*, is a lipase (Sato *et al.* 2003). Furthermore, the cytotoxin appears to require activation or modification by Eukaryotic factors.

Probiotic administration has been shown to affect functions, provided by our resident gut microbiota. The multi-probiotic VSL-3 (3×10^{11} CFU/g) was fed daily to 10 patients with IBS and 4 patients with functional diarrhoea for 20 days. Urease activity was significantly ($p < 0.01$) decreased and β -galactosidase activity was significantly ($p < 0.01$) increased through probiotic administration (Brigidi *et al.* 2001). Colon cancer model mice were fed daily with yoghurt containing *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (2×10^8 cells/mL), yoghurt supernatant or milk. Results showed that the probiotic yoghurt consuming mice had a significantly ($p < 0.01$) lower level of β -glucuronidase activity than mice consuming yoghurt supernatant or milk (De Moreno *et al.* 2005). Research such as this suggests that probiotic administration can have a significant impact on the functions expressed in our gut.

Changes to human diet in recent years have been implicated in the increase of diseases such as chronic kidney disease (Pecoits-Filho 2007), obesity and cardiovascular disease (CVD) (Fung *et al.* 2001). The "Western diet" has also been implicated in increasing the genotoxicity of faecal water (Rieger *et al.* 1999). Genotoxic compounds can cause DNA damage in the host and have been linked with colorectal cancer (Hughes *et al.* 2000). Genotoxicity testing was classically carried out using the Ames tester strain through screening for histidine reversion (Ames *et al.* 1975). Efforts have been made in order to make screening quantitative as opposed to qualitative e.g. the *E. coli* SOS test (Quillardet *et al.* 1982). This organism utilises β -galactosidase as a measurable colorimetric indicator of SOS induction and therefore, by proxy determines the genotoxicity of a given compound. Genotoxicity testing protocols, such as the comet assay, have been developed in order to make the process more biologically relevant to eukaryotic cells (Collins 2004).

With regards to probiotic administration, it has been shown that probiotics can decrease the genotoxicity of faecal water (Oberreuther-Moschner *et al.* 2004). The genotoxicity of faecal waters of individuals who consumed a daily dose of $\sim 10^9$ *L. acidophilus* 145 and *B. longum* 913 were compared to faecal waters of individuals who consumed control yoghurt. The probiotic yoghurt significantly ($p < 0.05$) reduced the induction of DNA strand breaks, as measured through a comet assay of HT-29 stem cells. There is therefore a precedent for probiotic organisms to modulate genotoxicity levels of faecal waters in humans. The research not only highlights the ability of the organisms to survive passage through the GIT but also their ability to exert anti-genotoxic effects.

4.1.2 Chapter Aims

The aims of this chapter are as follows:

1. to determine whether the numbers of bacteria expressing functions including protease, esterase/lipase, β -galactosidase, β -glucuronidase and cholesterol degrading activity are affected by probiotic administration;
2. to determine whether there is a difference in the numbers of bacteria expressing functions including protease, esterase/lipase, β -galactosidase, β -glucuronidase and cholesterol degrading activity between the two study centres;
3. to determine whether probiotic administration affects the genotoxicity of faecal waters from healthy individuals.

4.2 Methods and materials

4.2.1 Culture conditions and bacterial strains used for culture dependent analysis

During the course of the PROHEMI study many bacterial strains were used (**Table 4.1** and **Table 4.2**). These strains were used in the development of functional media and genotoxicity testing.

4.2.2 Functional screening media

4.2.2.1 Development of the screening media

4.2.1.1.1 Skimmed milk agar for the detection of extracellular protease activity

This agar was made as previously described (Jones *et al.* 2007a). Briefly, sterile skimmed milk was added to the appropriate base medium at a final concentration of 1% (w/v) after sterilising through autoclaving at 121°C for 15 min. *B. subtilis* MY2016 was used as a positive control for protease activity while *B. subtilis* WB800N was used as a negative control (**Table 4.1**). Tryptone soya agar (TSA) (see section 4.2.3) was used as the base medium for *B. subtilis*.

4.2.1.1.2 Buffered Protease Agar (BPA) for the detection of extracellular protease activity

Skimmed milk agar was made as previously described (Jones *et al.* 2007b). The pH of the medium was adjusted to 7.2 and buffered through the addition of a phosphate buffer to the agar base at a final concentration of 0.01 M. Phenol red, at a concentration of 100 mg/L, was also added to the agar base before sterilising through autoclaving at 121°C for 15 min. The phenol red served as a pH indicator changing from red to yellow when it becomes acidified. BPA plates were also made with a concentration of 0.1 M phosphate buffer, while all other components remained unchanged. In order to develop the agar *B. subtilis* MY2016 and *B. subtilis* WB800N were used as protease positive and negative controls respectively (**Table 4.1**). TSA was used as the base medium for *B. subtilis*. The 0.01 M phosphate BPA plate was also incubated following the spread of a faecal slurry. Luria Bertani (LB) agar (see section 4.2.3) was used as the base medium for the faecal slurry.

The ability of the 0.1 M BPA agar to withstand acidification of the medium was tested through hydrochloric acid. A 10-fold serial dilution of concentrated hydrochloric acid (~11.65 M) (Sigma-Aldrich company Ltd. Dorset, UK) was made and applied to the agar in a single drop.

Table 4.1| Bacterial strains and culture conditions used in the PROHEMI study

Species	Strain	Notable feature	Growth conditions	Reference
<i>Escherichia coli</i>	EPI300 (pCCFOS110 clone)	β -galactosidase activity		(Jones <i>et al.</i> 2007b)
	EPI300	Lipase/esterase activity	Luria Bertani (LB) broth + 12.5 μ g/mL chloramphenicol at 37°C	(Jones <i>et al.</i> 2007b)
	EPI300 (<i>choA</i>)	Cholesterol degrading activity		(Jones <i>et al.</i> 2007b)
	Nissle	β -glucuronidase activity		Lab collection
<i>Saccharomyces cerevisiae</i>	BY4741	Genotoxicity testing strain	TGA + 50 μ g/mL ampicillin at 37°C	(Zhang <i>et al.</i> 2008)
<i>Bacillus subtilis</i>	MY2016	Protease activity		Lab collection
	WB800N	No protease Activity	TSA/TSB at 30°C	(Murashima <i>et al.</i> 2002)
<i>Bifidobacterium bifidum</i>	CUL 20	β -galactosidase activity	MRS agar or MRS-X agar at 37°C	(Allen <i>et al.</i> 2012)

Table 4.2| Bacterial strains and culture conditions (continued from Table 4.1)

Species	Strain	Notable feature	Growth conditions	Reference
<i>Bifidobacterium lactis</i>	CUL 34		MRS agar or MRS-X agar at 37°C	(Allen <i>et al.</i> 2012)
<i>Lactobacillus acidophilus</i>	CUL 21	β-galactosidase activity	MRS agar or MRS-X agar at 37°C	(Allen <i>et al.</i> 2012)
	CUL 60	β-galactosidase activity		(Allen <i>et al.</i> 2012)
<i>Salmonella typhimurium</i>	TA1535/pSK1002	Genotoxicity testing strain	TGA + 50 µg/ml ampicillin	(Reifferscheid <i>et al.</i> 1991)

4.2.1.2.3 X-Gal (5-bromo-4-chloro-3-indolyl- β -d-galactopyranoside) agar for the detection of extracellular β -galactosidase activity

The appropriate medium was prepared and sterilised by autoclaving at 121°C for 15 min. The sterile medium was allowed to cool to 50°C before the addition of sterile X-Gal (Melford Laboratories Ltd, Ipswich, Suffolk, UK) at a final concentration 40 μ g per mL of agar. In order to develop the agar *E. coli* was used. The strain, from a metagenomic study, contained an EPI300 plasmid expressing β -galactosidase activity (**Table 4.1**). This strain was used as a positive control. LB agar (see section 4.2.3) was used as the base medium for *E. coli*.

4.2.1.3 X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid) agar for the detection of extracellular β -glucuronidase activity

The appropriate medium was prepared and sterilised by autoclaving at 121°C for 15 min. The sterile medium was allowed to cool to 50°C before the addition of sterile X-Gluc (Apollo Scientific Ltd, Stockport, Cheshire, UK) at a final concentration 40 μ g per mL of agar. The agar was developed through the use of *E. coli* Nissle. This strain expresses β -glucuronidase activity and was used as a positive control (**Table 4.1**). LB agar (see section 4.2.3) was used as the base medium for *E. coli*.

4.2.1.4 Cholesterol agar for the detection of extracellular cholesterol degrading enzymes

Cholesterol (1 g) (Sigma-Aldrich Company Ltd. Dorset, UK) was dissolved in 100 mL of ethanol to make a 1% w/v solution. This solution was added to the prepared appropriate medium at a concentration of 0.1% v/v. This was sterilised by autoclaving at 121°C for 15 min before the plates were poured. The agar was developed through the use of an *E. coli* strain from a metagenomic study. The strain contained an EPI300 plasmid expressing the cholesterol oxidase gene *choA* (Solaiman and Somkuti 1991) (**Table 4.1**). This strain is able to degrade cholesterol and was therefore used as a positive control. LB agar (see section 4.2.3) was used as the base medium for *E. coli*.

4.2.1.5 Tributyrin agar for the detection of extracellular lipases/esterases

Tributyrin agar consists of neutral tributyrin and a tributyrin agar base (Sigma-Aldrich company Ltd. Dorset, UK). The tributyrin agar base was prepared by following the manufacturer's guidelines and 10 g/L of neutral tributyrin was added to the base. The mixture was blended together for 1 min using a food processor before sterilisation by autoclaving at 121°C for 15 min. The agar was developed through the use of a lipase/esterase producing *E. coli*, from a metagenomic study (Jones *et al.* 2007b). This was used as a positive control as it contained an EPI300 plasmid expressing a lipase/esterase

gene (**Table 4.1**). *E. coli* expressing β -galactosidase activity was used as a negative control (**Table 4.1**). LB agar (see section **4.2.3**) was used as the base medium for *E. coli*.

4.2.2 Application of functional screening media

4.2.2.1 Functional screening of the probiotic organisms used in the PROHEMI study

The probiotic organisms used in the PROHEMI study were screened for expressed functions. The base media and culture conditions used to screen for these functions are shown in **Table 4.3**.

4.2.2.2 Functional screening of faecal samples from the PROHEMI study

Serial dilutions to 10^{-9} of faecal samples were made in maximum recovery diluent (MRD) containing L-cysteine (see section **4.2.4**). These dilutions were plated out onto the functional media. The base medium used for the functional media, growth conditions and dilutions plated are shown in **Table 4.4**. Colonies expressing the function of interest were counted and expressed as CFU/g of faeces at Cultech Ltd.

4.2.4 Media used in the study

4.2.4.1 TSA

TSA was prepared according to the manufacturer's guidelines (Oxoid Ltd, Hampshire, UK). The medium was sterilised by autoclaving at 121°C for 15 min.

4.2.4.2 LB agar

LB agar was prepared according to the manufacturer's guidelines (Sigma-Aldrich Company Ltd, Dorset, UK). The medium was sterilised by autoclaving at 121°C for 15 min.

4.2.4.3 de Man, Rogosa, Sharpe (MRS) agar

MRS agar was prepared following manufacturer's guidelines (Oxoid Ltd, Hampshire, UK). The medium was sterilised by autoclaving at 121°C for 15 min.

4.2.4.4 MRS agar with polymyxin

MRS agar was prepared following manufacturer's guidelines (Oxoid Ltd, Hampshire, UK). The medium was sterilised by autoclaving at 121°C for 15 min. Media was allowed to cool to 50°C in a waterbath before sterile polymyxin B was added at 120 units/mL of agar.

4.2.4.5 MRS-X agar

MRS agar was prepared following manufacturer's guidelines (Oxoid Ltd, Hampshire, UK). However, Lithium chloride, Sodium propionate and L-cysteine hydrochloride (Sigma-Aldrich Company Ltd. Dorset, UK) were added at 2 g/L, 3 g/L and 0.5 g/L respectively before sterilisation by autoclaving at 121°C for 15 min. The medium was allowed to cool to 45°C before the addition of defibrinated Sheep's blood (Oxoid Ltd, Hampshire, UK) at a final concentration of 5% v/v.

4.2.4.6 Plate count agar

Plate count agar was prepared following manufacturer's guidelines (Oxoid Ltd, Hampshire, UK). The medium was sterilised by autoclaving at 121°C for 15 min.

4.2.4.5 Maximum recovery diluent (MRD) containing 0.5% L-cysteine

MRD was prepared following manufacturer's guidelines (Oxoid Ltd, Hampshire, UK). L-cysteine hydrochloride (Sigma-Aldrich Company Ltd. Dorset, UK) was added at a concentration of 0.5% w/v before sterilisation by autoclaving at 121°C for 15 min.

Table 4.3 | Culture conditions used for functional screening of the probiotic organisms used in the PROHEMI study

Organism	Medium	Incubation conditions
<i>L. acidophilus</i> (Cul 21)	MRS agar	37°C for 72 h in aerobic and anaerobic conditions
<i>L. acidophilus</i> (Cul 60)	MRS agar	37°C for 72 h in aerobic and anaerobic conditions
<i>B. bifidum</i> (Cul 20)	MRS and MRS – X agars	37°C for 72 h in anaerobic conditions
<i>B. lactis</i> (Cul 34)	MRS and MRS – X agars	37°C for 72 h in anaerobic conditions

Table 4.4| Culture conditions used for functional screening of PROHEMI faecal samples

Functional screening plate	Base medium used for functional screening	Faecal dilutions plated	Culture conditions
BPA (Protease activity)	LB agar	10^{-1} to 10^{-6}	37°C for 48 h
X-gal agar (β-galactosidase activity)	Plate count agar	10^{-1} to 10^{-6}	37°C for 48 h
X-gluc agar (β-glucuronidase activity)	Plate count agar	10^{-1} to 10^{-6}	37°C for 48 h
Tributylin agar (Lipase/esterase activity)	Tributylin agar base	10^{-1} to 10^{-6}	37°C for 48 h
Cholesterol agar (Cholesterol degrading activity)	Plate count agar	10^{-1} to 10^{-6}	37°C for 5 days

4.2.5 Genotoxicity testing

Genotoxicity testing was carried out with

4.2.5.1 Genotoxicity testing using *Saccharomyces cerevisiae* BY4741

A genotoxicity testing protocol for this organism was followed (Jia and Xiao 2004; Zhang *et al.* 2008). *S. cerevisiae* BY4741 (0.5 mL) over-night culture was added to separate aliquots of 2.5 mL fresh YPD medium (20 g/L peptone, 10 g/L yeast extract and D-glucose 20 g/L) and incubated for a further 2 h. Methyl methanesulfonate (MMS) was added to the separate aliquots at final concentrations of 0.01 - 0.1% v/v; a control with no MMS was also used. These aliquots were further incubated for 4 h. 1 mL was taken from each of the aliquots and the cell density measured at OD_{600nm} . Remaining cells were collected by centrifugation at 13,000 g for 5 s and washed twice using distilled water. Supernatant was discarded and cells were resuspended in 1 mL of buffer Z (16.1 g/L $Na_2HPO_4 \cdot 7H_2O$, 5.5 g/L $NaH_2PO_4 \cdot H_2O$, 0.75 g/L KCl, 0.246 g/L $MgSO_4 \cdot 7H_2O$, 2.7 mL β -mercaptoethanol, pH 7.0) (Miller 1972) To this 50 μ L of a 0.1% w/v SDS solution and 50 μ L of chloroform were added before being vortexed for 10 s at high speed. To the now permeabilized cells 200 μ L of a 4 mg/mL *o*-nitrophenyl- β -galactoside (ONPG) in dimethyl sulfoxide (DMSO) solution was added followed by incubation in an orbital shaker set at 150 rpm for 20 min at 30°C. The reaction was stopped by the addition of 1 M Na_2CO_3 . Aliquots were centrifuged at 1,500 g for 5 min in order to obtain the supernatant, which was measured at OD_{420nm} . The level of β -galactosidase activity was determined by following the equation:

$$SA_{\beta-gal} = \frac{100 \cdot OD_{420 nm}}{Reaction\ time\ (minutes) \cdot culture\ volume\ (mL) \cdot OD_{600 nm}}$$

4.2.5.2 Genotoxicity testing using *Salmonella typhimurium* (TA1535/pSK1002)

The initial SOS/*umuC* test (Oda *et al.* 1985) using this organism was further developed (Reifferscheid *et al.* 1991) and this protocol was followed. The known mutagens methyl methanesulfonate (MMS) and 4-nitroquinoline-N-oxide (4-NQO) were used as mutation inducing agents to test the protocol. Briefly, *S. typhimurium* was revived from a freezer stock onto a TGA agar plate containing 50 µg/mL ampicillin (Amp). From this an over-night culture was set-up in TGA broth supplemented with 50 µg/mL Amp. The following morning the over-night culture was centrifuged at 3000 g for 5 min. The supernatant was discarded and the remaining bacterial pellet was resuspended in 1 mL of TGA. 20 mL of TGA broth was inoculated with 0.5 mL of the resuspended pellet and incubated at 37°C over-night until an optical density of 1.5 was obtained (OD₆₀₀). The over-night culture was diluted, through the addition of fresh TGA broth, and incubated at 37°C until an optical density of 0.15 (OD₆₀₀) was reached. The tester culture (290 µL) was distributed into microplates (Fisher Scientific UK Ltd, Loughborough, UK). Two-fold serial dilutions of MMS and 4-NQO in DMSO (10 - 0.15625 % v/v) were added to the tester culture and incubated at 37°C for 2 h in an orbital shaker. Absorbance at 420 nm and 600 nm was determined using a Tecan Infinite M200 Pro plate reader (Tecan UK Ltd, Reading, UK). The β-galactosidase activity was calculated as previously described (Miller 1972).

4.2.5.3 Genotoxicity testing using the SOS – Chromo Test™ system

The SOS – Chromo Test™ system was purchased from Environmental Bio-Detection Products Inc. (EBPI, Ontario, Canada). The test was carried out according to the manufacturer's protocol with faecal water as the test chemical. Genotoxicity of faecal waters was tested using faecal waters which were made as previously described (Marchesi *et al.* 2007). In order to ensure that the faecal water cytotoxicity was not masking its genotoxicity, faecal waters were also diluted 10-fold in PBS and tested. Test faecal waters and controls were incubated with the test organism in duplicate plates. The layout of the test plate is shown in **Fig. 4.1**. Induction of the SOS system in test plates was measured for the through the use of a Tecan Infinite M200 Pro plate reader (Tecan UK Ltd, Reading, UK) at 600 nm with the addition of faecal waters before (pre-incubation) and after (post-incubation) incubation at 37°C. Following SOS system induction measurement alkaline phosphatase activity was measured at 420 nm. This measure was achieved through the addition of a colorimetric substrate.

	1	2	3	4	5	6	7	8	9	10	11	12
	Control 4-NQO (µg/mL)											
A	10	PH1 1 (P)	PH2 2 (P)	PH6 9 (A)	PH11 1 (P)	PH24 7 (A)	PH26 11 (A)	PH1 16 (W)	PH2 17 (W)	PH9 2 (P)	PH11 16 (W)	PH26 1 (P)
B	5	PH1 2 (P)	PH2 9 (A)	PH6 13 (A)	PH11 2 (P)	PH24 11 (A)	PH26 14 (W)	PH1 17 (W)	PH6 1 (P)	PH9 13 (A)	PH11 17 (W)	PH26 2 (P)
C	2.5	PH1 9 (A)	PH2 13 (A)	PH6 16 (W)	PH11 9 (A)	PH24 14 (W)	PH26 15 (W)	PH2 1 (P)	PH6 2 (P)	PH9 17 (W)	PH24 2 (P)	PH26 7 (A)
D	1.25	PH1 13 (A)	PH2 16 (W)	PH9 1 (P)	PH11 13 (A)	PH24 15 (W)	PH1 1 (P)	PH2 2 (P)	PH6 9 (A)	PH11 1 (P)	PH24 7 (A)	PH26 11 (A)
E	0.625	PH1 16 (W)	PH2 17 (W)	PH9 2 (P)	PH11 16 (W)	PH26 1 (P)	PH1 2 (P)	PH2 9 (A)	PH6 13 (A)	PH11 2 (P)	PH24 11 (A)	PH26 14 (W)
F	0.313	PH1 17 (W)	PH6 1 (P)	PH9 13 (A)	PH11 17 (W)	PH26 2 (P)	PH1 9 (A)	PH2 13 (A)	PH6 16 (W)	PH11 9 (A)	PH24 14 (W)	PH26 15 (W)
G	0.16	PH2 1 (P)	PH6 2 (P)	PH9 17 (W)	PH24 2 (P)	PH26 7 (A)	PH1 13 (A)	PH2 16 (W)	PH9 1 (P)	PH11 13 (A)	PH24 15 (W)	Blank
H	DMSO	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Figure 4.1| Layout of test samples and controls for the SOS ChromoTest™ system – faecal water of participants are denoted by their respective PH numbers followed by the sample number. The period of the study is also shown, P = Pre-feeding, A = Active and W = Washout.

4.2.6 Statistical analysis

Box-plots of total bacterial counts, counts obtained from functional media and absorbances for genotoxicity screening were generated in R statistical software (R-Core-Team 2012). The plots were generated through a custom scripts which utilised `ggplot2` (Wickham 2009) and `reshape2` (Wickham 2007) packages (**Appendix II**). Box-plots show the median, 1st and 3rd quartiles and outliers of a sample or study period.

Normality testing, Kruskal-Wallis H tests and single pair-wise comparisons were carried out in IBM SPSS statistics 20 (IBM, Portsmouth, UK).

4.3 Results

4.3.1 Functional media testing

The media used in the study were developed and tested using positive and negative control organisms (Fig. 4.2 A-G).

4.3.1.1 Cholesterol degrading activity

Relevant media containing cholesterol were used as a screen for this activity. *E. coli* expressing the gene *choA* was used as a positive control (Fig. 4.2 A). It was possible to see zones of clearing around this organism where the cholesterol has been degraded. In order to ensure that the observed function was cholesterol degradation, a negative control was used. For this purpose *E. coli* was once again used. However, a frame-shift mutation was previously introduced into the inserted *choA* gene. Therefore, the gene was no longer expressed and the organism was no longer able to degrade cholesterol, producing no zone of clearing (Fig. 4.2 B).

4.3.1.2 β -galactosidase activity

The indoxyl glycoside X-gal was added to the relevant media in order to screen for β -galactosidase activity. *E. coli* expressing β -galactosidase activity was used as a positive control. Colonies which are positive for β -galactosidase activity appear blue in colour (Fig. 4.2 C).

4.3.1.3 β -glucuronidase activity

The indoxyl glucuronide X-gluc was added to the relevant media in order to screen for β -glucuronidase activity. In order to develop the medium *E. coli* Nissle 1917 was used. This organism expresses β -glucuronidase activity. Colonies which are positive for β -glucuronidase activity appear blue in colour (Fig. 4.2 D).

4.3.1.4 Lipase/esterase activity

The lipid tributyrin with a carbon chain length of 15 was used in order to screen for lipase/esterase activity. The suitability of the medium was tested through the use of a lipase/esterase expressing *E. coli*. Bacterial colonies which are positive for lipase/esterase activity produce a zone of clearing (Fig. 4.2 E).

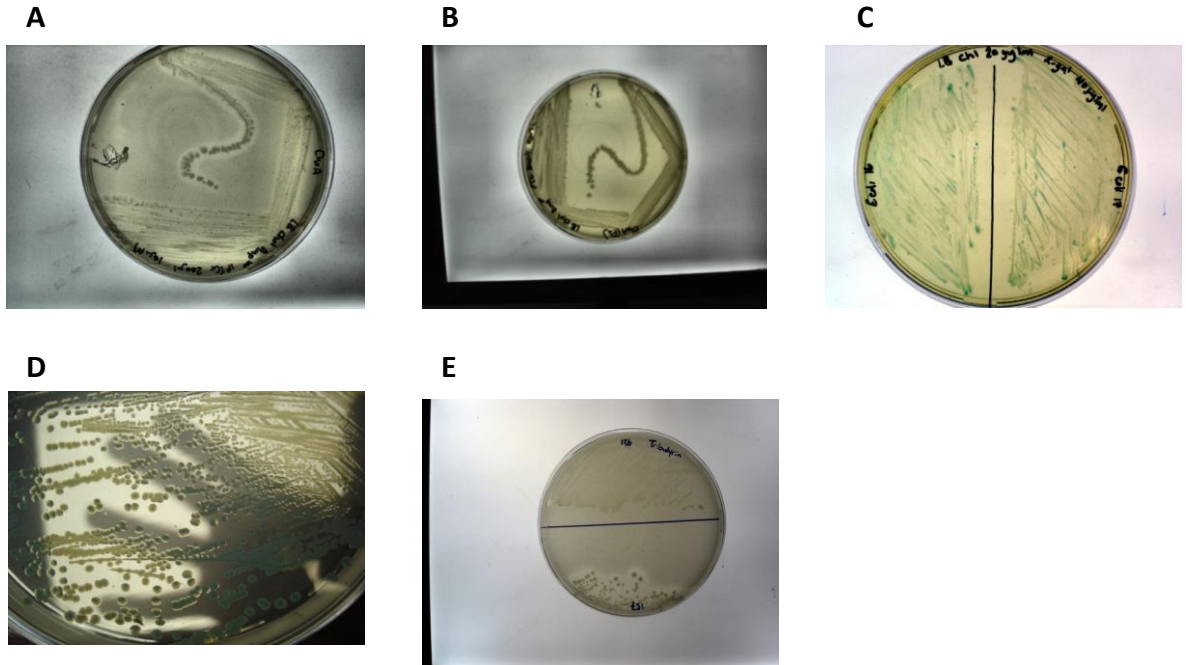


Figure 4.2| Functional media screens – (A) Cholesterol LB agar with *E. coli* expressing *choA* as shown by the zones of clearing around the colonies. **(B)** Cholesterol LB agar with no zones of clearing around the colonies. *E. coli* with a frame-shift mutation in the *choA* gene was used. **(C)** X-gal LB agar with two different *E. coli* strains expressing β -galactosidase activity as shown by the blue colour of the colonies. **(D)** X-gluc agar with blue colonies of *E. coli* due to the expression of β -glucuronidase. **(E)** Tributyrin agar with two strains of *E. coli*. The strain in the bottom half expresses a lipase/esterase gene and therefore produces zones of clearing around the colonies. The strain in the top half expresses a β -galactosidase gene and therefore does not produce a zone of clearing.

4.3.1.5 Protease activity

4.3.1.5.1 Skimmed milk agar

The protease positive *B. subtilis* MY2016 showed zones of clearing around its colonies on skimmed milk agar (**Fig. 4.3 A**). However, the agar was further developed in order to avoid false positives.

4.3.1.5.2 BPA

B. subtilis MY2016 was once again used in order to test the medium with a phosphate buffer at 0.01 M. The organism showed zones of clearing around its colonies, indicative of protease activity. The agar at 0.01 M buffering was also tested through the plating of a faecal slurry (**Fig. 4.3 B**). Some colonies showed zones of clearing suggesting protease activity. However, the colour of the pH indicator phenol red changed to orange-yellow. This suggests that the pH has dropped and therefore false positives are difficult to rule out.

To this end, the concentration of the phosphate buffer was increased to 0.1 M. The clearing of the plate, following the spread of an over-night culture of *B. subtilis* MY2016, shows that the plate detects protease activity (**Fig. 4.3 C**). Furthermore, the plate remains bright red and therefore shows that no drop in pH occurs. In contrast, the spreading of an over-night culture of *B. subtilis* WB800N does not clear the plate (**Fig. 4.3 D**). Therefore, for correct assignment of protease production in an organism or sample using BPA plates, a zone of clearing needs to be produced while the plate remains red. These plates show a strong colour change when concentrated hydrochloric acid (11.65 M) is placed on a BPA plate (**Fig. 4.3 E**). Colour change is also observable when a 1/10 dilution (~1.165 M) is dropped onto a BPA plate. However, there is no colour change when more dilute acid (~0.1165 M) is dropped onto the BPA plates. This further highlights the buffering capacity of the phosphate buffer.

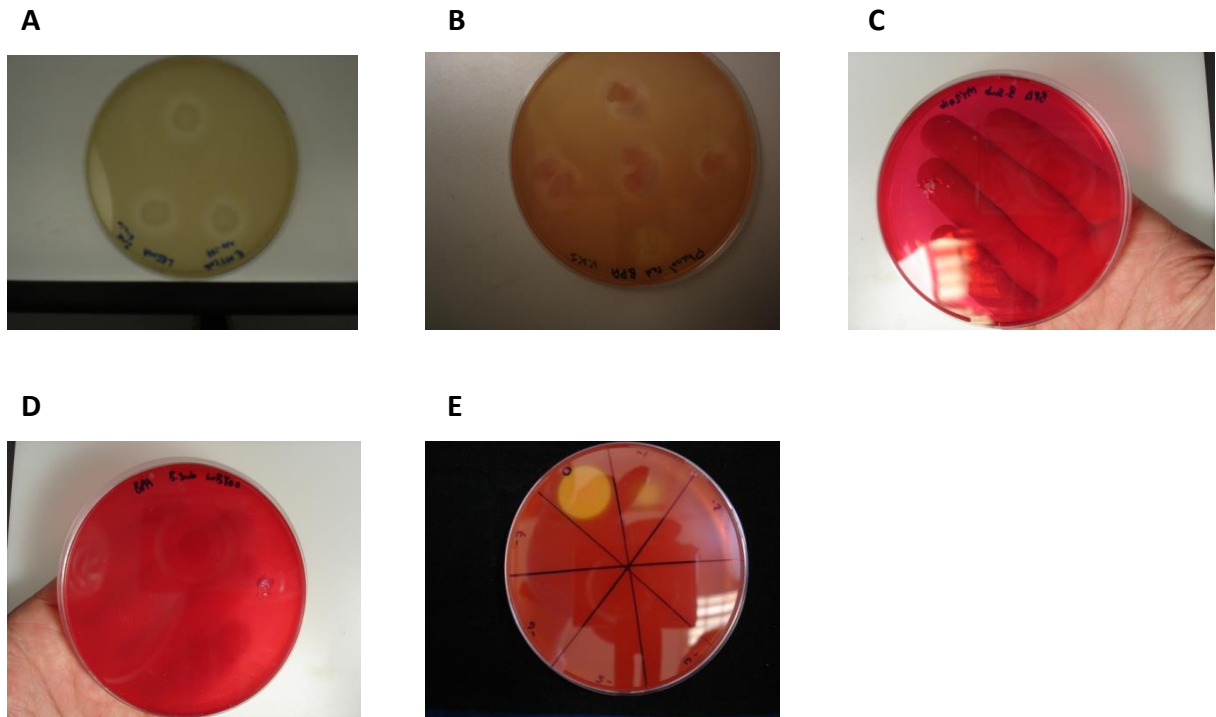


Figure 4.3| Functional media for the detection of extracellular proteases – (A) Skimmed milk LB agar with zones of clearing produced by *B. subtilis* MY2016. **(B)** BPA with 0.01 M phosphate buffer. The plates have been incubated with a faecal slurry and show a yellow/orange colour as well as zones of clearing around some of the colonies. **(C)** BPA with 0.1 M phosphate buffer. The plate was incubated with an over-night culture of the protease positive *B. subtilis* MY2016, clearing the plate while it remains red in colour. **(D)** BPA with 0.1 M phosphate buffer. The plate was incubated with an over-night culture of the protease deficient *B. subtilis* WB800N, producing no clearing. **(E)** Serial dilution of hydrochloric acid and drop application onto a 0.1 M BPA plate.

4.3.2 Functional screening of the probiotic mixture

The results of functional screening of the probiotic mixture are shown in **Table 4.5**. From the experiment it is possible to observe that both stains of *L. acidophilus* (Cul21 and Cul60) and *B. bifidum* (Cul20) produce extracellular β -galactosidase activity (**Fig. 4.4 A-C**). However, none of the other tested functions are exhibited by these probiotic organisms.

Table 4.5 | Functions expressed by the probiotic organisms used in the PROHEMI study

Function expressed	Organism			
	<i>B. bifidum</i> (CUL20)	<i>B. lactis</i> (CUL34)	<i>L. acidophilus</i> (CUL21)	<i>L. acidophilus</i> (CUL60)
Protease activity	-	-	-	-
Lipase/esterase activity	-	-	-	-
Cholesterol degrading activity	-	-	-	-
β -galactosidase activity	+	-	+	+
β -glucuronidase activity	-	-	-	-

* denotes that the function was positive when MRS and MRS-X were used as the base media.

**denotes the function was positive when *L. acidophilus* was cultured aerobically and anaerobically.

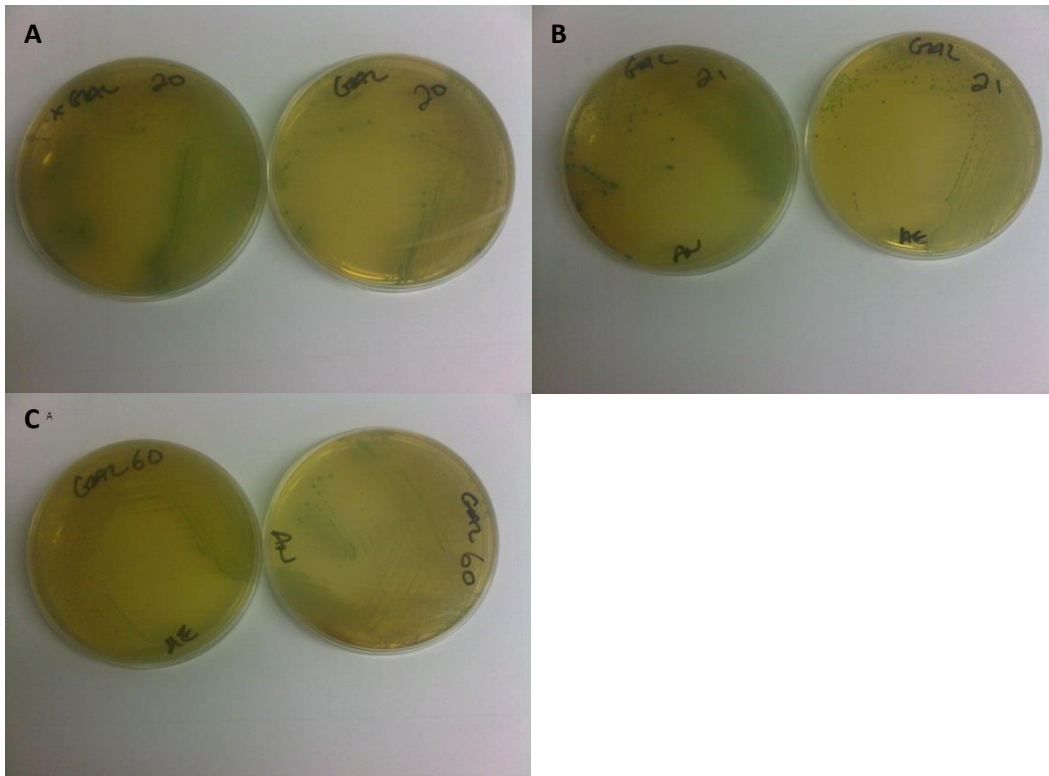


Figure 4.4 | β -galactosidase activity exhibited by the probiotic organisms used in the PROHEMI study – (A) shows *B. bifidum* (Cul20) growing on MRS-X and MRS (left to right). (B) shows *L. acidophilus* (Cul21) growing on MRS agar following anaerobic and anaerobic incubation (left to right). (C) shows *L. acidophilus* (Cul60) growing on MRS agar following aerobic and anaerobic incubation (left to right)

4.3.3 Functional media screening of faecal samples

The functions expressed, based on which study centre the samples come from, have been plotted. Box-plots show that there are outliers for the expression of tested functions (**Fig. 4.5**). It is possible to see that there are samples which express high levels of β -galactosidase and β -glucuronidase activity in both study centres. There are also outliers for protease production in both study groups and there are outliers also for lipase/esterase production but to a lesser extent. Cholesterol degrading bacteria were not detected in either of the study groups. Bacterial counts for functions expressed were not normally distributed therefore, a Kruskal – Wallis H test was carried out on the data. The test showed no significant difference between the mean ranks the two centres in terms of bacterial numbers expressing protease activity [χ^2 (1) = 0.569, p = 0.451] and lipase/esterase activity [χ^2 (1) = 0.05, p = 0.946]. However, there were significant differences between the mean ranks of the two centres in terms of β -galactosidase activity [χ^2 (1) = 33.36, p < 0.001] and β -glucuronidase activity [χ^2 (1) = 26.74, p < 0.001].

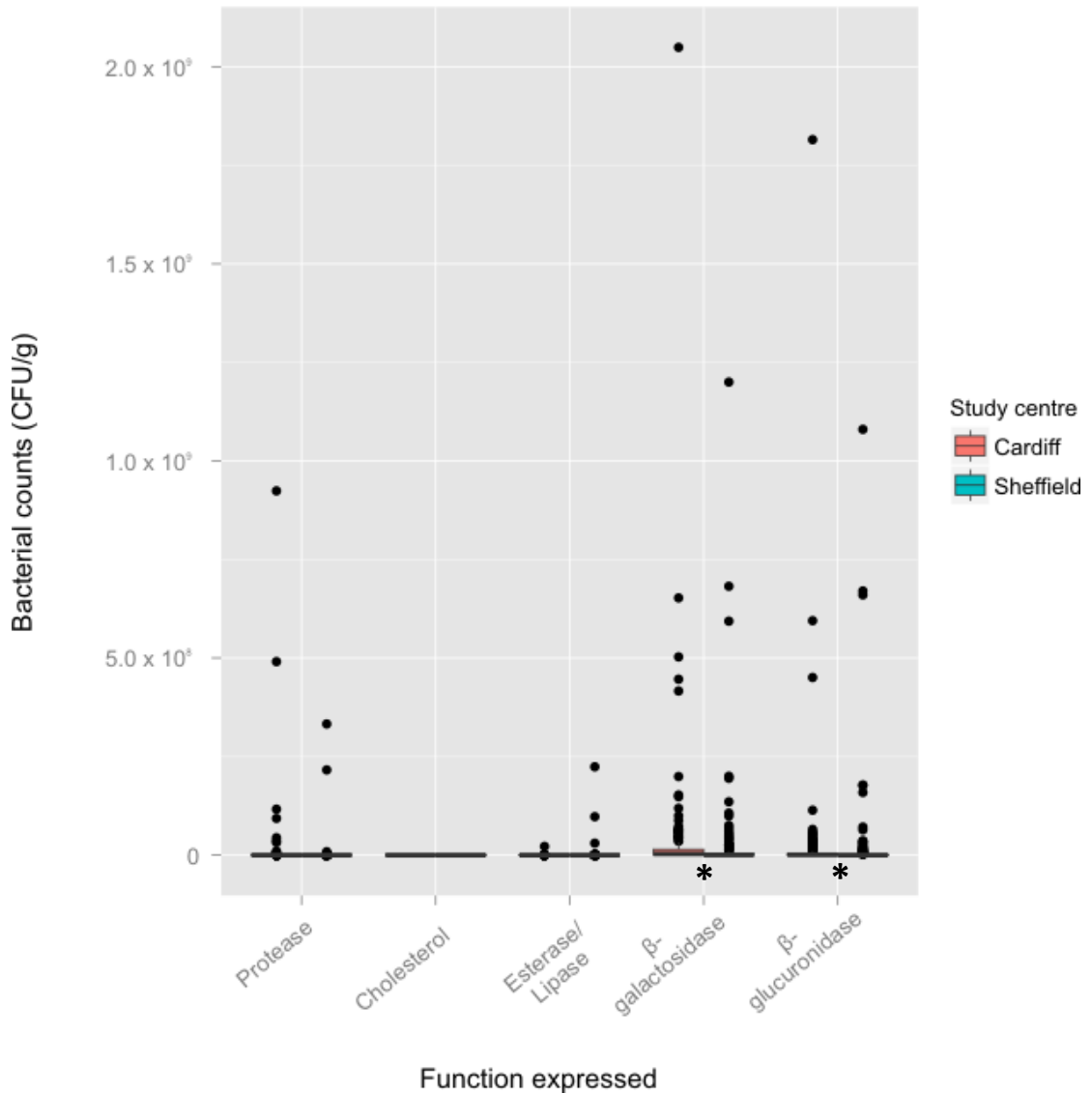


Figure 4.5| Box-plot of functions expressed by PROHEMI samples based upon the study centre – there are two box-plots for each tested expressed function, with Cardiff samples on the left of the two and Sheffield samples on the right. The box-plot shows the median, 1st and 3rd quartiles. Outliers are represented by •. The numbers of bacteria expressing each function are shown in CFU/g of faecal material. *denotes a significant difference between the ranked means of the two centres ($p < 0.001$).

Functions expressed by bacteria were also analysed by study period separately for each study centre. Box-plots of the tested functions expressed by Sheffield samples show outliers once again (**Fig. 4.6**). Due to the outliers it is difficult to discern differences between the study periods. It is especially difficult as outliers for a given function such as β -galactosidase activity are much higher than outliers from other functions such as esterase/lipase activity. Therefore, box-plots were generated for protease (**Fig. 4.7**), β -galactosidase and β -glucuronidase activity (**Fig. 4.8**). Bacterial counts for functions expressed from Sheffield were not normally distributed so a Kruskal – Wallis H test was carried out on the data. The test showed significant differences between the mean ranks of the three study periods in terms of protease [$\chi^2 (2) = 10.27, p = 0.006$], β -galactosidase [$\chi^2 (2) = 9.85, p = 0.007$] and β -glucuronidase [$\chi^2 (2) = 30.15, p < 0.001$] expression by bacteria. Post-hoc analysis through single pairwise comparisons showed that the mean rank of bacteria expressing protease activity was significantly higher in the Pre-feeding group than both the Active and Washout groups at the 0.05 level. This difference can be viewed in the box-plot (**Fig. 4.7**) where the median and 3rd quartile are higher in the Pre-feeding period than the Active and Washout periods. Through post-hoc analysis it was shown that the mean ranks of bacteria expressing β -galactosidase and β -glucuronidase activities were significantly higher in the Active and Washout groups than the Pre-feeding group at the 0.05 level. Box-plots show that the medians and 3rd quartiles for β -galactosidase and β -glucuronidase activities are higher in the Active and Washout periods than the Pre-feeding period (**Fig. 4.8**). Furthermore, the numbers of bacteria expressing these activities are highest during the Active period.

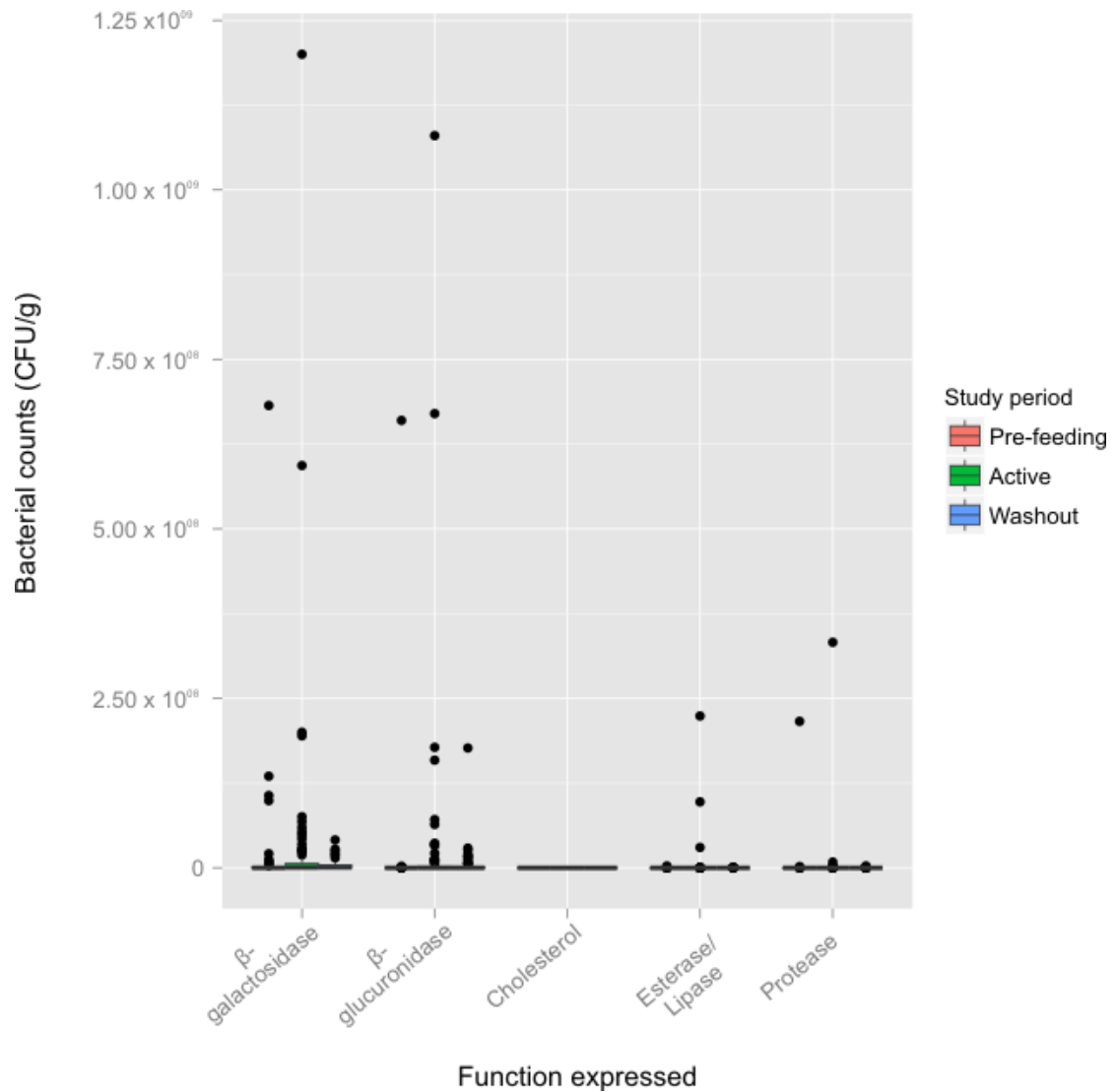


Figure 4.6| Box-plot of functions expressed by PROHEMI samples obtained from Sheffield based upon study period - there are three box-plots for each tested expressed function, with **Pre-feeding**, **Active** and **Washout** samples shown left, middle and right respectively for each function. The box-plot shows the median, 1st and 3rd quartiles. Outliers are represented by •. The numbers of bacteria expressing each function are shown in CFU/g of faecal material.

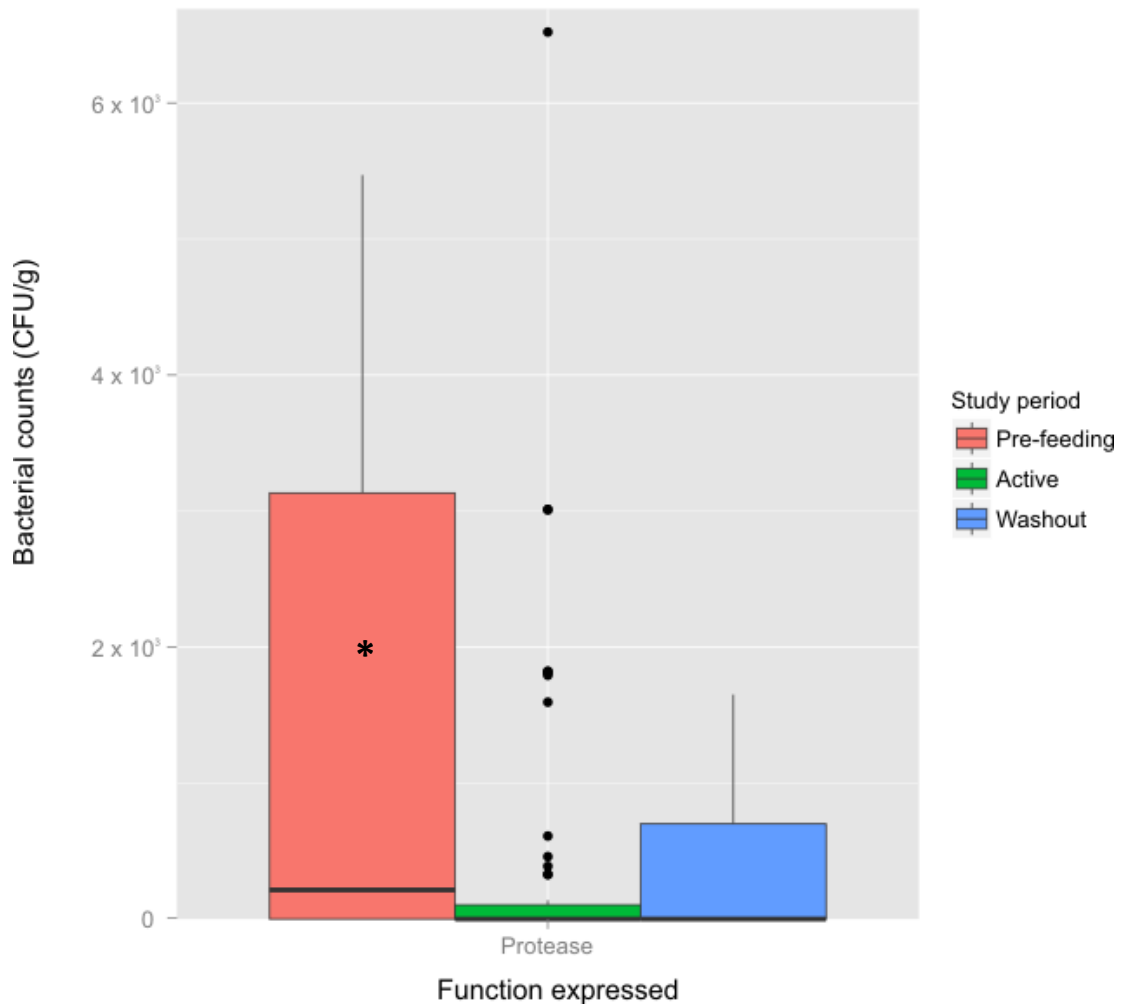


Figure 4.7| Box-plot of protease activity expressed by PROHEMI samples obtained from Sheffield based upon study period – three boxplots are shown with a corresponding key. The median, 1st and 3rd quartiles and outliers are represented by •. The numbers of bacteria expressing each function are shown in CFU/g of faecal material. *denotes a significantly higher ranked mean than the Active and Washout period (p<0.05).

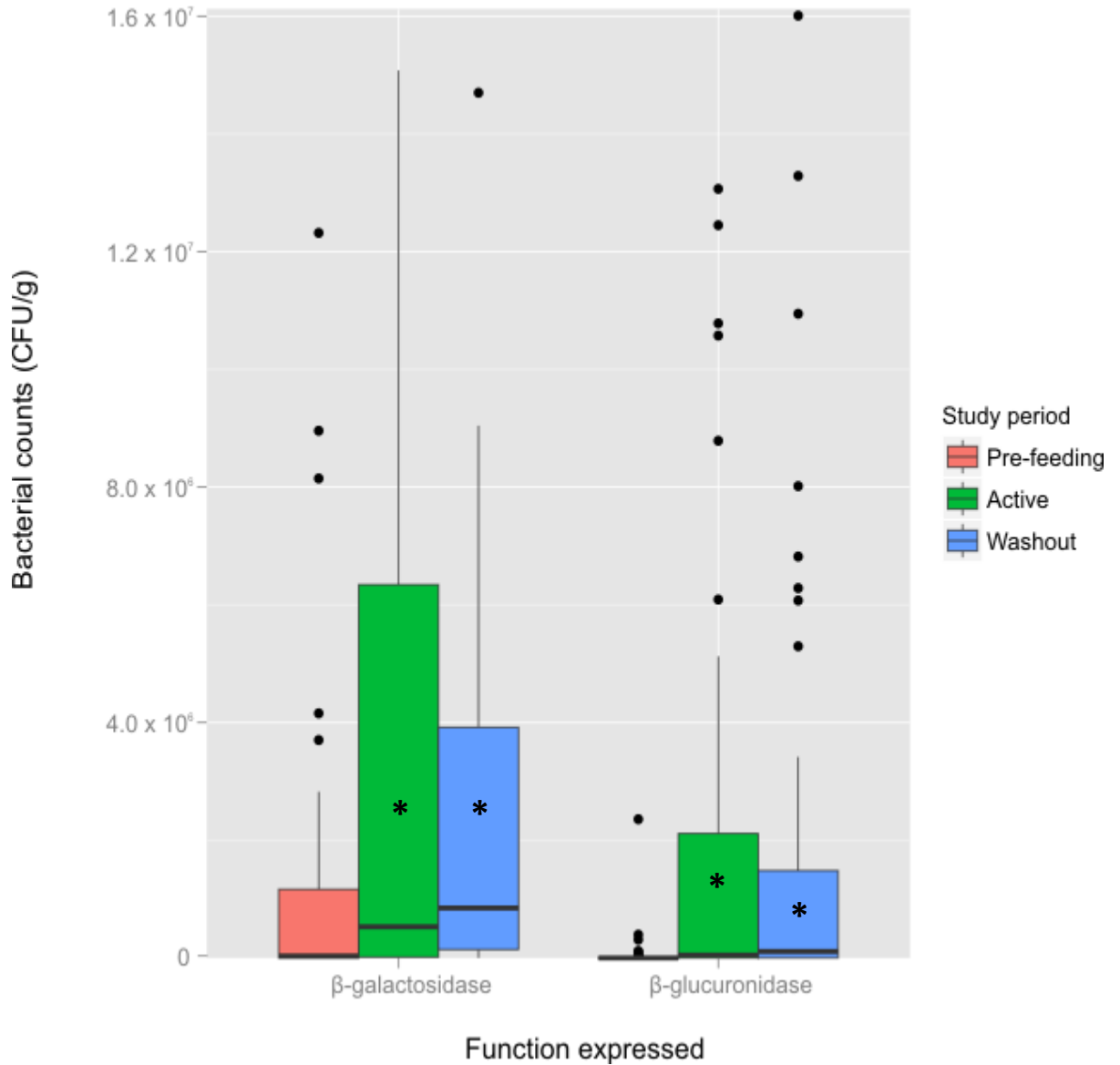


Figure 4.8| Box-plot of β -galactosidase and β -glucuronidase activity expressed by PROHEMI samples obtained from Sheffield based upon study period – three boxplots are shown for each function with a corresponding key. The median, 1st and 3rd quartiles and outliers are represented by •. The numbers of bacteria expressing each function are shown in CFU/g of faecal material. *denotes a significantly higher ranked mean than the Pre-feeding period ($p < 0.05$).

Samples from Cardiff were also analysed separately. In a similar fashion to Sheffield samples, outliers for expressed functions were observed (**Fig. 4.9**). These outliers made it difficult to discern difference in bacterial counts expressing functions between the study periods. Therefore, box-plots of protease activity (**Fig. 4.10**), β -galactosidase and β -glucuronidase activities (**Fig. 4.11**) were plotted separately. Bacterial counts for functions expressed were not normally distributed. Therefore, a Kruskal-Wallis H test was carried out on the data. The test showed significant differences between the mean ranks of the four study periods in terms of protease [$\chi^2(3) = 13.67, p = 0.003$], and β -glucuronidase activities gluc [$\chi^2(3) = 28.06, p < 0.001$]. Through post-hoc analysis it was shown that the Pre-feeding group had a significantly higher mean rank of bacteria expressing protease activity than Active group 1, Active group 2 and Washout periods at the 0.05 level. It is possible to see that the median and 3rd quartile are higher in the Pre-feeding group in the box-plot (**Fig 4.10**). Post-hoc analysis showed that the mean rank of bacteria expressing β -glucuronidase activity was significantly higher in the Active group 1, Active group 2 and Washout periods than the Pre-feeding period at the 0.05 level (**Fig 4.11**).

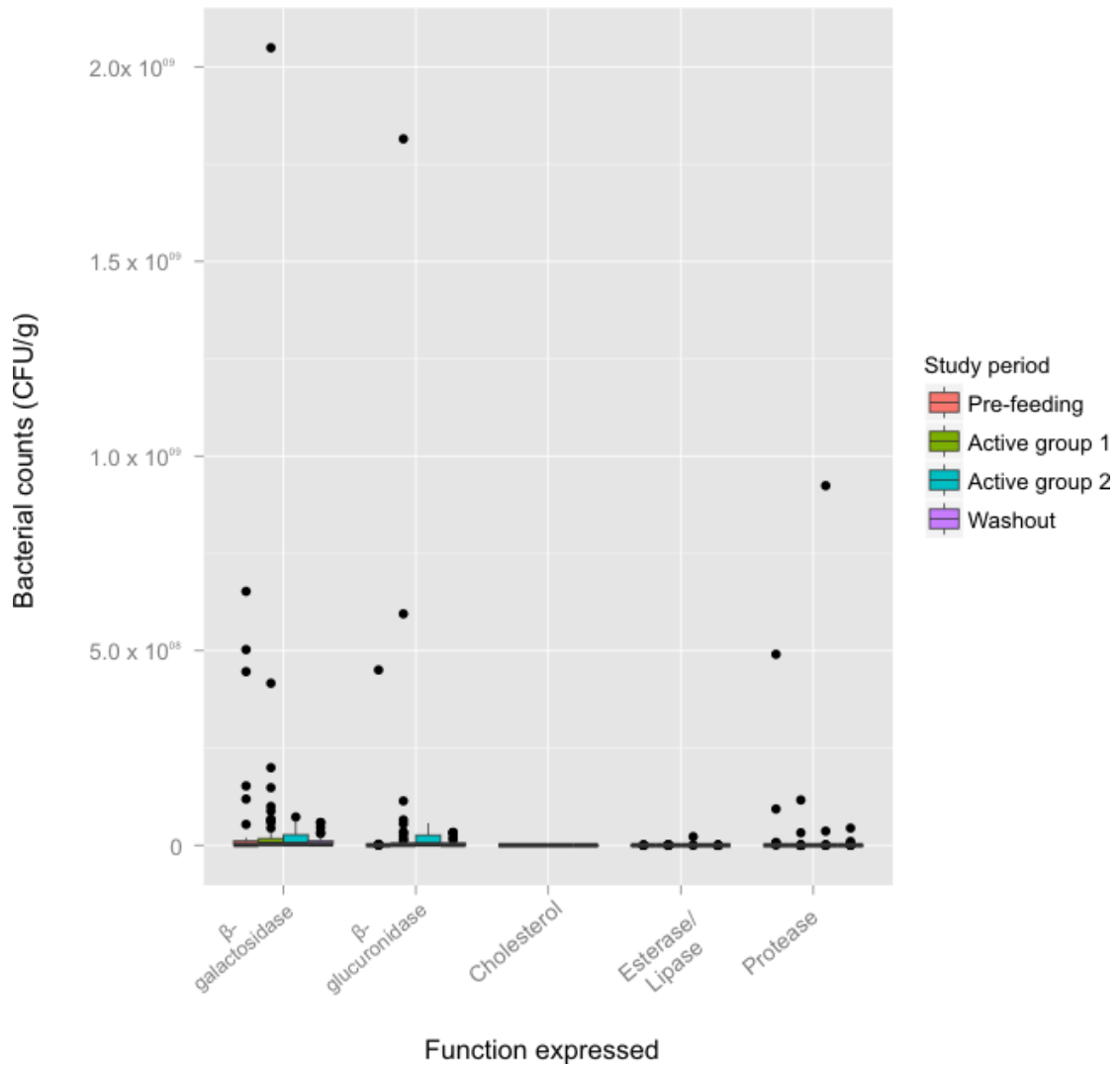


Figure 4.9|Box-plot of functions expressed by PROHEMI samples obtained from Cardiff based upon study period - there are four box-plots for each tested expressed function, with Pre-feeding, Active group 1, Active group 2 and Washout samples shown left, middle left, middle right and right respectively for each function. The box-plot shows the median, 1st and 3rd quartiles. Outliers are represented by •. The numbers of bacteria expressing each function are shown in CFU/g of faecal material

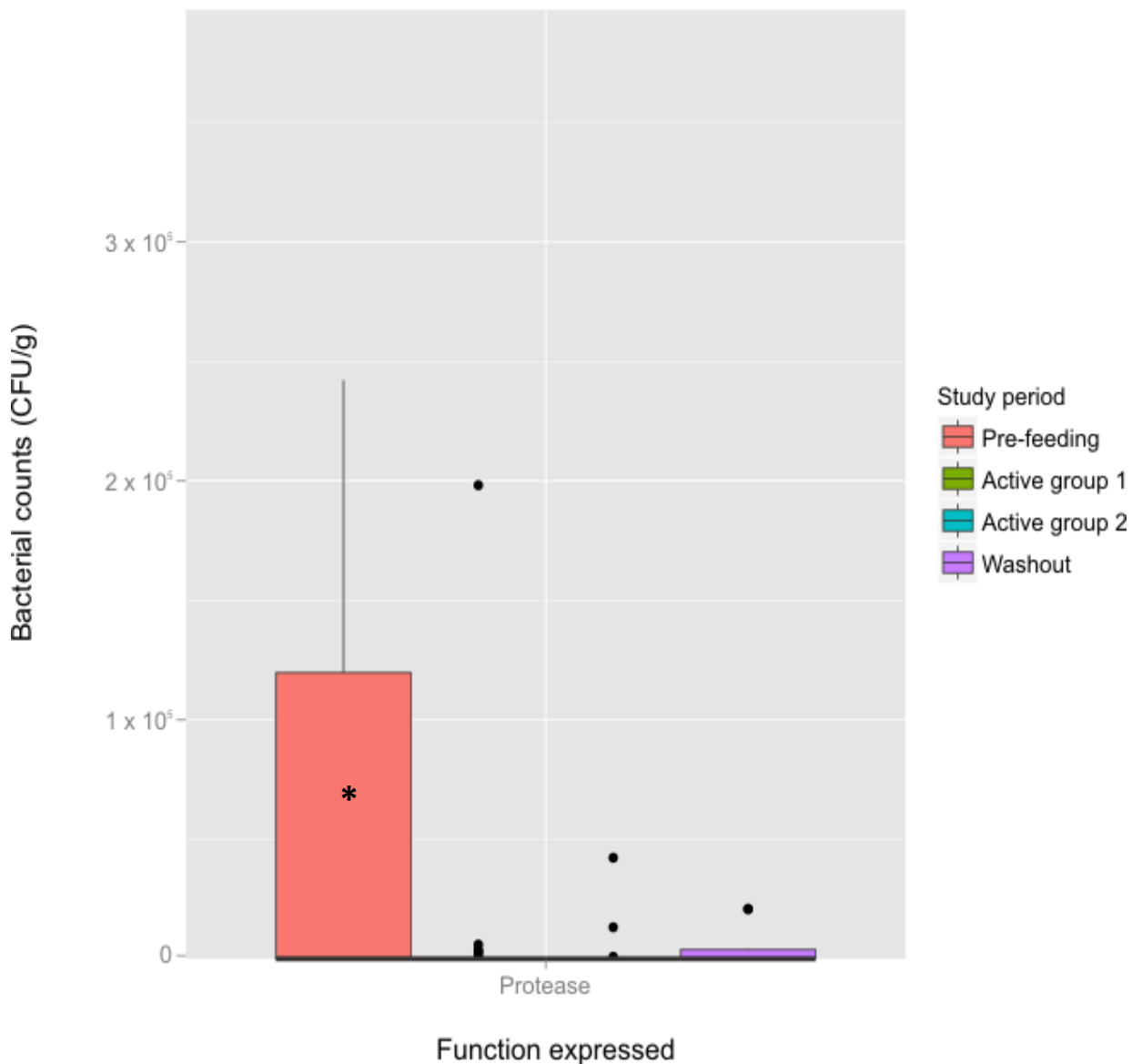


Figure 4.10| Box-plot of protease activity expressed by PROHEMI samples obtained from Cardiff based upon study period – four boxplots are shown for each function with a corresponding key. **Pre-feeding, Active group 1, Active group 2** and **Washout** samples are shown from left to right respectively. The median, 1st and 3rd quartiles and outliers are represented by •. The numbers of bacteria expressing each function are shown in CFU/g of faecal material. *denotes a significantly higher ranked mean than the Active group 1, Active group 2 and Washout periods ($p < 0.05$). **Active group 1** and **Active group 2** cannot be seen as the protease level is significantly lower than the **Pre-feeding** period.

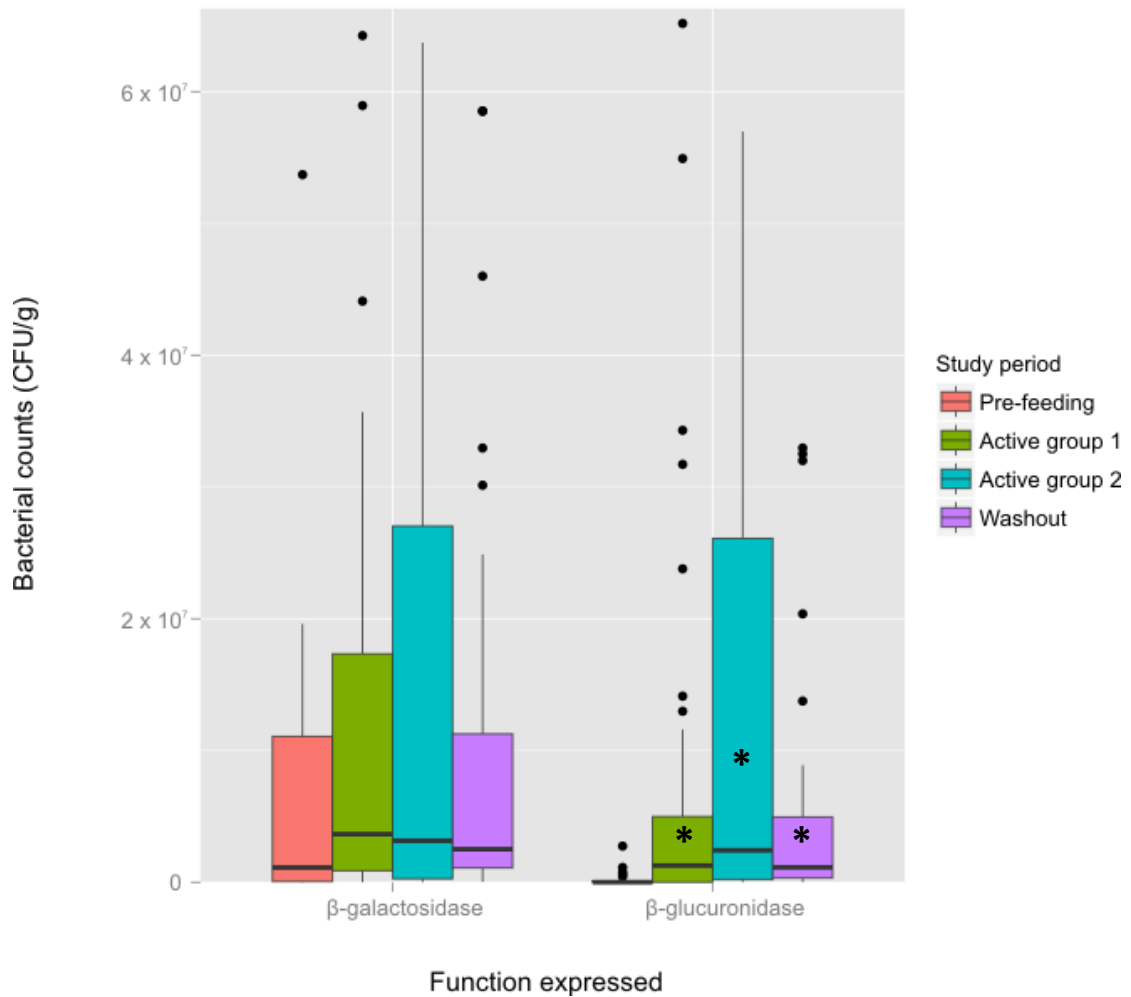


Figure 4.11| Box-plot of β -galactosidase and β -glucuronidase activity expressed by PROHEMI samples obtained from Cardiff based upon study period – four boxplots are shown for each function with a corresponding key. The median, 1st and 3rd quartiles and outliers are represented by •. The numbers of bacteria expressing each function are shown in CFU/g of faecal material. *denotes a significantly higher ranked mean than the Pre-feeding period ($p < 0.05$).

4.3.4 Genotoxicity testing of faecal waters

Genotoxicity testing was carried out using *Saccharomyces cerevisiae* BY4741 and *Salmonella typhimurium* (TA1535/pSK1002). However, the results were inconclusive and will not be discussed in this thesis.

4.3.4.3 Genotoxicity testing using the *E. coli* SOS – ChromoTest™ system

The genotoxic agent 4-NQO was used as a positive control and illustrated the SOS induction response of this *E. coli* strain (Fig. 4.12). The absorbance at 605 nm increased as the concentration of 4-NQO increased. However, the curve begins to plateau between 5-10 µg/mL of 4-NQO.

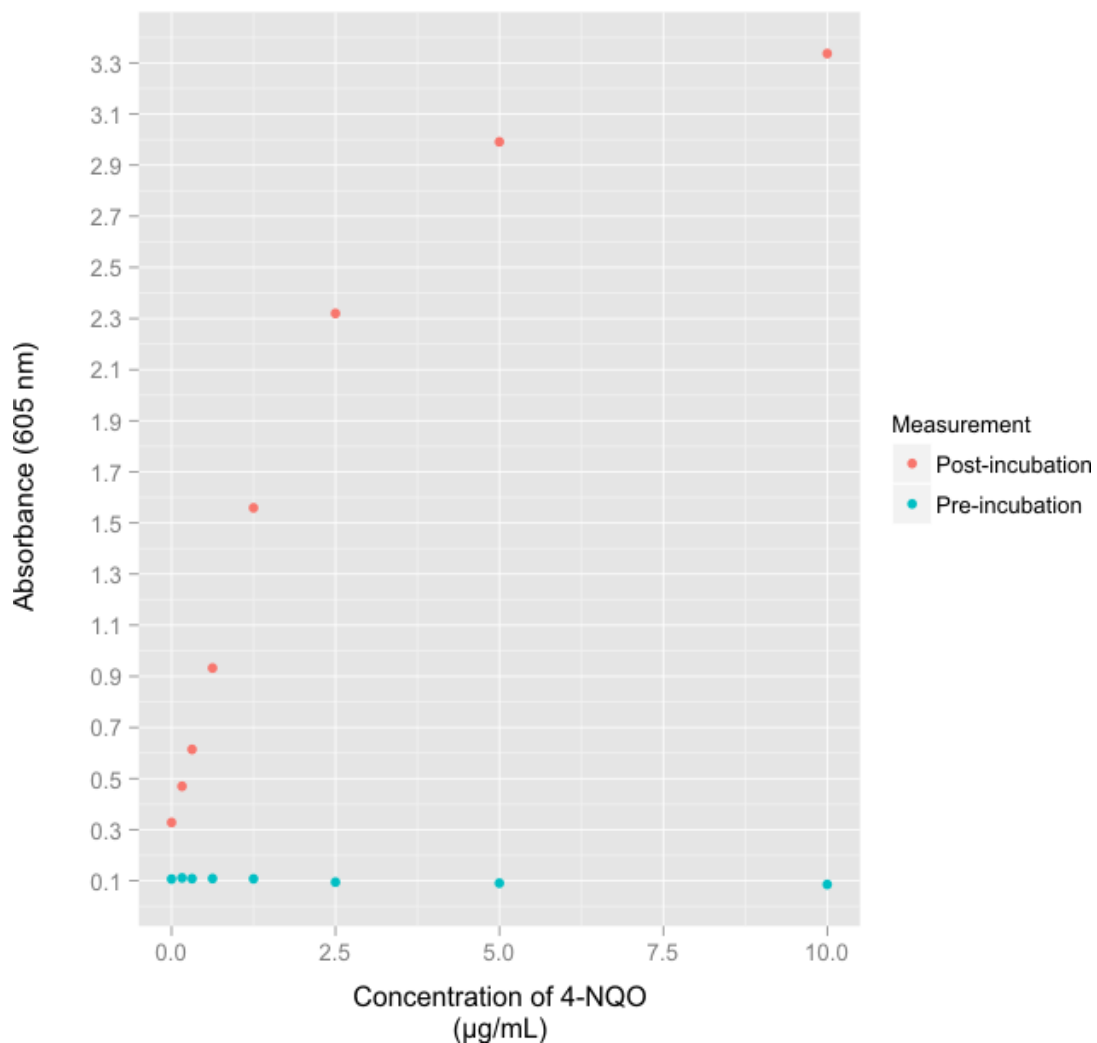


Figure 4.12| The response of the SOS system and alkaline phosphatase activity to increasing concentrations of 4-NQO – the absorbance at 605 nm is plotted, before and after incubation, against 4-NQO at increasing concentrations.

Analysis of the data through comparison of study periods shows that faecal waters from Pre-feeding, Active and Washout period induce a response from the SOS system. Neat and diluted faecal waters from the 3 study periods show no significant difference in SOS induction from the control, prior to incubation (**Fig. 4.13** and **Fig 4.14** respectively). However, a significant difference in SOS induction was observed post-incubation (**Fig. 4.13** and **Fig. 4.14** respectively). The data were not normally distributed; therefore, a Kruskal-Wallis H test was carried out on the data. Analysis of neat samples in this manner showed that there was a significant difference in the mean ranks of the 3 study periods and the control group [$\chi^2 (3) = 23.653, p < 0.001$]. Post-hoc analysis through pairwise comparison showed that Pre-feeding, Active and Washout periods had a significantly higher ranked mean than the control samples at the 0.05 level. Although not statistically significant, analysis showed that the median absorbance at 605 nm was higher in the Active and Washout groups than the Pre-feeding group (0.518, 0.514 and 0.463 respectively). Kruskal-Wallis H test analysis of the diluted faecal waters showed a similar pattern; with no significant difference between the study periods and control samples prior to incubation. However, there was a significant difference between the 3 study groups and control samples following incubation [$\chi^2 (3) = 19.774, p < 0.001$]. Pairwise comparison post-hoc analysis showed that the ranked means of Pre-feeding, Active and Washout samples were significantly higher than the control groups at the 0.05 level. The observation of higher absorbance medians was emulated in this dataset; with the Active and Washout groups exhibiting a higher median absorbance than the Pre-feeding group (0.454, 0.437 and 0.422 respectively).

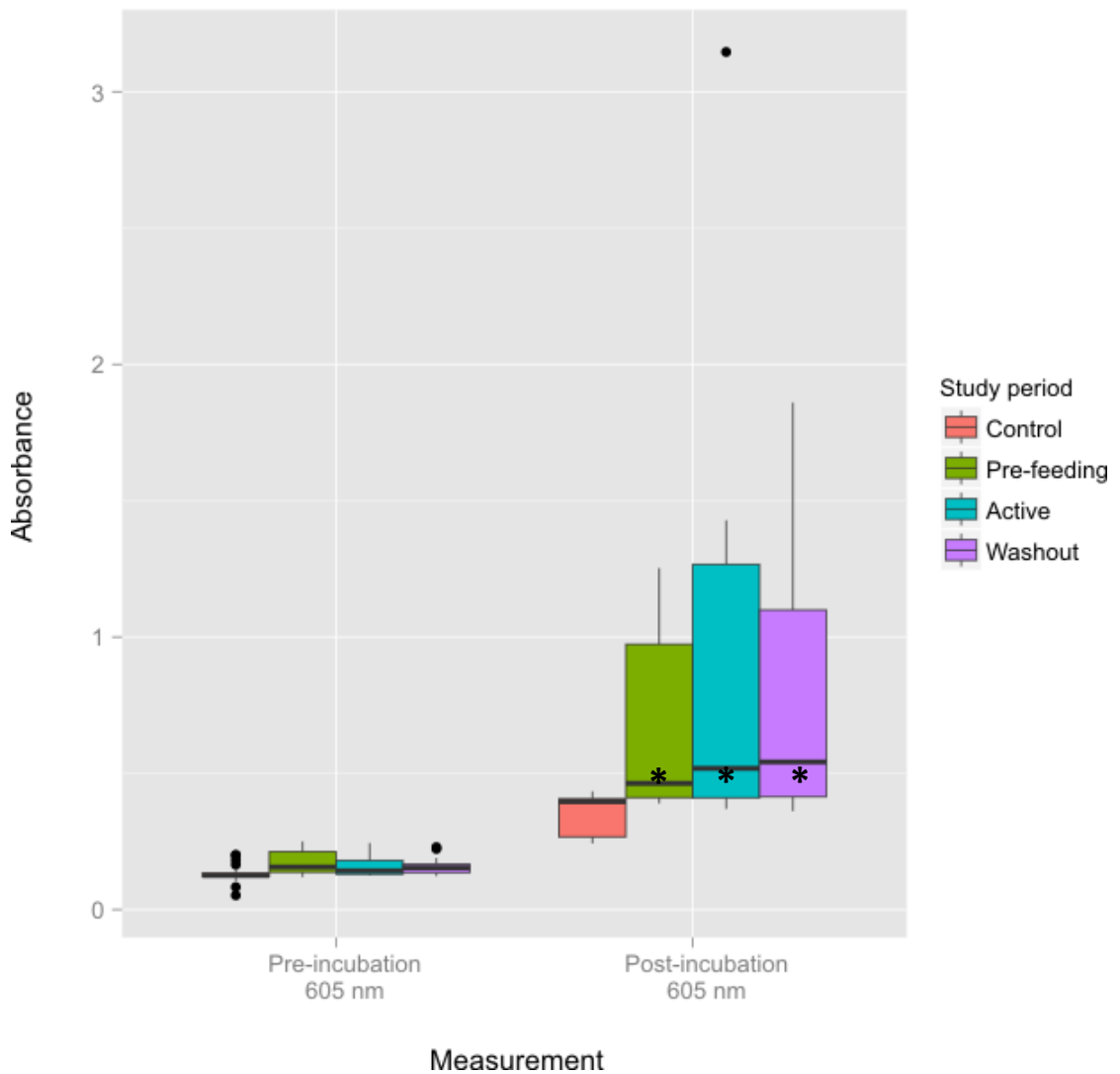


Figure 4.13| The effect of neat faecal waters on SOS induction in the SOS ChromoTest™ system – a box-plot of the absorbance at 605 nm pre-incubation and post-incubation is shown. The absorbance of neat faecal waters from the 3 study periods is shown along with the absorbance of control samples. Outliers are represented by •.*denotes a significant ($p = 0.05$) difference between the study period and control.

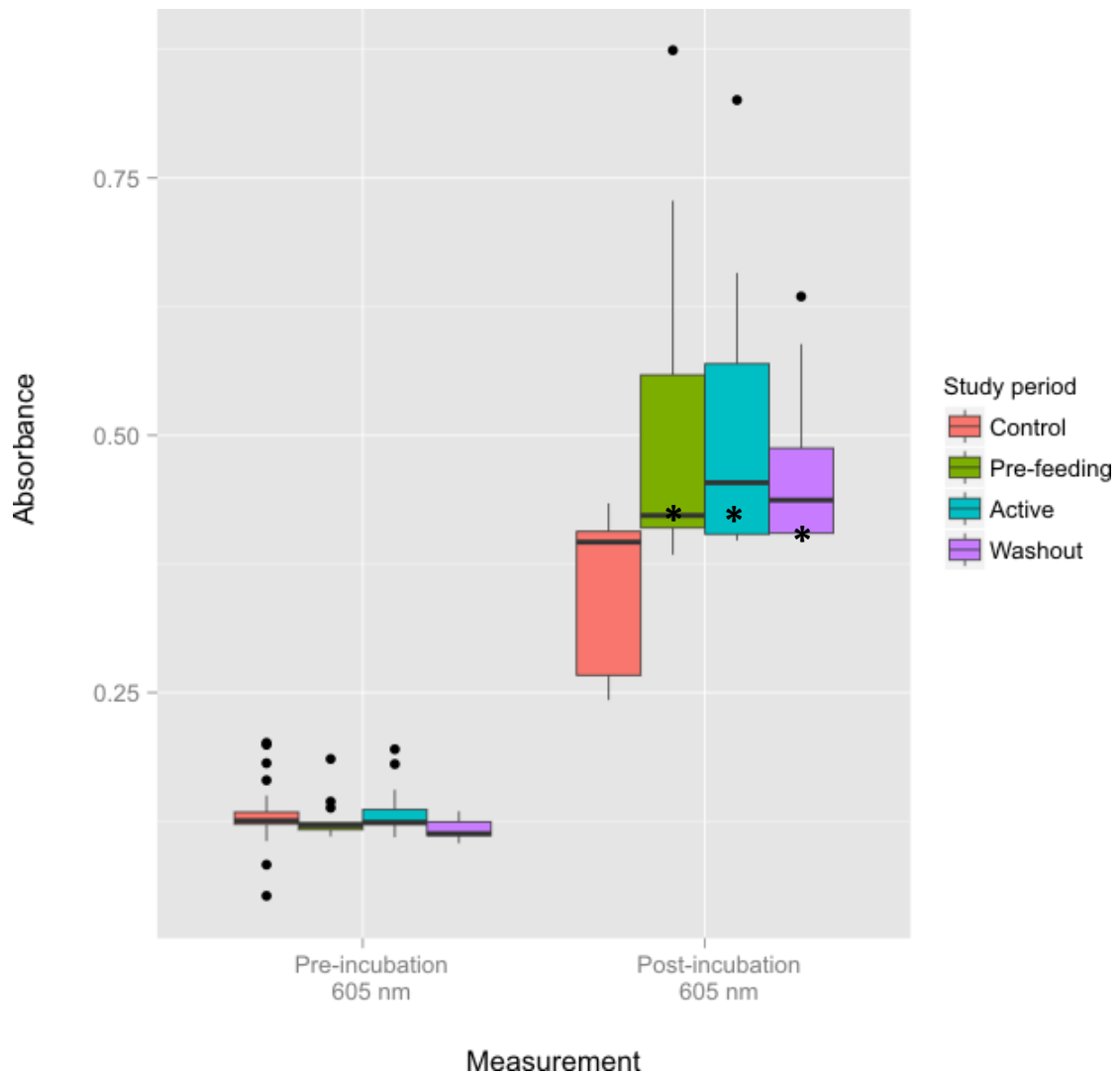


Figure 4.14 | The effect of diluted faecal waters on SOS induction in the SOS ChromoTest™ system - a box-plot of the absorbance at 605 nm pre-incubation and post-incubation is shown. The absorbance of diluted faecal waters from the 3 study periods is shown along with the absorbance of control samples. Outliers are represented by •*denotes a significant ($p = 0.05$) difference between the study period and control.

Neat and diluted faecal waters were also analysed by individual (**Fig.4.15** and **Fig. 4.16**). The data was not normally distributed; therefore a Kruskal-Wallis H test analysis was carried out. Analysis of neat faecal water prior to incubation showed that there was a significant difference between the absorbance of participants' faecal water and the controls at 605 nm [$\chi^2 (7) = 26.327, p < 0.001$] (**Fig. 4.15**). Post-hoc analysis through pairwise comparison showed that the ranked mean absorbance, at 605 nm, of PH11, PH26 and PH6 was significantly higher than the control ranked means at the 0.05 level. Analysis of neat faecal waters, following incubation, showed a significant difference between participants and control faecal waters [$\chi^2 (7) = 40.073, p < 0.001$]. Pairwise comparison post-hoc analysis showed that PH26 and PH11 had a significantly higher ranked mean absorbance at 605 nm than the controls at the 0.05 level. Furthermore, PH11 had a significantly higher ranked mean than PH1 at the 0.05 level. Analysis of the diluted faecal waters (**Fig 4.16**) gave different results to the neat faecal water (**Fig 4.15**). There was no significant difference between the ranked means of participants and controls at 605 nm prior to incubation. However, there was a significant difference between the ranked mean absorbance at 605 nm of participants and controls following incubation [$\chi^2 (7) = 38.204, p < 0.001$]. Post-hoc analysis through pairwise comparison showed that PH1, PH24 and PH26 had a significantly higher ranked mean absorbance, at 605 nm, than controls. In addition, PH1 and PH24 had a significantly higher ranked mean absorbance at 605 nm than PH11. The results of the 1/10 dilution show that the faecal waters of PH1 are genotoxic while the SOS system was not induced by its respective neat faecal water.

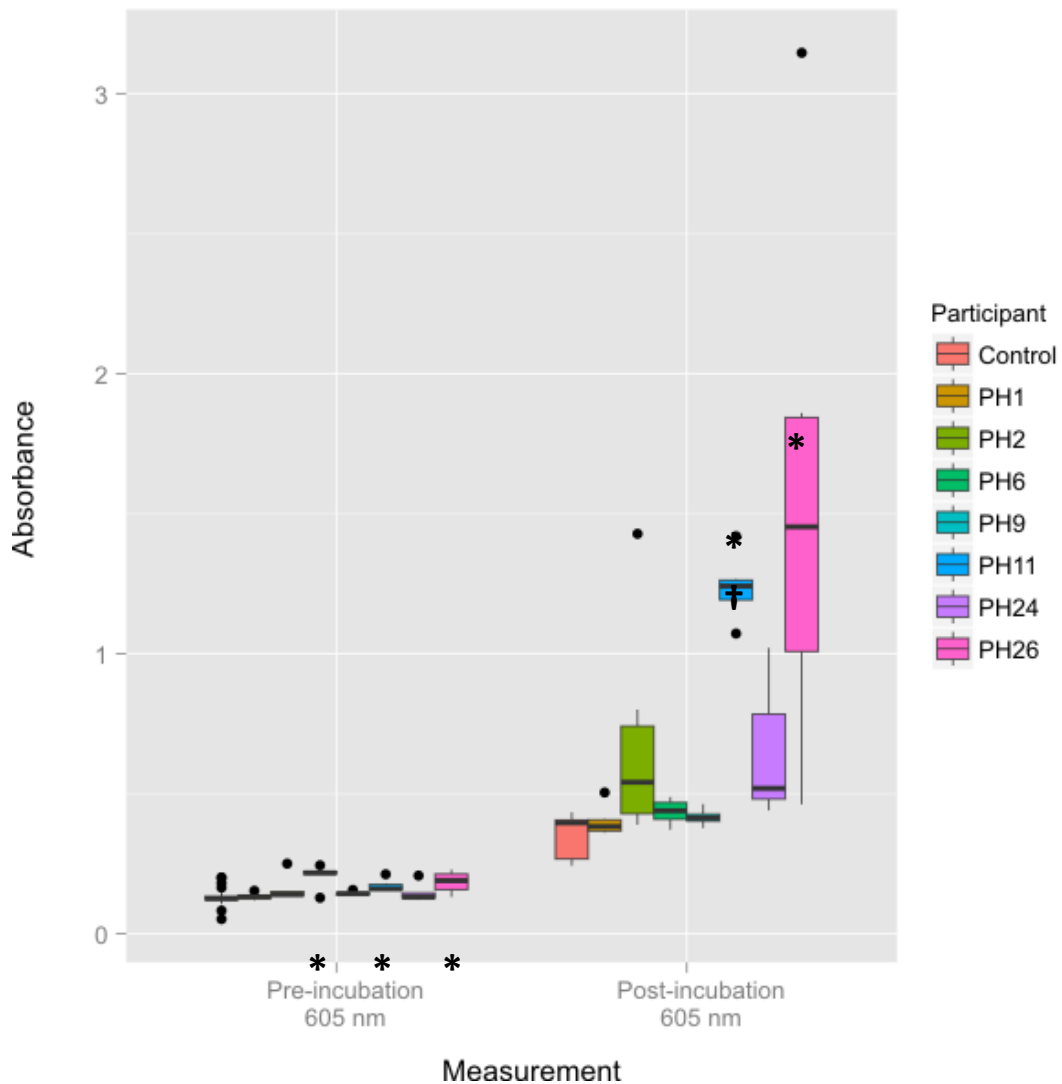


Figure 4.15| The effect of neat faecal waters on SOS induction in the SOS ChromoTest™ system - a box-plot of the absorbance at 605 nm pre-incubation and post-incubation is shown. The absorbance of neat faecal waters from PROHEMI participants is shown along with the absorbance of control samples. Outliers are represented by •. *denotes a significant ($p = 0.05$) difference between the study period and control, †denotes a significant difference between the sample and PH1.

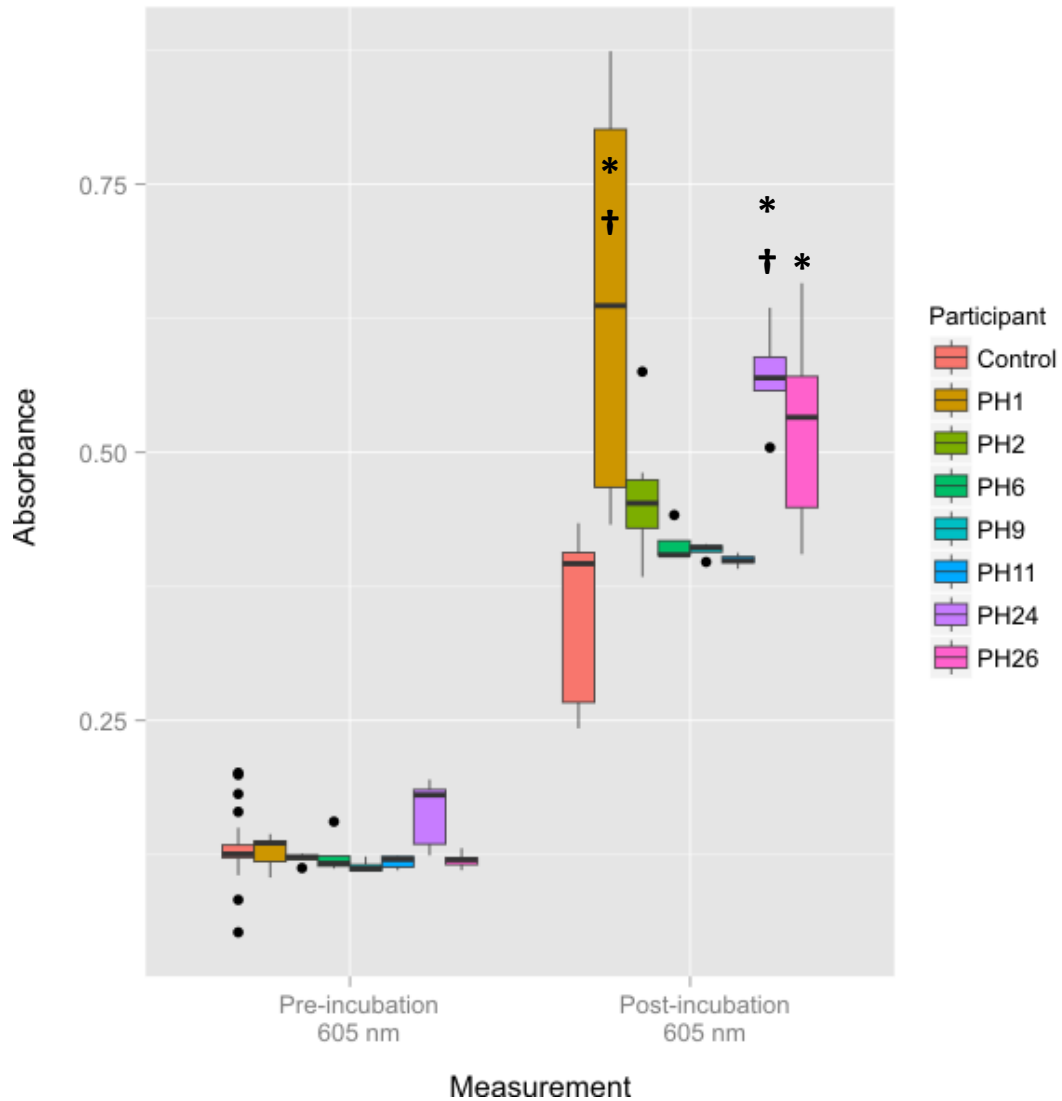


Figure 4.16| The effect of diluted faecal waters on SOS induction in the SOS ChromoTest™ system - a box-plot of the absorbance at 605 nm pre-incubation and post-incubation is shown. The absorbance of diluted faecal waters from PROHEMI participants is shown along with the absorbance of control samples. Outliers are represented by •. *denotes a significant difference between the study period and control, †denotes a significant difference between the sample and PH11.

4.4 Discussion

Culture dependent methods provide a means to interrogate a microbial community for functions of interest. Through the use of specialised agars, we have screened faecal material and the probiotic organisms used in the PROHEMI study for such functions. It has previously been shown that *B. bifidum* and some strains of *L. acidophilus* express β -galactosidase activity (de Vrese *et al.* 2001; Møller *et al.* 2001). Furthermore, the consumption of a probiotic organism, capable of increasing β -galactosidase activity, has been linked with alleviating clinical symptoms of lactose maldigestion (de Vrese *et al.* 2001). Previous studies are in accordance with my results, whereby *B. bifidum* (Cul20, NCIMB 30153) and *L. acidophilus* (Cul21, NCIMB 30156) and (Cul60, NCIMB 30157), express β -galactosidase activity. The results show a significant difference in β -galactosidase activity following probiotic administration in the Sheffield cohort, where activity increased. This difference may be due to expression of β -galactosidase activity by the two strains of *L. acidophilus* and *B. bifidum*. It has previously been shown that the consumption of *L. acidophilus* and *B. bifidum* for a 3 week period, in healthy individuals, did not increase β -galactosidase expression (Marteau *et al.* 1990). On the one hand the results suggest that probiotic consumption, for a much longer period, does increase β -galactosidase expression. However, in agreement with the findings of Marteau and colleagues Active Group 1, Active Group 2 and Washout period, from the Cardiff cohort did not show an increase in β -galactosidase activity. This result seems counter-intuitive. I would expect to see an increase in the activity of this enzyme in one of the groups, as one of the groups received the Active treatment. While there appears to be no evidence for geographical differences in response to probiotic administration, this may be the underlying reason for a differential response.

Bacterial β -glucuronidases are now receiving more attention, as they are becoming increasingly recognised as problem enzymes during drug therapy. Bacterial forms of the enzyme can have a significant impact upon the human body's efforts to excrete toxic compounds. The Phase I clinical trial of the anti-cancer compound CPT-11 (irinotecan) was linked with granulocytopenia, nausea, vomiting and diarrhoea (Shinkai *et al.* 1994). It has since been argued that the toxicity of this compound in the GIT, and therefore its side-effect of diarrhoea, is due to bacterial β -glucuronidase activity (Takasuna *et al.* 1996). Following this finding, research published this year determined the efficacy of an *E. coli* β -glucuronidase inhibitor in the prevention of CPT-11 induced diarrhoea (Roberts *et al.* 2013). One such inhibitor was found to protect against CPT-11 induced diarrhoea in mice. The probiotic strains used in the PROHEMI study do not express β -glucuronidase activity.

However, all Active groups from Sheffield and Cardiff as well as both Washout groups showed a significant increase in β -glucuronidase activity compared to their respective Pre-feeding levels. It has previously been shown that the feeding of *L. acidophilus* and *B. bifidum*, in the short-term, non-significantly increased β -glucuronidase activity (Marteau *et al.* 1990). The PROHEMI study used a longer Active feeding period of 6 months. Therefore, if *B. bifidum* and *L. acidophilus* have the means to increase β -glucuronidase activity, the longer feeding period may explain the significant increase. The diet of the individual can impact the level of β -glucuronidase activity. Through the use of a murine model, it has been shown that a diet high in fat and/or protein can significantly increase β -glucuronidase activity (Reddy *et al.* 1977). The authors further suggest the toxicity, excretion and reabsorption of carcinogens can be impacted by an increase in β -glucuronidase activity. Bacterial encoded glucuronidases can deconjugate these carcinogenic substances from inactive glucuronide conjugates bound for excretion. The role of probiotic administration in protection against colorectal cancer has recently been reviewed (Uccello *et al.* 2012). One discussed mechanism of protection was the ability of some probiotic bacteria to decrease β -glucuronidase activity. Uccello and colleagues discussed the potential for *L. acidophilus* to decrease β -glucuronidase activity in humans. However, my results show the potential for an increase in the activity of this enzyme; with the potential to detrimentally affect host-health. Furthermore, probiotic administration in patients receiving particular drug therapies will need to be considered carefully, as an increase in β -glucuronidase activity may lead to drug toxicity.

Bacterially encoded proteases can be detrimental to human health. These enzymes have been implicated in the progression of IBD and IBS (Steck *et al.* 2012). It has been shown that the metalloproteinase gelatinase (GelE) from *Enterococcus faecalis* contributes to the development of colitis in an experimental mouse model (Steck *et al.* 2011). It is believed that GelE cleaves E-cadherin leading to loss of epithelial barrier function. In addition, bacterial proteases are believed to play a pivotal role in immune evasion by pathogenic bacteria. The proteases produced to this end are various, numerous and have been discussed previously (Potempa and Pike 2009). One such mechanism is the resistance of pathogens to antimicrobial peptides through the degradation of such compounds by proteases (Nizet 2006). The importance of proteases in the progression of inflammatory disease of the gut is now being recognised. Through this recognition it has been suggested that the inhibition of proteases should be used as a novel therapy for inflammatory diseases of the GIT (Sałaga *et al.* 2013). The effect of probiotic consumption on the protease activity of the distal gut is

sparse. My results suggest that probiotic consumption can decrease protease activity with the potential to positively affect host health. The positive effects of some probiotic species and strains in the treatment of IBD and IBS have been discussed in **Chapter 1**. In addition to this discussion, the results hint at a potential mechanism for the beneficial effects exerted in this disease state. Probiotic bacteria may decrease the inflammatory potential of pathogenic bacteria through decreasing their pro-inflammatory effector molecules.

The potential for modulation of the gut microbiota to promote degradation of dietary cholesterol has attracted attention. *L. plantarum* has been shown to exhibit hypocholesterolemic effects (Jeun *et al.* 2010). Following a 4 –week intervention, where 6-week old C57BL/6 mice were fed 10^9 CFU/day, total cholesterol levels were significantly ($p < 0.05$) lower in the probiotic group than the control. The authors suggest that this lowering is not as a direct result of cholesterol degradation by the bacteria. It was suggested that the cholesterol lowering potential of this probiotic organism is mediated through elevation of bile acid excretion and decreased cholesterol uptake in the intestine. As previously discussed, some bacterial species possess cholesterol degrading enzymes. The *Streptomyces* cholesterol oxidase enzyme ChoA has been expressed in *E. coli* (Solaiman and Somkuti 1991). Further to this work, this enzyme has also been expressed in *B. longum* (Park *et al.* 2008). The authors suggest that the system used to express *choA* in this organism can be used in order to produce dairy products with useful proteins in them. Whether the authors aim to produce dairy products containing ChoA is unclear. Throughout the PROHEMI study, no cholesterol degrading activity was observed from any sample, at any time-point. This hints at the rarity/non-existence of direct cholesterol degradation, at least at a detectable level, in the human gut.

The importance of lipase/esterase activity has been discussed previously and probiotic administration can affect the expression of these enzymes also. Research has shown that in children receiving ceftriaxone treatment probiotic administration with a range of organisms/strains increased lipase/esterase activity (Zoppi *et al.* 2001). The data, however, were not shown. Furthermore, to what extent probiotic administration in this cohort increased lipase/esterase was not discussed either. My results show that lipase/esterase activity was unaffected by probiotic consumption. The general level of this activity was low throughout samples from all study periods.

Assessing the genotoxicity of compounds through a bacterial strain has been used for many years. Ames developed histidine auxotrophic *Salmonella typhimurium* strains in order to screen compounds for mutagenic properties (Ames *et al.* 1975). Three published strains were used in order to screen for different types of mutagenic potential. Strain TA1535 was utilised to screen for base-pair substitutions while strains TA1537 and TA1538 detected frameshift mutations. In addition, an *E. coli* strain, which is auxotrophic for tryptophan, has also been used in order to screen for mutagenic compounds (Tarmy *et al.* 1973). While these tester strains were useful for qualitative purposes, a given compound has mutagenic potential or not, they were not useful for direct quantification of the mutagenic/genotoxic potential of a compound. To this end, new bacterial strains with a means to directly quantify the genotoxic potential of a compound were developed. Quillardet and colleagues developed the PQ37 strain of *E. coli* (Quillardet *et al.* 1982). This strain contained a *sfiA::lacZ* fusion operon, whereby the expression of β -galactosidase was regulated by the cell division inhibitor SfiA (also known as Sula) (Huisman and D'Ari 1981). The *sfiA/sulA* gene is part of the SOS response in *E. coli* and is expressed when DNA is damaged (Mizusawa *et al.* 1983). Quillardet and colleagues utilised this strain to screen for genotoxic compounds. This was achieved through the induction of β -galactosidase activity, and its measurement through the enzymatic cleavage of the colorimetric substrate ONPG, by genotoxic agents. Sensitivity to genotoxic agents was further increased by making *E. coli* PQ37 deficient for the gene *uvrA* and therefore, unable to carry out excision repair. It can be argued that the genotoxic potential of a compound in a prokaryote model is not directly applicable to eukaryotic cells

With this in mind, a yeast genotoxicity reporter strain was developed (Jia *et al.* 2002). This strain once again utilises *lacZ*, in conjunction with the ribosomal component encoding *RNR3* gene, for colorimetric determination of the genotoxic potential of a compound. The authors argue that this system is a more "faithful" model of the genotoxic effect of compounds in mammalian cells. The responses of a single-cell eukaryote will be closer to a mammalian cell than the responses of a prokaryotic cell. Sensitivity of this model was further increased through the deletion of *cwp* genes encoding cell wall mannoproteins (Zhang *et al.* 2008). In addition to the utilisation of β -galactosidase activity as a proxy measurement for the genotoxic potential of a compound, *lux* genes have also been used. Yagur-Kroll and colleagues have developed a *sulA::luxCDABE E. coli* strain (Yagur-Kroll *et al.* 2010). For their experiments the SOS inducing quinolone nalidixic acid was used. In response to the induced DNA damage, *E. coli* become bioluminescent and this bioluminescence acts as a proxy for the quantification of genotoxic potential of a compound.

Bacterial reporter strains allow high-throughput and relatively simple screening of compounds in order to determine their genotoxic potential. While efforts have been made in order to make the results of these experiments applicable to humans, studies using human and animal cell populations are difficult to surpass. The comet assay and its methodological variations have previously been discussed (Fairbairn *et al.* 1995). One advantage of this assay is the ability to test the genotoxicity of a compound on a particular cell-line of interest. The immortal cell-line HT-29 was first isolated from a colon tumour in 1964 (Fogh *et al.* 1977) and can be used in the comet assay. Faecal water genotoxicity has been tested using HT-29 cells in the comet assay. It is possible to argue that detected genotoxic potentials of faecal waters in this system are biologically relevant to humans, as genotoxic faecal waters will come into contact with cells of the colon.

Reviewing the literature highlights the impact on diet upon the cytotoxicity and genotoxicity of faecal water. It has been shown that a reduction in dairy product consumption is linked with a significant lower HT-29 cell survival (34% vs. 20% respectively, $p = 0.025$) in the HT-29 cytotoxicity test (Glinghammar *et al.* 1997). However, the genotoxicity of faecal waters following dairy rich and dairy free diets was not significantly different. Other research however, has shown that a diet high in fat but low in dietary fibre increases the genotoxic potential of faecal water (Rieger *et al.* 1999). Tail intensity, following the incubation of faecal waters from two diets (rich in fat and low in fibre vs. high in dietary fibre and low in fat) with HT-29 cells in a comet assay, were significantly different (28.7% vs. 17.5% respectively, $p = 0.02$). In an effort to negate the potential of diet induced faecal water genotoxicity, the effect of probiotic supplementation has been studied. AD sufferers and healthy subjects received a placebo yoghurt drink or a probiotic drink containing *L. paracasei* Lpc-37, *L. acidophilus* 74-2 and *B. lactis* 420 (7.8×10^{10} , 5.8×10^6 and 1.2×10^7 CFU/day respectively) for 8 weeks. While there was no significant difference in the genotoxic activity of faecal waters of healthy individuals, there was a significant difference between AD sufferers. Patients with AD who received the probiotic yoghurt had a significantly ($p = 0.029$) lower level of faecal water genotoxicity (Roessler *et al.* 2012).

While the genotoxicity experiment used only a limited number of samples, probiotic administration showed no significant effect upon faecal water genotoxicity. Neat and diluted faecal waters from all study periods showed significant SOS induction compared to the control. In a study where various LAB strains were incubated with faecal water prior to incubation with HT-29 cells in a comet assay, a significant difference was observed (Burns

and Rowland 2004). Burns and colleagues did not incubate the HT-29 cells directly with the bacterial faecal water suspension due to bacterial cell interference with the comet assay. *B. bifidum* BB12, *B. lactis* 420, *L. plantarum* and *L. bulgaricus* showed a significant reduction in genotoxicity compared to control faecal water incubated with PBS (68%, 24%, 63% and 37% reduction respectively, $p < 0.05$). While this research shows that probiotic organisms can decrease the genotoxicity of faecal water, it is not entirely clear how applicable the results are to probiotic supplementation. The authors selected the bacterial strains as previous research showed that these strains could survive GIT transit. However, their effects *in vivo* still need to be corroborated as my results suggest that probiotic supplementation *in vivo* does not decrease the genotoxic potential of faecal water.

My results highlight that there are inter-individual differences in the genotoxicity of faecal water. This has previously been shown where the genotoxic potential of faecal waters from 6 individuals induced varying levels of genotoxicity (Oßwald *et al.* 2000). Furthermore, faecal waters from one individual over 6 weeks showed varying levels of genotoxicity with significant differences ranging from no significant difference to the control to highly significant ($p < 0.001$). Results from the PROHEMI study show that the neat faecal waters of PH1, PH2, PH6, PH9 and PH24 are not statistically different from the controls while PH11 and PH26 are different. However, once diluted the faecal waters of PH1 and PH24 do become significantly more genotoxic than controls. This suggests that while neat, the faecal waters of PH1 and PH24 were too cytotoxic for the SOS ChromoTest™ reporter strain and therefore, the SOS system was not induced. However, once diluted the cytotoxicity of the samples decreased and therefore, SOS system induction could take place. The SOS ChromoTest™ system utilises the *E. coli* PQ37 strain developed by Quillardet and colleagues (Quillardet *et al.* 1982). This strain is constitutive for alkaline phosphatase activity and can be measured through the use of a colorimetric substrate. Spill-over from the colour produced through the β -galactosidase reaction interferes with this readout and therefore, makes it difficult to attribute whether absorbance is linked with alkaline phosphatase activity (viability) or β -galactosidase activity (genotoxicity). The concept of responders to a treatment has been discussed previously. Due to the inter-participant variation seen, it would be of interest to stratify the results based on participant and to then determine whether probiotic administration has a genotoxic reducing effect. It would be particularly interesting to determine the effect of probiotic administration on those with particularly high genotoxic faecal water. Unfortunately, the effect of probiotic administration on faecal water genotoxicity in this instance was underpowered. While there are enough numbers to carry

out study period and participant analysis, I was unable to stratify the results based upon individual in a given study period. However, repeating the experiment with faecal waters from participants with high genotoxicity alone may give rise to a responder response to probiotic administration.

4.5 Conclusions

The main conclusions for this chapter are as follows:

1. screening for β -galactosidase, β -glucuronidase, protease, esterase/lipase and cholesterol degrading activities can be carried out on faecal material using functional media;
2. probiotic administration reduces the numbers of bacteria expressing protease activity in healthy males;
3. the numbers of bacteria expressing β -galactosidase and β -glucuronidase activity in healthy males increases during probiotic supplementation;
4. there are geographical differences between the two study centre in terms of the numbers of bacteria expressing β -galactosidase and β -glucuronidase activity
5. the genotoxicity of faecal waters are unaffected by probiotic supplementation in healthy males;
6. there are individual differences in faecal water genotoxicity levels in healthy males.

5. CULTURE INDEPENDENT ANALYSIS OF FAECAL MATERIAL AND FAECAL WATERS FROM THE PROHEMI STUDY

5.1 Introduction

While microbiology has historically utilised classical culture dependent techniques, there has been a shift in recent years to the use of culture independent techniques. Researchers have been aware of the bias that culture dependent analysis can introduce since Staley and Konopka coined the phrase, "the great plate count anomaly" (Staley and Konopka 1985). This notion has previously been discussed by Rappé and Giovannoni. Meta-analysis of published GenBank 16S *rRNA* gene sequences from 1993 – 2002 showed that deposition of sequences from culture independent studies were over 2-fold greater than those from culture dependent studies (Rappe and Giovannoni 2003).

Many culture independent techniques rely on the use of the 16S *rRNA* gene as a molecular chronograph (Woese 1987) and biomarker for organisms through their subsequent sequencing. With regards to sequencing, there have been significant advances since the advent of Sanger sequencing by Frederick Sanger (Sanger *et al.* 1977). The NGS technology 454 pyrosequencing, through the use of emulsion PCR, has been applied to sequence and assemble the genome of *Mycoplasma genitalium* (Margulies *et al.* 2005). The method has also been applied to analyse complex microbial communities such as the deep sea "rare biosphere" (Sogin *et al.* 2006), the soil (Roesch *et al.* 2007) and the gut microbiome in obese and lean twins (Turnbaugh *et al.* 2009). NGS technologies are still evolving with sequencing platforms such as the Ion Torrent, which utilises protons (Rothberg *et al.* 2011) and the further development of optical based platforms such as Illumina-Solexa sequencing (Bentley *et al.* 2008). Illumina-Solexa sequencing, through the use of HiSeq2000 and MiSeq platforms, has been applied to sequence complex microbial communities (Caporaso *et al.* 2012). These complex communities included the human oral cavity, the skin and faecal material. Sequencing technologies allow researchers to interrogate the importance of both culturable and as yet uncultivated unculturable bacteria that make up the human distal gut microbiome.

Metabonomics, as defined by Nicholson and colleagues, is "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" (Nicholson *et al.* 1999). This technique can be considered culture independent as biological fluids are generally the source of information and are analysed through spectroscopic methods. The technique has been applied in order to analyse urine and plasma (Beckonert *et al.* 2007) and has been successfully applied in the detection of epithelial ovarian cancer (Odunsi *et al.* 2005).

With regards to the gut, it is understood that the commensal gut microbiota play a pivotal role in the metabolism of compounds. Therefore, it follows that the dysbiosis of the gut microbial community, and the subsequent impact upon host health, will give rise to different metabonomic signatures. Antibiotic treatment with enrofloxacin in mice affected their faecal and urinary metabonomic profiles (Romick-Rosendale *et al.* 2009). Analysis through principal component analysis (PCA) showed that samples prior to antibiotic treatment clustered separately from post-treatment samples. In humans ¹H nuclear magnetic resonance (NMR) analysis of faecal waters obtained from IBD patients, showed significant differences to healthy controls. Namely, there was a significant ($p < 0.05$) depletion in the SCFAs acetate and butyrate in UC patients (Marchesi *et al.* 2007). This research highlights that disruptions in normal gut function can have pronounced effects upon the metabonomic profiles of an individual.

Novel high-throughput methods such as NGS and metabonomic analysis shed light on our complex gut microbial ecosystem. However, there is very little in the literature as to the effect of probiotic administration on metabonomic profiles in humans. In addition there is very little research into the effect of probiotic administration into the gut bacterial community of healthy individuals. The response of the infirm to various probiotic strains and prebiotics has been well documented (Tuohy *et al.* 2003). However, there is a gap in knowledge with respect to the changes, if any, in the gut microbiota of healthy individuals during probiotic intervention.

5.1.1 Chapter Aims

The aims of this chapter are as follows:

1. to determine whether probiotic administration affects the bacterial community of healthy males;
2. to determine whether there is a difference between the bacterial community of healthy males from the two study centres;
3. to answer whether the gross metabonomic profiles of healthy individuals are affected by probiotic administration;
4. to answer whether probiotic administration in healthy males affects chemical shifts associated with SCFAs;
5. to determine whether there are differences in the gross metabonomic profiles and SCFA profiles of healthy individuals from the two study centres.

5.2 Methods and materials

5.2.1 Illumina MiSeq sequencing of PROHEMI faecal DNA

Previously extracted faecal DNA from the PROHEMI study was diluted to a concentration of 5 ng/ μ L. The primers, 515F (5'- GTGCCAGCMGCCGCGGTAA) and 806R (5'- GGACTACHVGGGTWTCTAAT), were synthesised with the necessary Illumina adapters as previously described (Caporaso *et al.* 2012) in order to amplify the V4 region of the 16S *rRNA* gene. Multiplex sequencing of the DNA samples was achieved through the synthesis of a unique sample barcode in the reverse primer. Each DNA sample was amplified in triplicate 25 μ L reactions through the PCR. Briefly, each reaction contained 5 ng of template DNA, 0.7 μ M of total primers, 0.7 μ M mixed dNTPs, 2.5 U KAPA HiFi *Taq* DNA polymerase (Kapa Biosystems Inc., Boston, US) and 1 X accompanying reaction buffer (2 mM MgCl₂). The thermal cycler (Bioer Gene Pro, Bioer Technology, China) was programmed with an initial denaturing step of 95°C for 5 min, followed by 25 cycles of 98°C for 20 s, 60°C for 30 s and 72°C for 40 s with a final extension step of 72°C for 1 min. Triplicate reactions were pooled and visualised through gel electrophoresis. Visualised bands of correct size were excised and purified using the QIAquick gel extraction kit (QIAGEN, Crawley, West Sussex, UK) following manufacturer's guidelines. Product concentration was quantified using the Qubit® 2.0 fluorometer system (Life Technologies Ltd, Paisley, UK) following manufacturer's guidelines. All samples were diluted to a final concentration of 2.5 ng/ μ L and were pooled in equal volumes. One final visual check was carried out through gel electrophoresis, where the desired result was a single clear product. Following the final visual check, the equimolar pooled samples were subjected to 250 bp paired end Illumina MiSeq sequencing (Illumina United Kingdom, Essex, UK) following manufacturer's guidelines.

5.2.2 Bioinformatic analysis of MiSeq results

Mothur, an open-source programme was used for the majority of the analysis (Schloss *et al.* 2009). In order to carry out the analysis a tab separated file, which contained the sample ID and file names of both paired end reads for a given sample, was created (**Appendix III**). This served to associate the relevant sequence files with the correct sample.

Sequences were processed using Mothur through custom batch files (**Appendix IV**) based upon a protocol developed by Schloss and colleagues (Schloss *et al.* 2011). Mothur utilised a current version of the RDP training set (training set 9) and the Silva 16S *rRNA* gene FASTA reference sequences (Pruesse *et al.* 2007) in order to carry out the analysis. Briefly, contigs were constructed from the two paired-end reads for a given sample and associated with the respective sample. Sequences were shortened to a maximum length of 275 bp and any sequences with ambiguous bases removed. In order to reduce the computational power needed to carry out the analysis, unique sequences were kept and multiples of unique sequences removed. Unique sequences were aligned to the reference Silva 16S *rRNA* FASTA sequences. The need for computational power was once again reduced by selecting unique sequences and discarding multiples of the unique sequence. Chimeras were identified and removed before an OTU table was generated using an 80% cut-off. Sequences which were not bacterial, including archaeal, mitochondrial and chloroplast DNA, were removed. OTUs were classified using a 97% similarity cut-off to the genus level. Normalisation of OTUs was achieved through sub-sampling to 1370 and 10197 sequences. Samples were initially sub-sampled to 1370 sequences in order to maintain a high level of samples for analysis. Sub-sampling to 10197 sequences was carried out to maintain a higher level of sequences for analysis. However, sub-sampling to 10197 sequences removed some samples with sequence counts below 10197 from further analyses. Computations including the inverse Simpson's diversity and Jaccard similarity coefficient indices, Yue and Clayton θ similarity coefficients, weighted and unweighted UniFrac (Lozupone and Knight 2005) distances were carried out in Mothur. Ordination through non-metric multi-dimensional scaling (nMDS) and principal co-ordinate analysis (PCoA) of some of the previous indices were also carried out in Mothur. Generated axes from the ordination techniques were plotted using R statistical software (R-Core-Team 2012) through the use of a custom script (**Appendix II**). The script utilised the R packages, labdsv (Roberts 2010), vegan (Oksanen *et al.* 2011) and calibrate (Graffelman 2012) packages. Normality, homogeneity of variance testing and Kruskal – Wallis H tests were carried out in IBM statistics 20 (IBM, Portsmouth, UK).

Extended error bar plots of mean proportions of phyla and families were created using STAMP: Statistical Analysis of Metagenomic Profiles v2.0.0 software (Parks and Beiko 2010). Study centres and periods were analysed through a two-sided Welch's t-test with Bonferroni multiple test correction.

5.2.3 Metabonomic analysis of PROHEMI faecal waters

5.2.3.1 Generation of faecal waters from PROHEMI faecal samples

Faecal waters were generated as previously described (see **Chapter 2.5**).

5.2.3.2 ^1H NMR analysis of PROHEMI faecal waters

Metabonomic analysis of faecal waters was carried out through proton NMR spectroscopy at Imperial College London. The protocol followed has previously been described (Marchesi *et al.* 2007). Briefly, 180 μL of faecal water was added to 320 μL of deuterium oxide and 100 μL of 0.2 M sodium phosphate buffer (pH 7.4). This buffer contained 100% deuterium oxide, for the magnetic field lock, and 0.01% 3-(trimethylsilyl)-[2,2,3,3- $^2\text{H}_4$] propionic acid sodium salt, for the spectral calibration. The buffer also contained 3 mM of sodium azide as a precautionary antibacterial measure. The faecal water, deuterium oxide and sodium phosphate buffer mixture was centrifuged at 10,000 g for 5 min before 580 μL was dispensed into 5 mm thickness, 7 inch borosilicate NMR tubes (Cortecnet, France). These tubes were subjected to NMR analysis. ^1H NMR spectra of faecal waters were obtained using a Bruker 600 MHz spectrometer (Bruker, Rheinstetten, Germany) at the operating ^1H frequency of 600.13 MHz at a temperature of 300 K. A standard NMR pulse sequence (recycle delay -90°- t_1 -90°- t_m -90°-acquisition) was applied to acquire one-dimensional ^1H NMR spectral data, where t_1 was set to 3 μs and t_m (mixing time) was set to 100 ms. The water peak suppression was achieved using selective irradiation during a recycle delay of 2 s and t_m . A 90° pulse was adjusted to 10 μs . A total of 256 scans were collected into 64 k data points with a spectral width of 20 ppm.

5.2.3.3 Assignment of SCFAs to chemical shifts

Chemical shifts for acetate, propionate, butyrate and valerate were obtained from the Madison Metabolomics Consortium Database (Cui *et al.* 2008). Chemical shifts corresponding to these compounds were manually selected from the dataset and exported.

5.2.3.4 Statistical analysis of the ^1H NMR data

The proportional abundances of the chemical shifts for NMR spectra were calculated. AGNES, using Ward's method, of the calculated Euclidean distances of the proportional abundances of NMR chemical shifts and export in Newick format was carried out in R statistical software. In order to carry out AGNES a custom script was used. The script utilised the R packages, ape (Paradis *et al.* 2004), BiodiversityR (Kindt and Coe 2005) and cluster (Maechler *et al.* 2005) packages (**Appendix II**). Once exported in Newick format, the tree was manipulated using iTol software online (Letunic and Bork 2011). PCoA, using calculated Euclidean distances of the proportional abundances of chemical shifts, was carried out in R statistical software. A custom script was used which utilised vegan (Oksanen *et al.* 2011) calibrate (Graffelman 2012) and labdsv (Roberts 2010) R packages (**Appendix II**).

5.3 Results

5.3.1 Illumina MiSeq DNA sequencing of 16S *rRNA* genes obtained from PROHEMI DNA samples

5.3.1.1 The effect of normalisation on the dataset

Sequence counts for a given sample are shown in **Appendix V**. The lowest number of sequences was 33 while the highest was 279,029 sequences. Samples were sub-sampled to 1370 and 10197 with sequence counts below the two cut-offs removed from β -diversity, phylum and family level analyses.

5.3.1.2 α – diversity of samples

Samples were compared by study centre and study period. Inverse Simpson diversity results were plotted based upon the study period (**Fig. 5.1**) and study centre (**Fig. 5.2**) prior to and following normalisation. Analysis through the Kruskal – Wallis H test showed no significant difference between the Simpson diversity indices of non-normalised and both sets of normalised sequences. Furthermore, there was no significant difference in the Simpson diversity indices of samples based upon study period. However, Kruskal – Wallis H test analysis showed a significant difference between the mean ranks of samples based upon study centre, [χ^2 (1) = 33.104, $p < 0.001$].

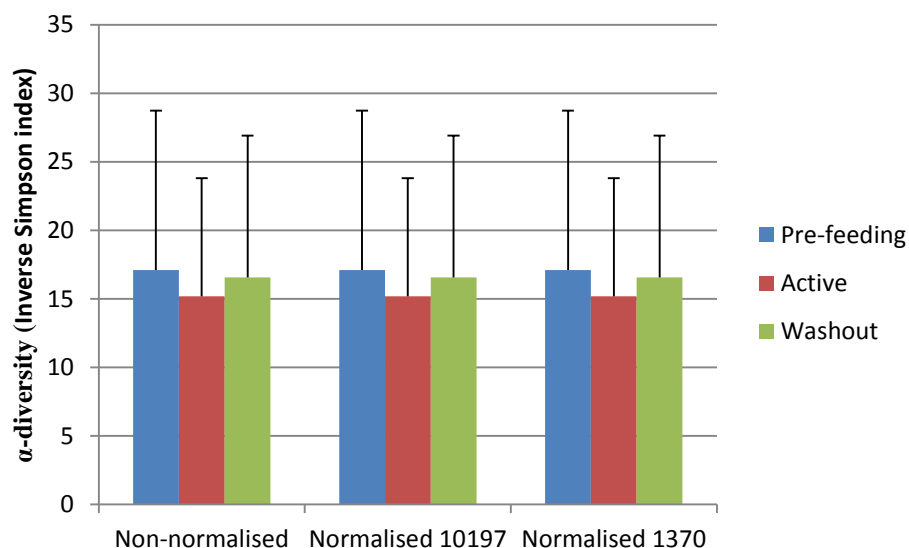


Figure 5.1| Study period inverse Simpson diversity indices of PROHEMI faecal DNA – non – normalised, normalised through sub-sampling to 10197 sequences and 1370 sequences are shown based upon study period

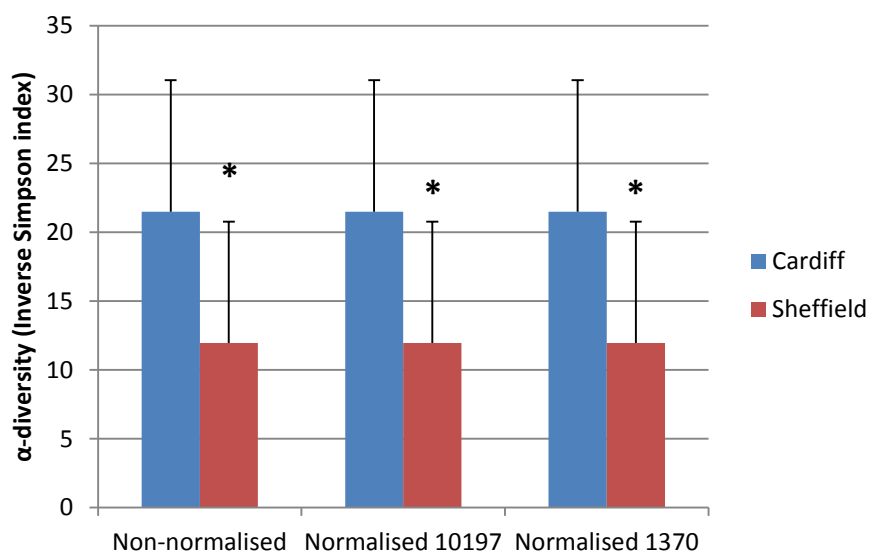


Figure 5.2| Study centre inverse Simpson diversity indices of PROHEMI faecal DNA – non – normalised, normalised through sub-sampling to 10197 sequences and 1370 sequences are shown based upon study centre. * denotes a significant difference between Sheffield and Cardiff samples ($p < 0.05$).

5.3.1.2 β – diversity analysis of PROHEMI faecal DNA samples

β -diversity ordination plots for the 1370 sub-sample are not shown as the results were similar to those obtained from sub-sampling to 10197 sequences.

5.3.1.2.1 Analysis of all PROHEMI faecal DNA samples by study period

PROHEMI faecal samples were subjected to PCoA analysis of Jaccard similarity coefficient indices and Yue and Clayton θ similarity coefficients. Data, which were normalised through sub-sampling to 1370 and 10197 sequences, showed no separate clustering of Pre-feeding, Active and Washout periods (**Fig. 5.3 A-B**).

In addition, nMDS analysis of both unweighted and weighted UniFrac distances of the two sub-samples showed no separate clustering of samples based upon study period (**Fig. 5.4 A-B**).

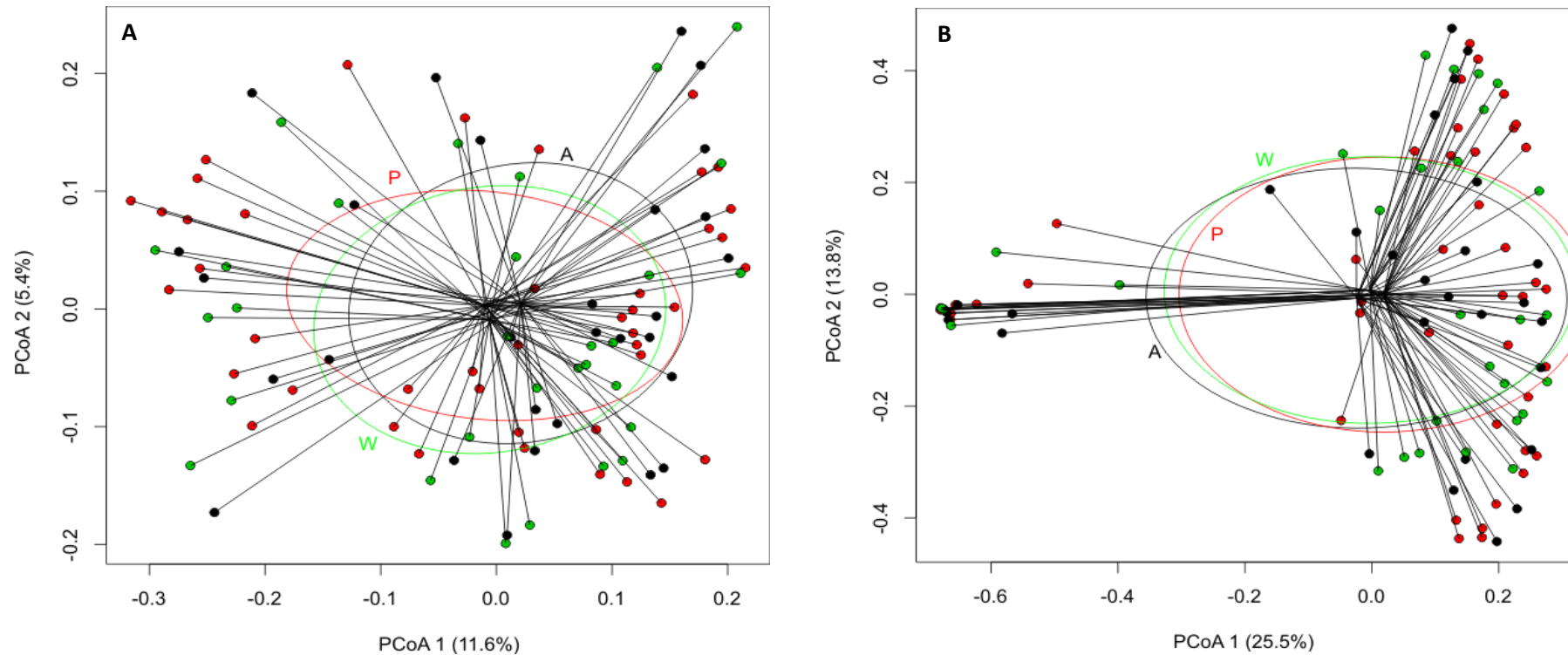


Figure 5.3| The effect of probiotic administration on the faecal DNA β -diversity of all individuals from the PROHEMI study following normalisation through sub-sampling to 10197 sequences – In both plots P = Pre-feeding, A = Active and W = Washout periods. The percentage of variation explained is shown for each axis. (A) shows PCoA of Jaccard similarity coefficient indices of PROHEMI samples. (B) shows PCoA of Yue and Clayton θ similarity coefficients of PROHEMI samples.

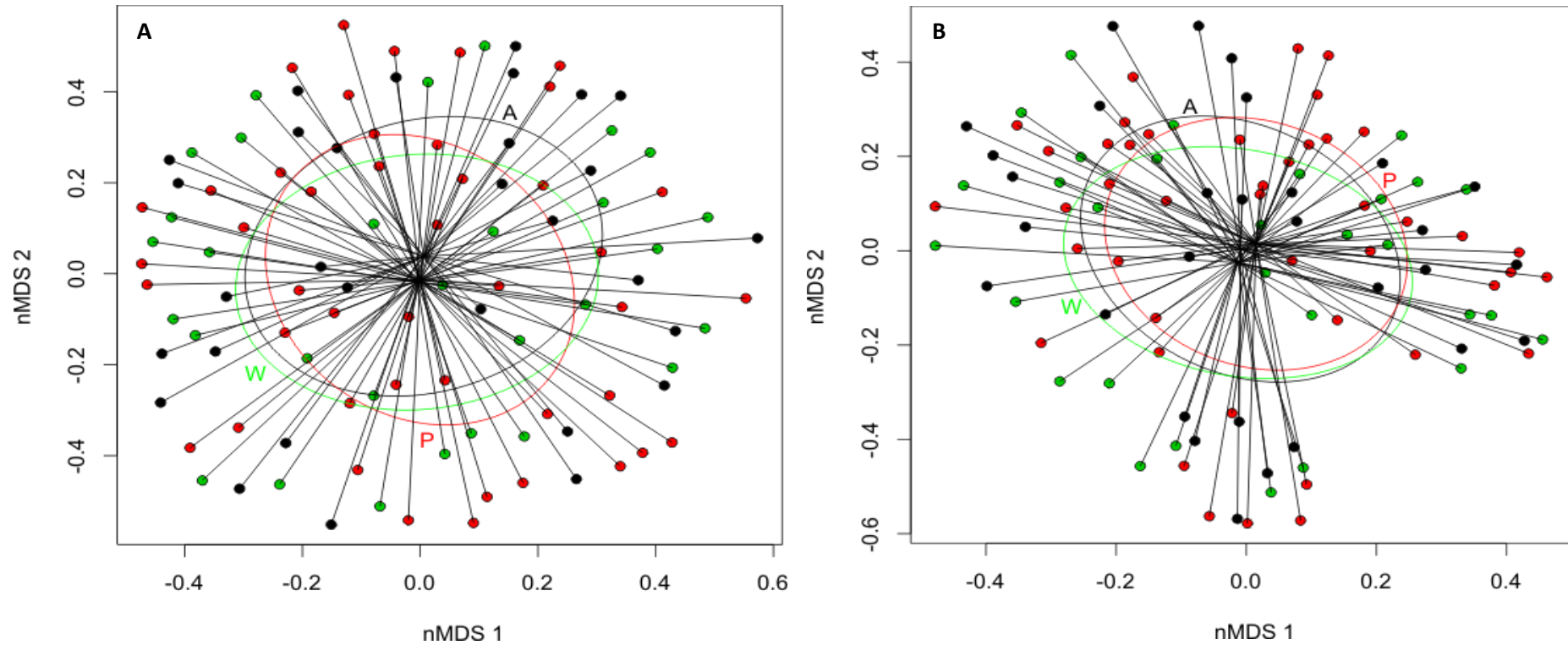


Figure 5.4| The effect of probiotic administration on the faecal DNA UniFrac distances of all individuals from the PROHEMI study following normalisation through sub-sampling to 10197 sequences – In both plots **P** = Pre-feeding, **A** = Active and **W** = Washout periods. **(A)** shows nMDS of unweighted UniFrac distances of PROHEMI samples. **(B)** shows nMDS of weighted UniFrac distances of PROHEMI samples.

5.3.1.2.2 Analysis of Cardiff PROHEMI faecal DNA samples by study period

β -diversity analysis was also carried out by study period through splitting the data into their respective study centres. With respect to the Cardiff study centre, PCoA of Jaccard similarity coefficient indices, using both sub-sample sets, shows samples clustered together regardless of the study period (**Fig. 5.5 A**). Analysis through PCoA of Yue and Clayton θ similarity coefficients of the two sub-sample sets showed similar results (**Fig. 5.5 B**). However, the Pre-feeding group did not cluster tightly with the other groups as was observed in PCoA analysis of the Jaccard similarity coefficient indices.

Analysis through nMDS of weighted and unweighted UniFrac distances was also carried out. Through analysis of the 1370 sequence and 10197 sequence sub-samples it was shown that samples from all 4 study periods clustered together (**Fig. 5.6 A-B**).

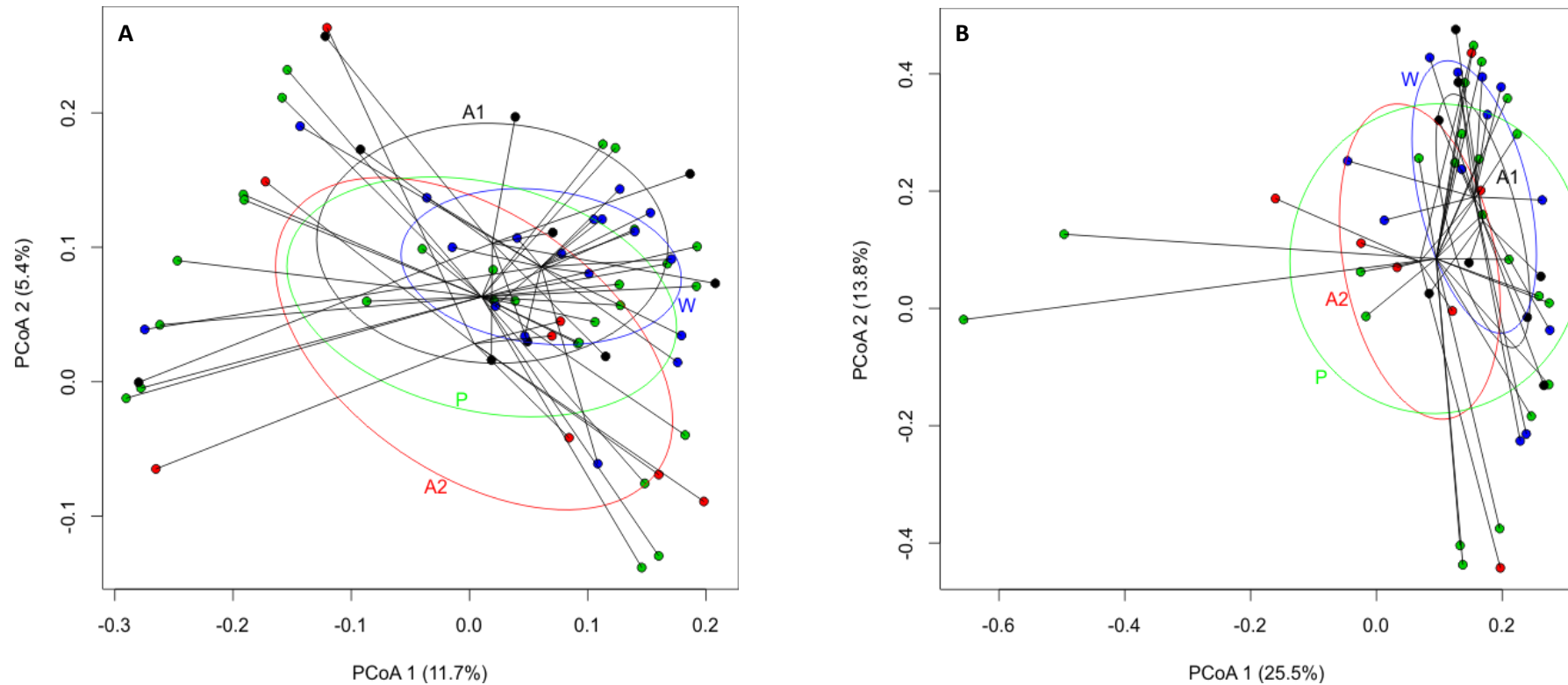


Figure 5.5] The effect of probiotic administration on the β -diversity of Cardiff/Port Talbot faecal DNA from the PROHEMI study following normalisation through sub-sampling to 10197 sequences – In both plots **P = Pre-feeding, **A1** = Active group 1, **A2** = Active group 2 and **W** = Washout periods. The percentage of variation explained is shown for each axis. **(A)** shows PCoA of Jaccard similarity coefficient indices of Cardiff/Port Talbot PROHEMI samples. **(B)** shows PCoA of Yue and Clayton θ similarity coefficients of Cardiff/Port Talbot PROHEMI samples.**

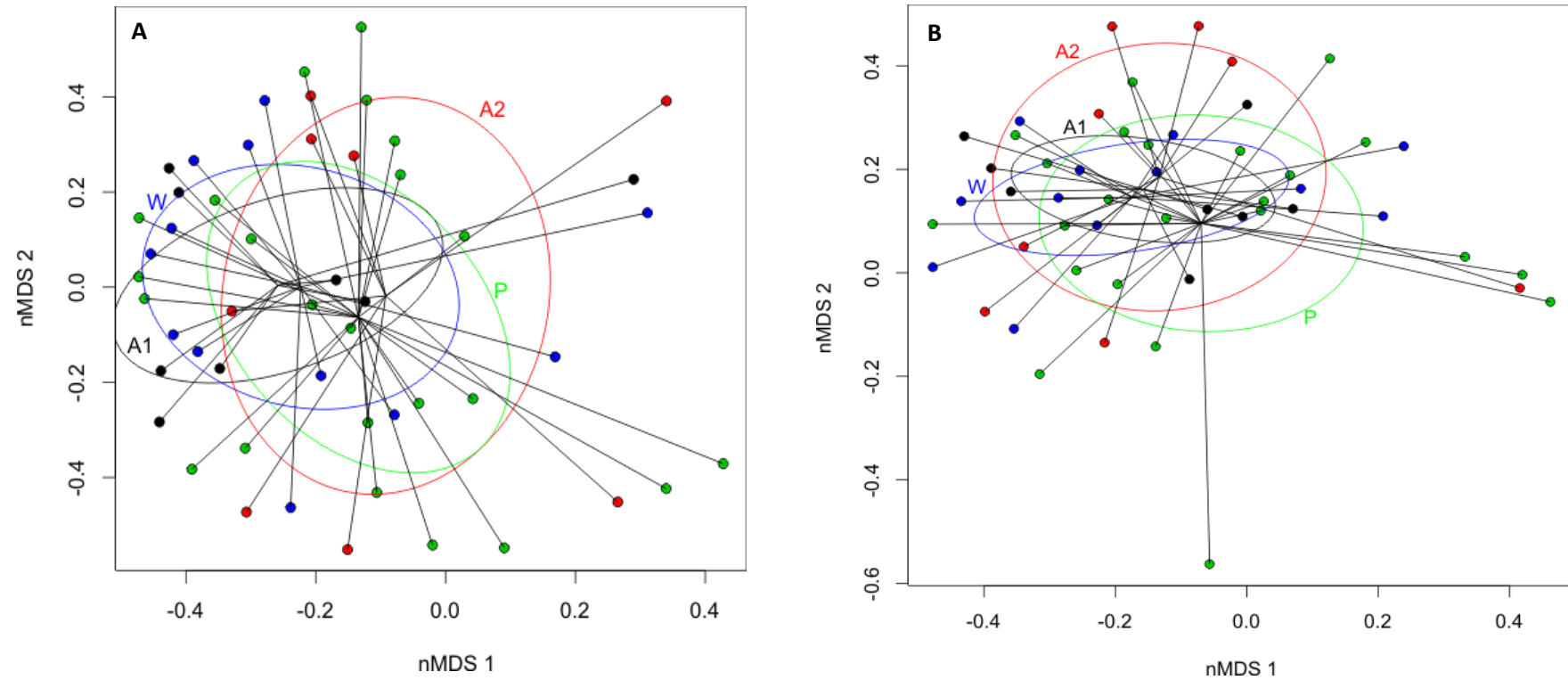


Figure 5.6| The effect of probiotic administration on the faecal DNA UniFrac distances of Cardiff/Port Talbot individuals from the PROHEMI study following normalisation through sub-sampling to 10197 sequences – In both plots P = Pre-feeding, A1 = Active group 1, A2 = Active group 2 and W = Washout periods. (A) shows nMDS of unweighted UniFrac distances of Cardiff/Port Talbot PROHEMI samples. (B) shows nMDS of weighted UniFrac distances of Cardiff/Port Talbot PROHEMI samples.

5.3.1.2.3 Analysis of Sheffield PROHEMI faecal DNA samples by study period

Analysis of Sheffield samples alone through PCoA analysis of Jaccard similarity coefficient indices of the two sub-sample datasets showed no effect of probiotic administration (**Fig. 5.7 A**). Samples from the Pre-feeding, Active and Washout periods clustered together. PCoA analysis of Yue and Clayton θ similarity coefficients also showed no effect of probiotic administration on the β -diversity of Sheffield PROHEMI faecal DNA, as samples from all 3 study periods clustered together (**Fig 5.7B**).

Through nMDS analysis of unweighted and weighted UniFrac distances of both sub-samples, no probiotic driven change in β -diversity of Sheffield samples was observed (**Fig. 5.8 A-B**). Samples from all three study periods clustered together once again.

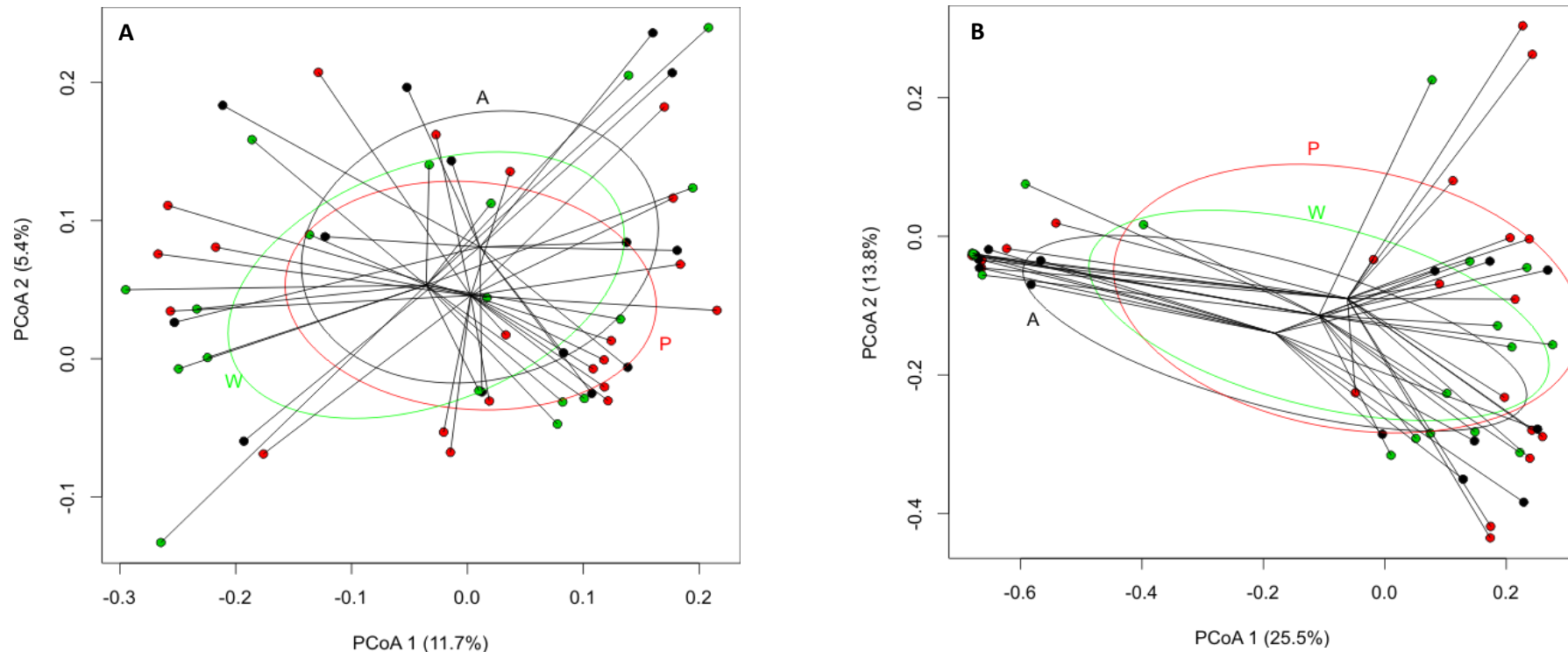


Figure 5.7 | The effect of probiotic administration on the faecal DNA β -diversity of Sheffield individuals from the PROHEMI study following normalisation through sub-sampling to 10197 sequences – In both plots P = Pre-feeding, A= Active and W = Washout periods. The percentage of variation explained is shown for each axis. (A) shows PCoA of Jaccard similarity coefficient indices of Sheffield PROHEMI samples. (B) shows PCoA of Yue and Clayton θ similarity coefficients of Sheffield PROHEMI samples.

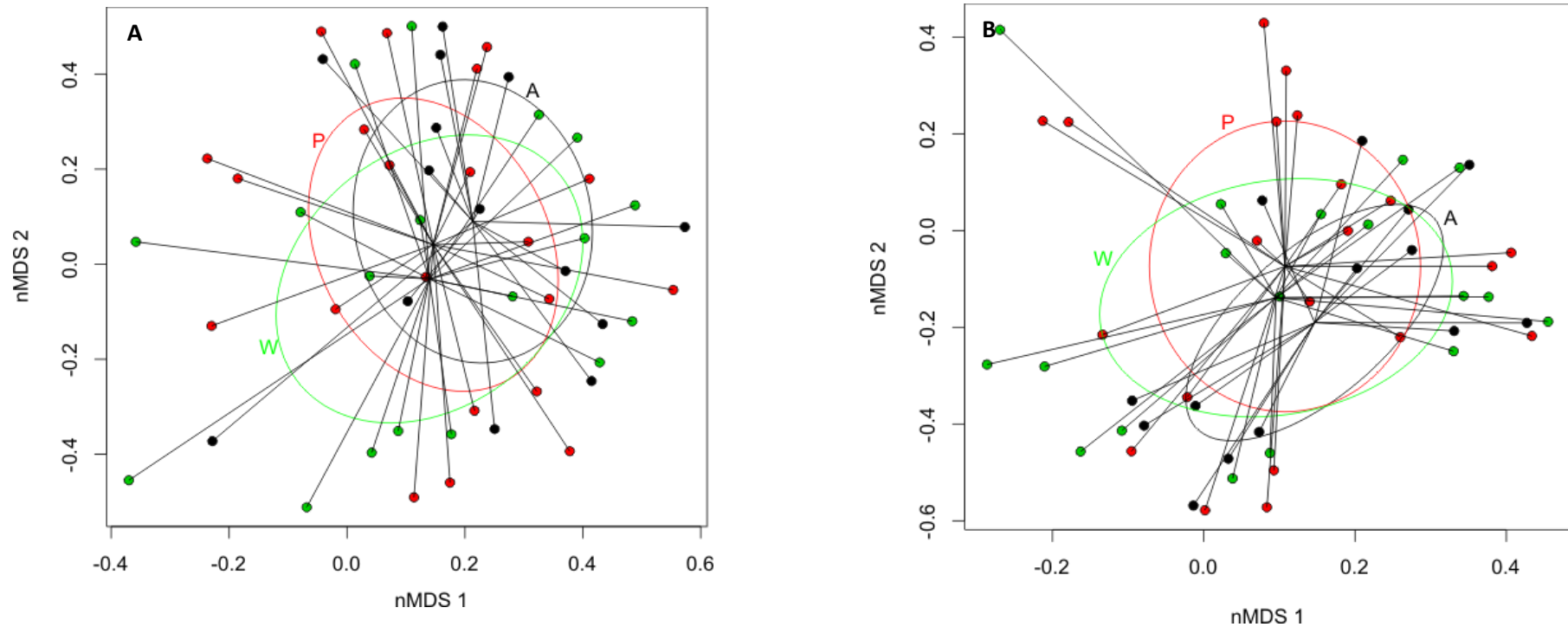


Figure 5.8| The effect of probiotic administration on the faecal DNA UniFrac distances of Sheffield individuals from the PROHEMI study following normalisation through sub-sampling to 10197 sequences – In both plots P = Pre-feeding, A= Active and W = Washout periods. The percentage of variation explained is shown for each axis. (A) shows nMDS of unweighted UniFrac distances of Sheffield PROHEMI samples. (B) shows nMDS of weighted UniFrac distances of Sheffield PROHEMI samples.

5.3.1.2.4 Analysis of all PROHEMI faecal DNA samples by study centre

In addition to analysis by study period, DNA samples from the PROHEMI study were also analysed by study centre. PCoA analysis of Jaccard similarity coefficient indices and Yue and Clayton θ similarity coefficients of 1370 and 10197 sequence sub-samples was carried out (**Fig. 5.9 A-B**). Results showed samples clustering based upon study centre, with Sheffield and Cardiff samples clustering relatively separately. However, there is a degree of overlap between the two study centres with samples from Cardiff clustering with Sheffield samples and vice versa.

Analysis through nMDS of unweighted and unweighted UniFrac distances based on study period showed similar results. Results of weighted and unweighted UniFrac distances of the 1370 and 10197 sequence sub-samples showed separate clustering of the samples based upon study centre (**Fig. 5.10 A-B**). It must be noted that both sub-samples show a degree of overlap between the two study centres, with samples from Cardiff clustering with Sheffield samples and vice versa.

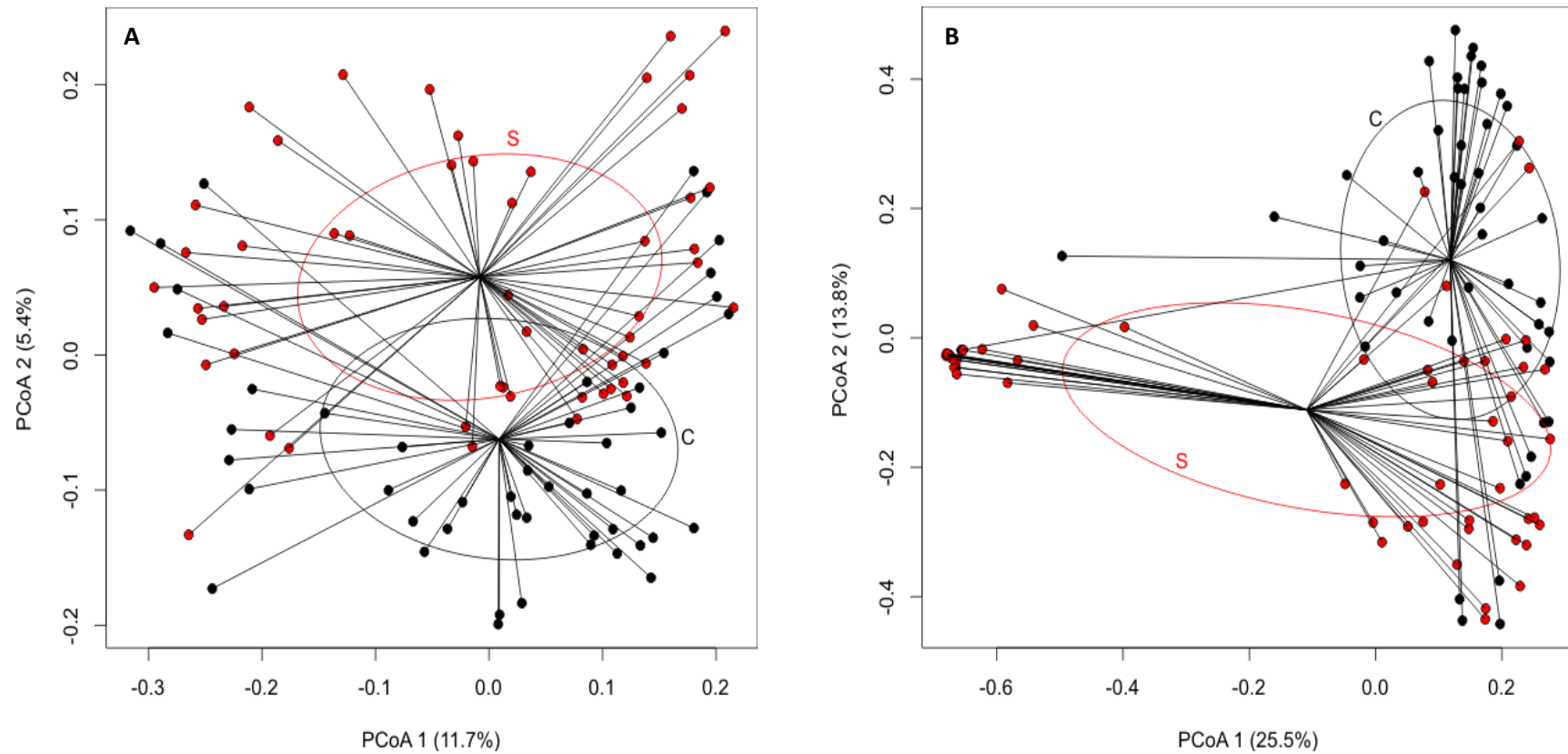


Figure 5.9] The β -diversity of PROHEMI study faecal DNA samples by study centre following normalisation through sub-sampling to 10197 sequences – In both plots **C** = Cardiff and **S** = Sheffield study centres. The percentage of variation explained is shown for each axis. **(A)** shows PCoA of Jaccard similarity coefficient indices of PROHEMI samples. **(B)** shows PCoA of Yue and Clayton θ similarity coefficients of PROHEMI samples.

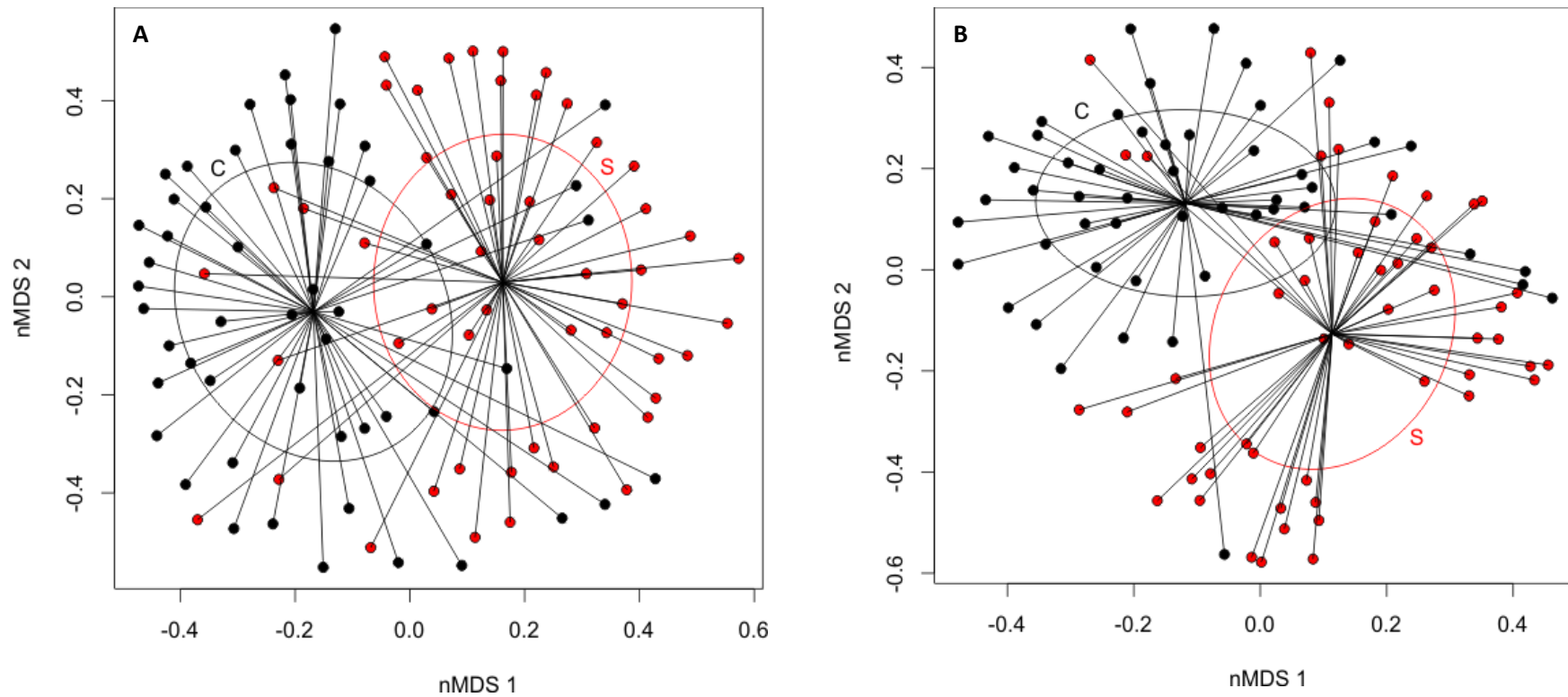


Figure 5.10| UniFrac distances of PROHEMI study faecal DNA samples by study centre following normalisation through sub-sampling to 10197 sequences – In both plots **C** = Cardiff and **S**= Sheffield study centres. The percentage of variation explained is shown for each axis. **(A)** shows nMDS of unweighted UniFrac distances of PROHEMI samples. **(B)** shows nMDS of weighted UniFrac distances of PROHEMI samples.

5.3.1.2.5 Phylum level analysis of all PROHEMI faecal DNA samples by study period

Although there was no observed separate clustering of samples based upon study period, the samples were analysed at the phylum level. Extended error bar plots of phyla from each study period, after sub-sampling to 1370 and 10197 sequences, were compared (**Fig. 5.11 A-C** and **Fig. 5.12 A-C**). Comparisons of the 1370 sequence sub-sample showed a significant difference. The *Proteobacteria* were significantly ($p < 0.05$) higher in the Active group than the Pre-feeding group (**Fig. 5.11 A**). No significant differences in the mean proportions of all other phyla were observed between study periods in both sets of sub-samples.

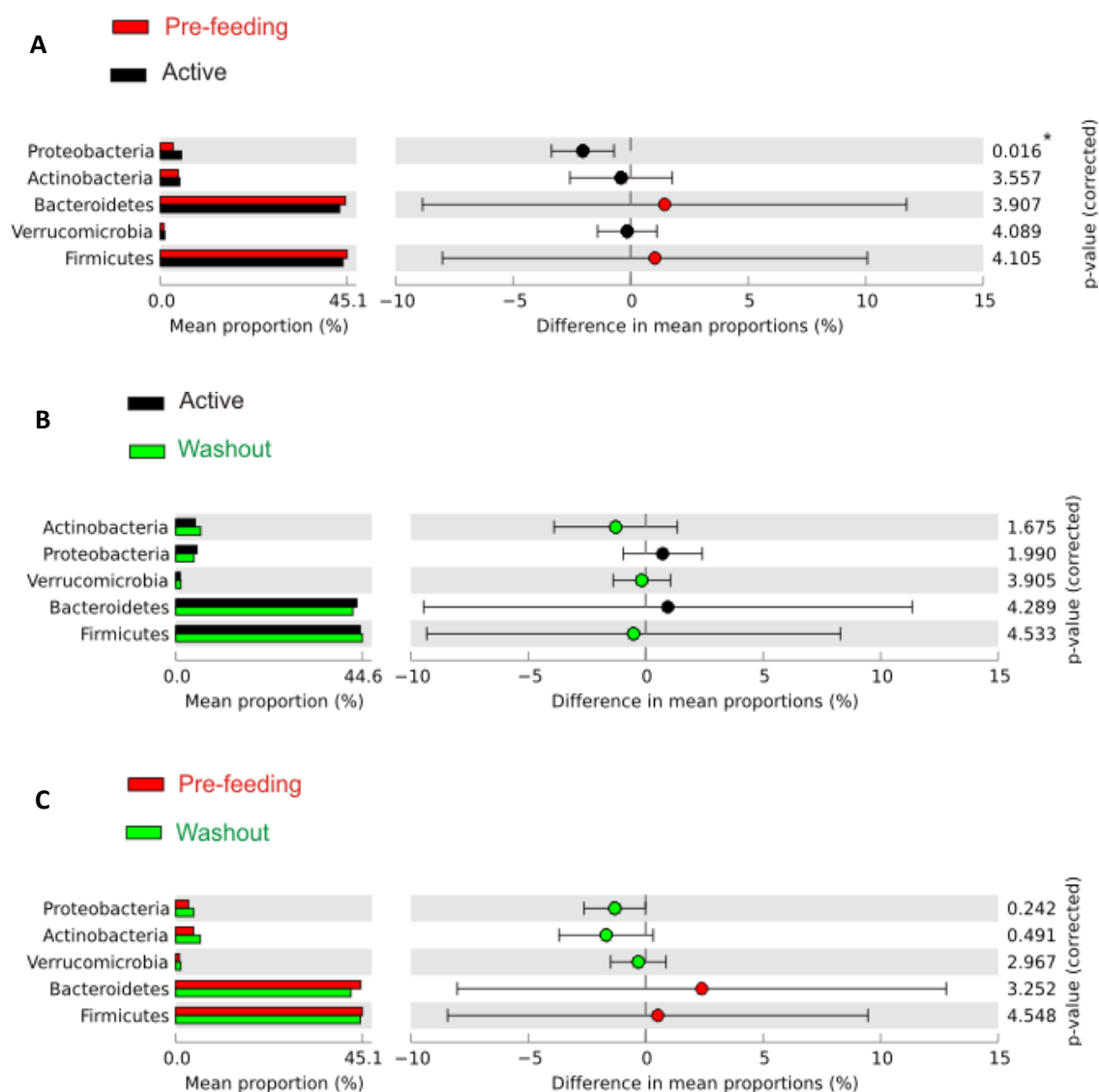


Figure 5.11| Extended error bar plot of phylum level abundance in PROHEMI samples based upon study period from the 1370 sequence sub-sample – for each plot the mean proportional abundances are shown for the phyla and the differences between the mean proportions of phyla with 95% confidence interval error bars are shown. Respective p-values for a given phylum have been displayed with * denoting a significant ($p < 0.05$) difference through Welch's t-test with Bonferroni correction for multiple comparisons. **(A)** shows the mean proportional abundances of phyla in both Pre-feeding and Active groups as well as the differences in the mean proportions of phyla between Pre-feeding and Active groups. **(B)** shows the mean proportional abundances of phyla in both Active and Washout groups as well as the differences in the mean proportions of phyla between Active and Washout groups. **(C)** shows the mean proportional abundances of phyla in both Pre-feeding and Washout groups as well as the differences in the mean proportions of phyla between Pre-feeding and Washout groups.

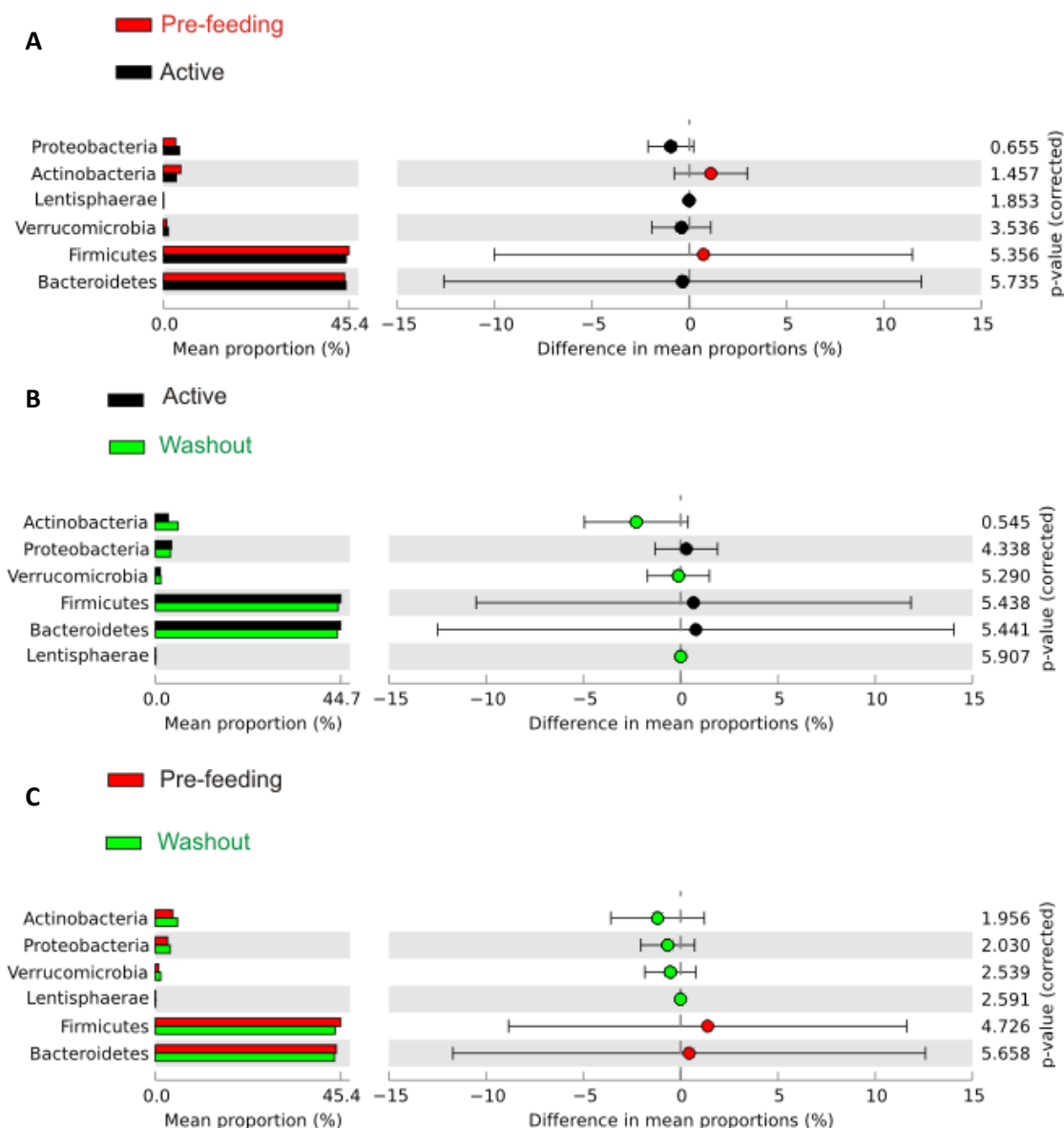


Figure 5.12| Extended error bar plot of phylum level abundance in PROHEMI samples based upon study period from the 10197 sequence sub-sample – for each plot the mean proportional abundances are shown for the phyla and the differences between the mean proportions of phyla with 95% confidence interval error bars are shown. Respective p-values for a given phylum have been displayed with * denoting a significant ($p < 0.05$) difference through Welch's t-test with Bonferroni correction for multiple comparisons. **(A)** shows the mean proportional abundances of phyla in both Pre-feeding and Active groups as well as the differences in the mean proportions of phyla between Pre-feeding and Active groups. **(B)** shows the mean proportional abundances of phyla in both Active and Washout groups as well as the differences in the mean proportions of phyla between Active and Washout groups. **(C)** shows the mean proportional abundances of phyla in both Pre-feeding and Washout groups as well as the differences in the mean proportions of phyla between Pre-feeding and Washout groups

5.3.1.2.6 Phylum and family level analysis of all PROHEMI faecal DNA samples by study centre

Due to the observed separate clustering of samples based upon study centre, as previously discussed in section 5.3.1.2.4, samples were analysed at the phylum and family level. Analysis of the 1370 sub-sample showed that Cardiff samples have a significantly ($p < 0.05$) higher mean proportion of *Firmicutes* and *Actinobacteria* than Sheffield samples (Fig. 5.13). However, Sheffield samples have a significantly ($p < 0.05$) higher mean proportion of *Bacteroidetes* and *Proteobacteria* than Cardiff samples (Fig. 5.13). Family level analysis of the 1370 sub-sample shows that in terms of the significant difference observed in the mean proportions of *Bacteroidetes* from the two study centres there are differences. The mean proportion of *Prevotellaceae* and *Porphyromonadaceae* are significantly higher in the Sheffield samples than Cardiff samples (Fig. 5.14). However, no significant difference was observed in the mean proportions of *Bacteroidaceae* and *Rikenellaceae* between the two study centres (Fig. 5.14). In terms of the difference in the mean proportions of *Firmicutes* differences were also observed at the family level. There was a significantly higher proportion of *Ruminococcaceae*, *Lachnospiraceae*, *Peptostreptococcaceae* and *Streptococcaceae* in Sheffield samples than Cardiff samples (Fig. 5.14). However, no significant difference in the mean proportions of *Clostridiaceae*, *Veillonellaceae* nor *Acidaminococcaceae* was observed between the two study centres (Fig. 5.14).

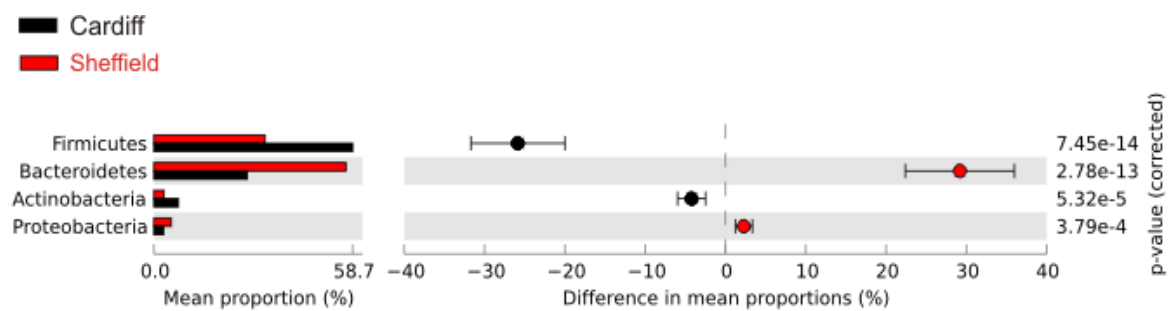


Figure 5.13| Extended error bar plot of phylum level abundance in PROHEMI samples based upon study centre from the 1370 sequence sub-sample – the mean proportional abundances of phyla are shown for Cardiff/Port Talbot and Sheffield samples. The differences in the mean proportions of phyla between Cardiff/Port Talbot and Cardiff with 95% confidence interval error bars are also shown. Respective p-values for a given phylum have been displayed, calculated using Welch's t-test with Bonferroni correction for multiple comparisons. All differences in mean proportions were highly significant ($p < 0.001$).

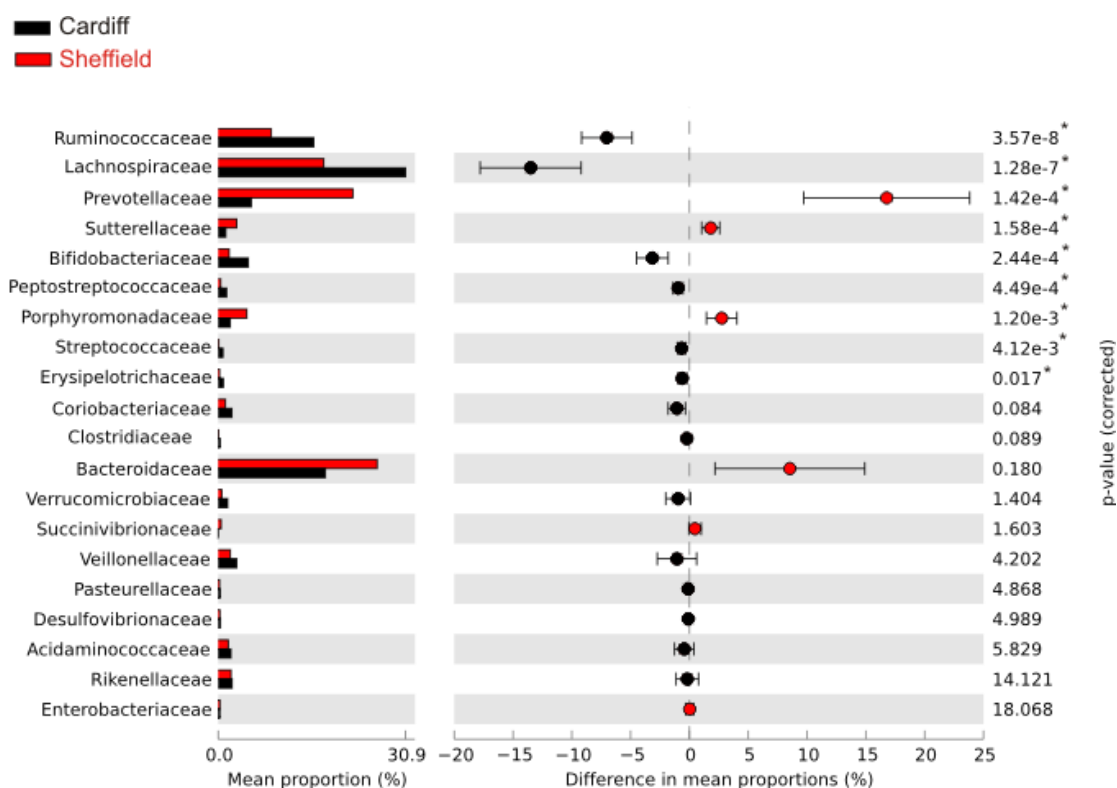


Figure 5.14 | Extended error bar plot of family level abundance in PROHEMI samples based upon study centre from the 1370 sequence sub-sample – the mean proportional abundances of families are shown for Cardiff/Port Talbot and Sheffield samples. The differences in the mean proportions of families between Cardiff/Port Talbot and Cardiff with 95% confidence interval error bars are also shown. Respective p-values for a given family have been displayed, calculated using Welch's t-test with Bonferroni correction for multiple comparisons where * denotes a significant difference ($p < 0.05$).

Analysis of the 10197 sub-sample at the phylum level shows the same trend as the 1370 sub-sample. Cardiff samples show a significantly ($p < 0.05$) higher mean proportion of *Firmicutes* than the Sheffield samples (**Fig. 5.15**). The mean proportion of *Bacteroidetes* is significantly higher in the Sheffield samples than Cardiff samples (**Fig. 5.15**). Analysis at the family level showed that there were differences in the mean proportions of members of the *Firmicutes* and *Bacteroidetes* at the family level. The mean proportion of *Ruminococcaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, *Streptococcaceae*, *Erysipelotrichaceae* and *Clostridiaceae* families were significantly ($p < 0.05$) higher in the Cardiff group than the Sheffield group (**Fig. 5.16**). This finding is slightly different as the mean proportion of *Clostridiaceae* was not significantly higher in the Cardiff group than the Sheffield group in the 1370 sub-sample. However, there was no significant difference between the mean proportions of *Veillonellaceae*, *Acidaminococcaceae*, *Peptococcaceae*, *Lactobacillaceae* nor *Leuconostocaceae* of the two study centres (**Fig. 5.16**). In a similar fashion to the *Firmicutes*, family level differences were observed in the *Bacteroidetes*. The mean proportions of the *Prevotellaceae*, *Porphyromonadaceae* and *Bacteroidaceae* were significantly ($p < 0.05$) higher in the Sheffield samples than Cardiff samples. Unlike the 1370 sub-sample, the 10197 sub-sample showed a significantly ($p < 0.05$) higher mean proportion of *Bacteroidaceae* in Sheffield samples. The mean proportions of both *Rikenellaceae* and *Flavobacteriaceae* showed no significant difference between the two study centres (**Fig. 5.16**).

While there are observable differences in the phyla and families of bacteria within samples from Cardiff and Sheffield, differences were also observed between the two sub-samples. Differences in the mean proportions of families, such as the *Clostridiaceae* and *Bacteroidaceae*, which did not reach significance in the 1370 sub-sample, reach significance in the 10197 sub-sample. Furthermore, the mean proportion of *Proteobacteria* between the two study centres does not exhibit a significant difference in the 10197 sub-sample while the phylum does in the 1370 sub-sample. Through sub-sampling to 10197 sequences, there is an increase in observable families over sub-sampling to 1370 sequences e.g. the *Lactobacillaceae* and *Leuconostocaceae* families.

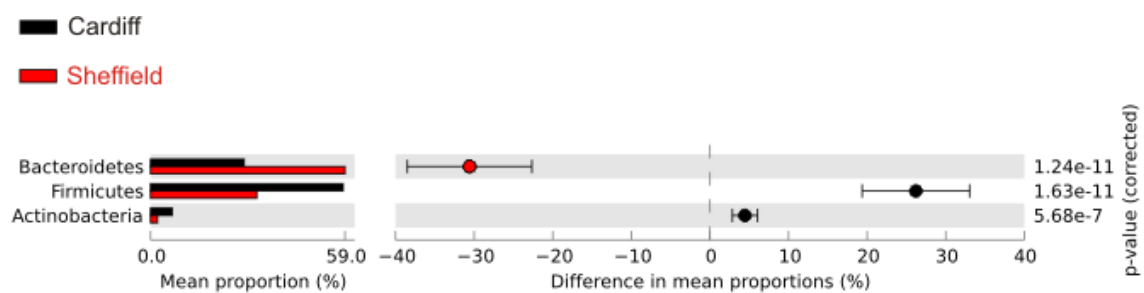


Figure 5.15| Extended error bar plot of phylum level abundance in PROHEMI samples based upon study centre from the 10197 sequence sub-sample – the mean proportional abundances of phyla are shown for Cardiff/Port Talbot and Sheffield samples. The differences in the mean proportions of phyla between Cardiff/Port Talbot and Cardiff with 95% confidence interval error bars are also shown. Respective p-values for a given phylum have been displayed, calculated using Welch's t-test with Bonferroni correction for multiple comparisons. All differences in mean proportions were highly significant ($p < 0.001$).

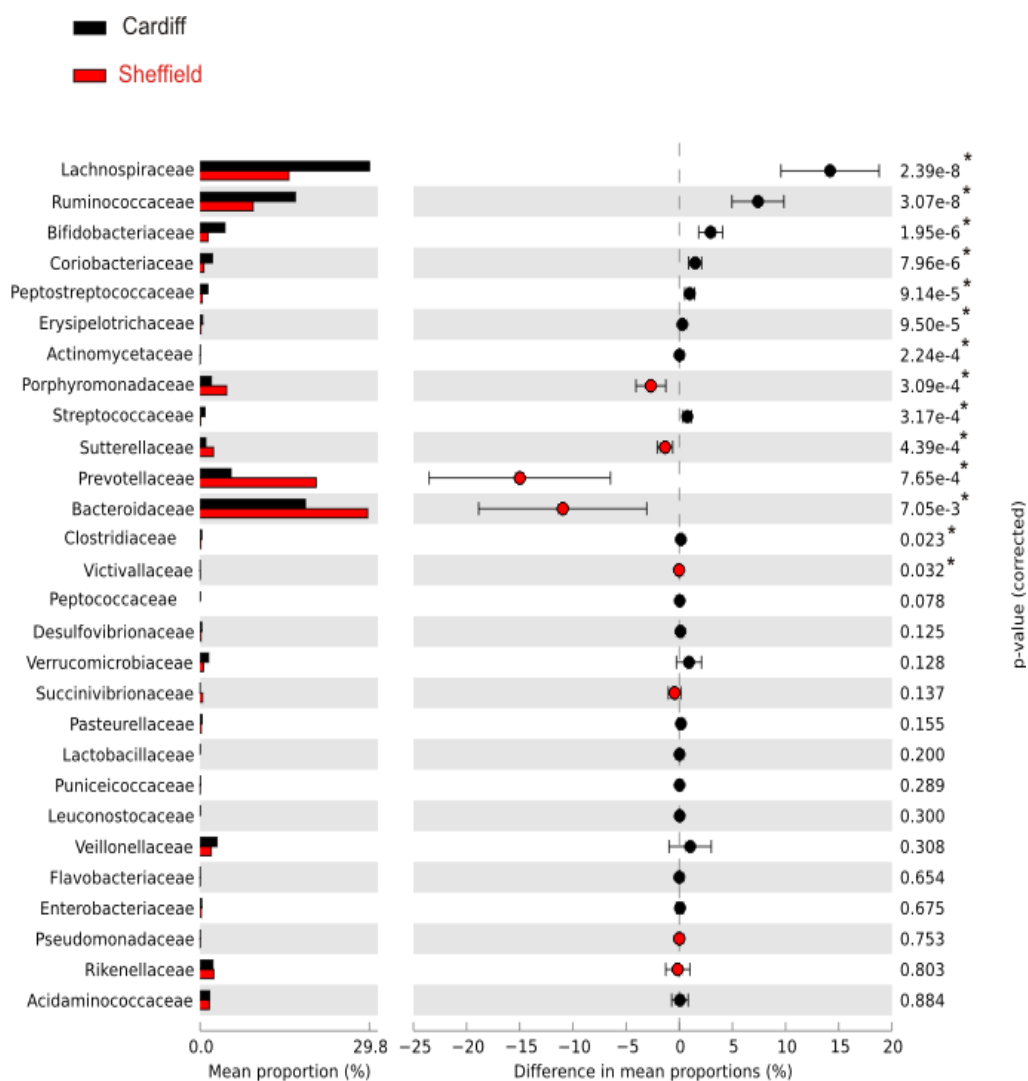


Figure 5.16 | Extended error bar plot of family level abundance in PROHEMI samples based upon study centre from the 10197 sequence sub-sample – the mean proportional abundances of families are shown for Cardiff/Port Talbot and Sheffield samples. The differences in the mean proportions of families between Cardiff/Port Talbot and Cardiff with 95% confidence interval error bars are also shown. Respective p-values for a given family have been displayed, calculated using Welch's t-test with Bonferroni correction for multiple comparisons where * denotes a significant difference ($p < 0.05$).

5.3.2 Metabonomic analysis of PROHEMI faecal waters

5.3.2.1 Analysis of gross metabonomic profiles

Cluster analysis of all gross metabonomic profiles, obtained from the faecal waters of PROHEMI participants, appears to show no effect of probiotic administration (**Fig. 5.17**). Samples from all 3 study periods appear to cluster together randomly. Furthermore, analysis of the gross metabonomic profiles through PCoA showed no separate clustering between the 3 study periods (**Fig. 5.18**)

In order to ensure that a response to probiotic administration was not being masked by differential study centre responses, gross metabonomic profiles were also analysed separately. Analysis, through PCoA, of Cardiff/Port Talbot faecal water metabonomic profiles showed no separate clustering due to study period (**Fig. 5.19**). The figure shows that samples from the Pre-feeding, Active group 1, Active group 2 and Washout periods cluster together. PCoA of Sheffield faecal water metabonomic profiles showed a similar result, with Pre-feeding, Active and Washout periods clustering together (**Fig. 5.20**). Therefore, the results suggest that probiotic administration has no effect on the gross faecal metabonomic profiles of healthy individuals.

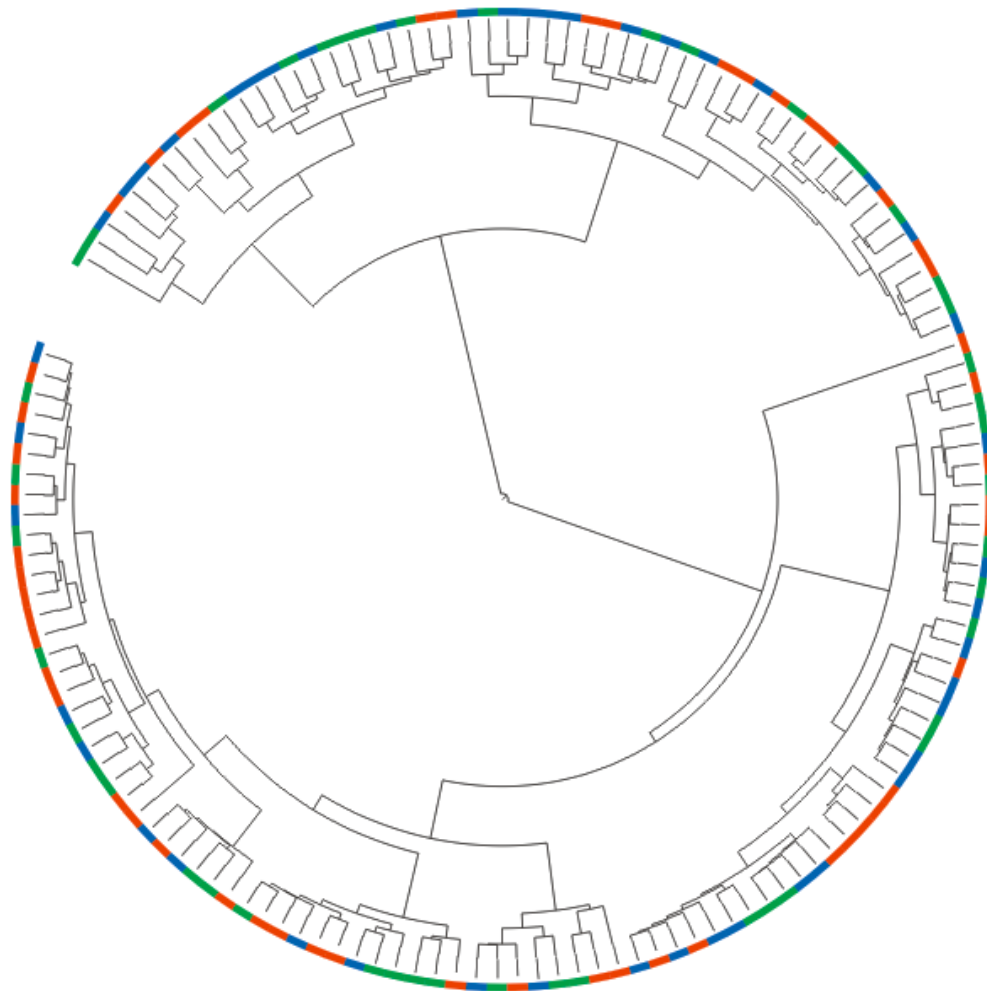


Figure 5.17| Cluster analysis of PROHEMI faecal metabonomic profiles based on study period – the study period is represented by a coloured ring on the outside of the dendrogram. Samples from the Pre-feeding period are **red**, Active period are **green** and Washout period are **blue**.

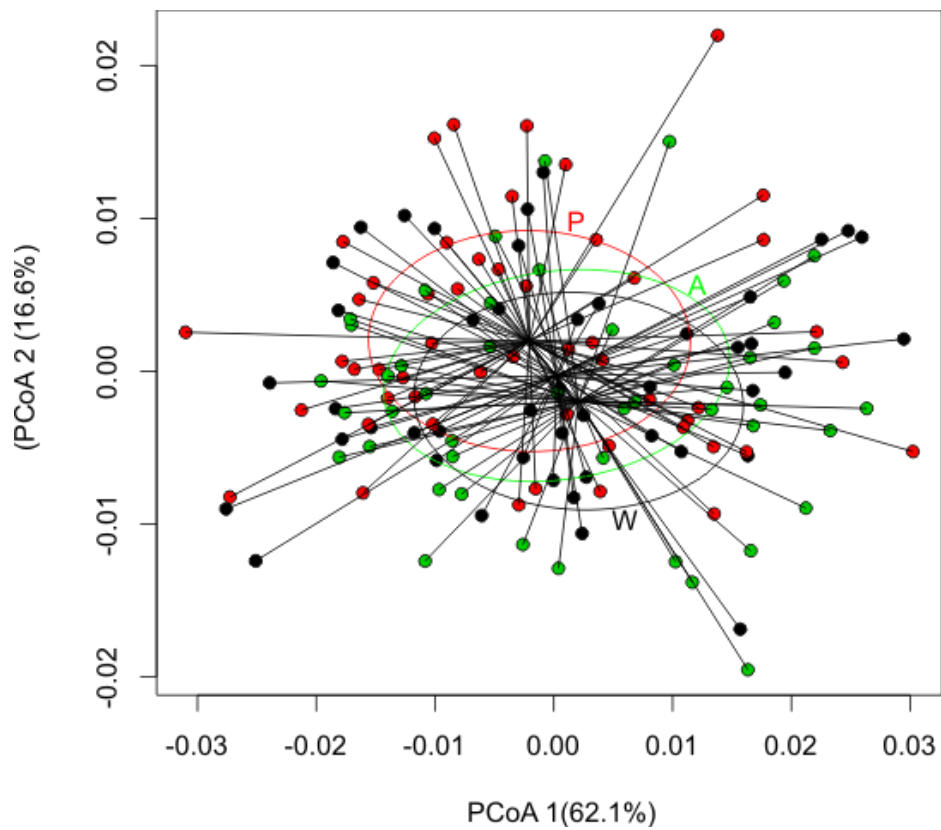


Figure 5.18 | PCoA through Euclidean distance of all PROHEMI faecal metabonomic profiles based on study period – the first two principal co-ordinates have been plotted with the percentage of variation explained shown. Pre-feeding samples are shown in **red**, Active samples are shown in **green** and Washout samples are shown in **black**.

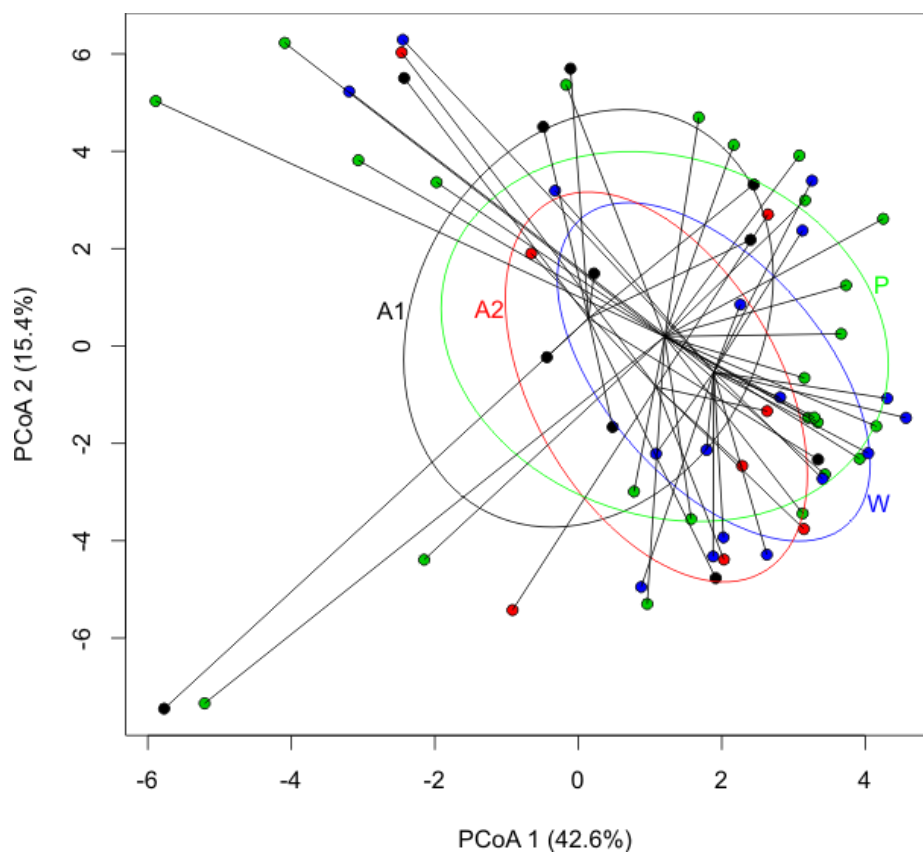


Figure 5.19| PCoA through Euclidean distance of Cardiff PROHEMI faecal metabonomic profiles based on study period – the first two principal co-ordinates have been plotted with the percentage of variation explained shown. Pre-feeding samples are shown in **green**, Active group 1 samples are shown in **black**, Active group 2 samples in **red** and Washout samples are shown in **blue**.

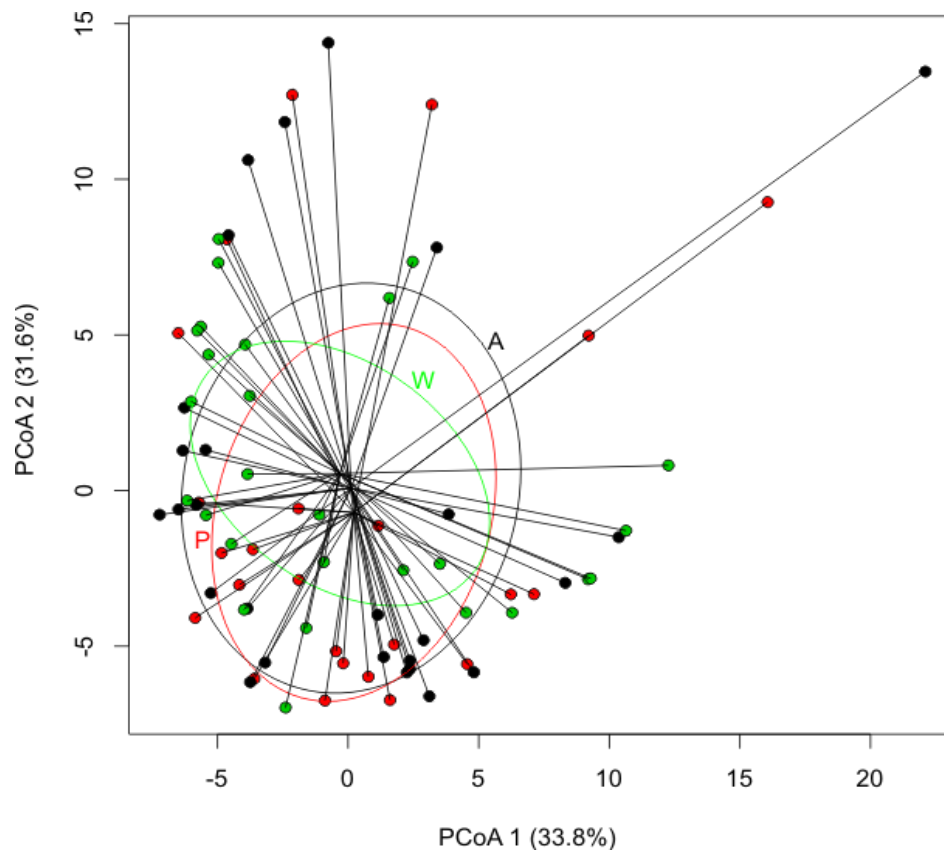


Figure 5.20|PCoA through Euclidean distance of Sheffield faecal metabonomic profiles based on study period – the first two principal co-ordinates have been plotted with the percentage of variation explained shown. Pre-feeding samples are shown in red, Active samples are shown in black and Washout samples are shown in green.

In contrast to cluster analysis of PROHEMI faecal metabonomic profiles by study period, analysis by study centre shows a degree of clustering by study centre (**Fig. 5.21**). The dendrogram shows large clusters of samples from a given study centre interspersed with samples from the other study centre.

Gross faecal metabonomic profiles were also subjected to PCoA analysis by study centre (**Fig. 5.22**). Through plotting the first two components it is possible to see that the metabonomic profiles of the two study centres cluster separately. It is likely that the observed interspersed study centre samples observed in **Fig. 5.21** are those samples which appear close together in **Fig. 5.22**. These results suggest a geographical difference in the gross metabonomic profiles of PROHEMI participants as Sheffield and Cardiff/Port Talbot samples appear to cluster separately.

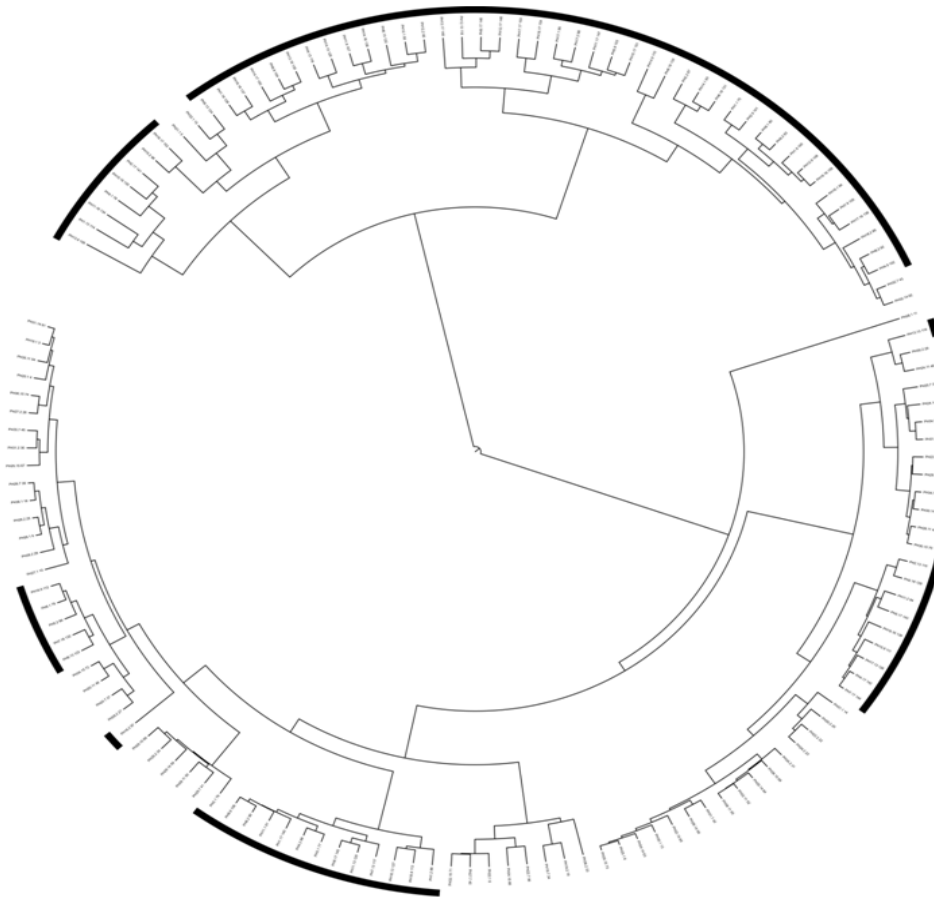


Figure 5.21 | Cluster analysis of PROHEMI faecal metabonomic profiles based on study centre – the study centre is represented by a ring on the outside of the dendrogram with Sheffield in **black** and Cardiff samples with no colouring.

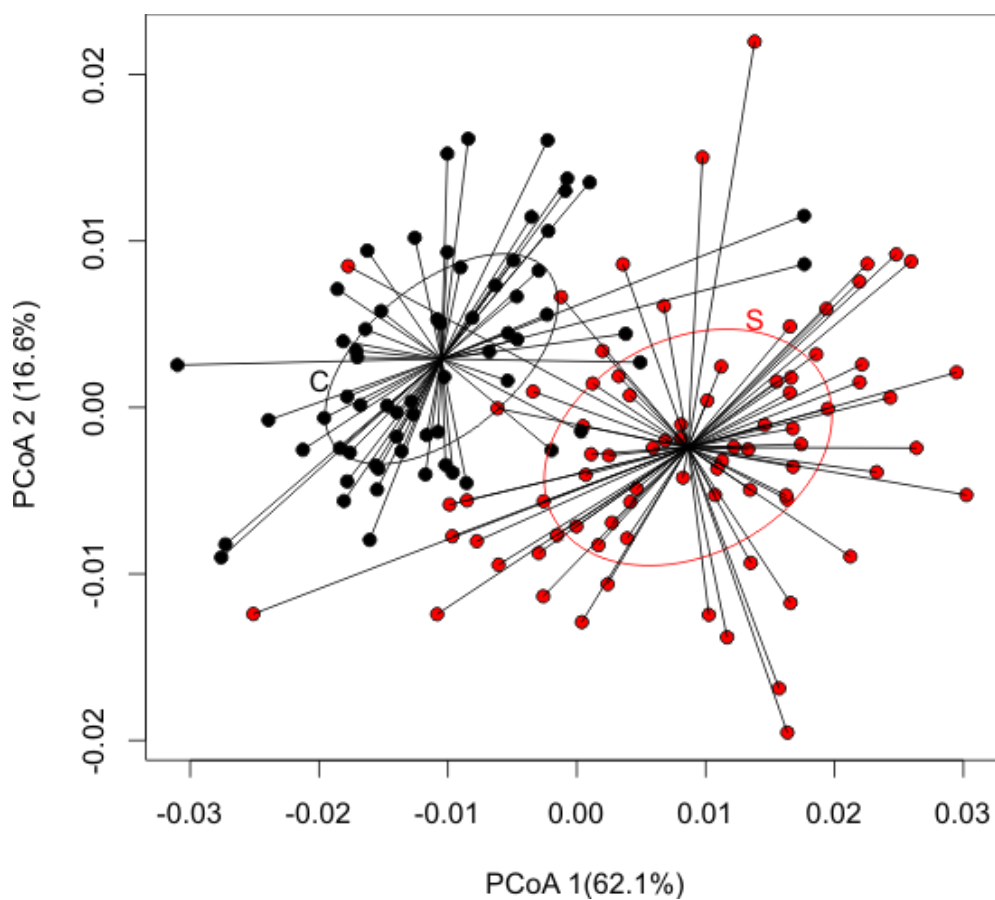


Figure 5.22 | PCoA through Euclidean distance of all PROHEMI faecal metabonomic profiles based on study centre – the first two principal co-ordinates have been plotted with the percentage of variation explained shown. Sheffield samples are shown in red and Cardiff samples are shown in black.

5.3.2.2 Analysis of SCFA metabonomic profiles

Exported chemical shifts of acetate, propionate, butyrate and valerate were analysed by study period through cluster analysis. Samples appeared to cluster together irrespective of study period (**Fig. 5.23**). The data were also analysed through PCoA based upon study period (**Fig. 5.24**). However, samples from all 3 study periods clustered together once again.

SCFA metabonomic profiles were also analysed by study period for each study centre. Analysis through PCoA showed no separate clustering due to probiotic administration in the Cardiff/Port Talbot samples (**Fig. 5.25**). In accordance with this observed result, samples from Sheffield clustered together irrespective of study period also (**Fig. 5.26**). These results suggest that probiotic administration has no effect upon the SCFA profiles of healthy male subjects.

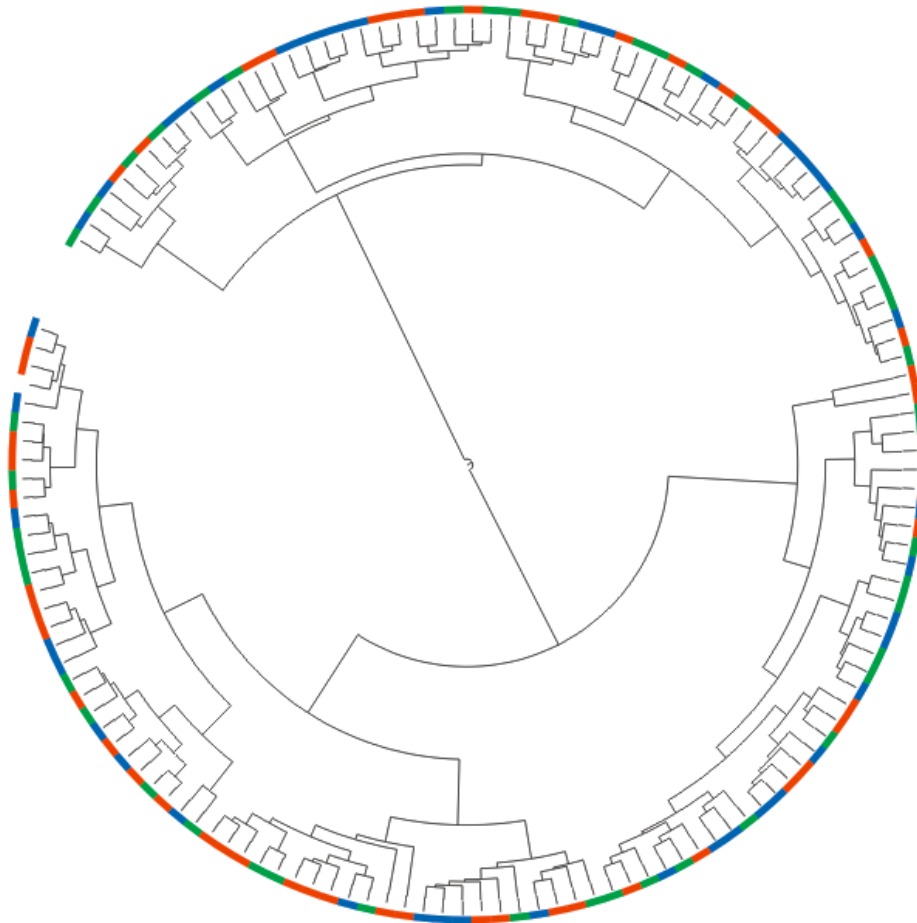


Figure 5.23| Cluster analysis of PROHEMI faecal SCFA metabonomic profiles based on study period – the study period is represented by a coloured ring on the outside of the dendrogram. Samples from the Pre-feeding period are **red**, Active period are **green** and Washout period are **blue**.

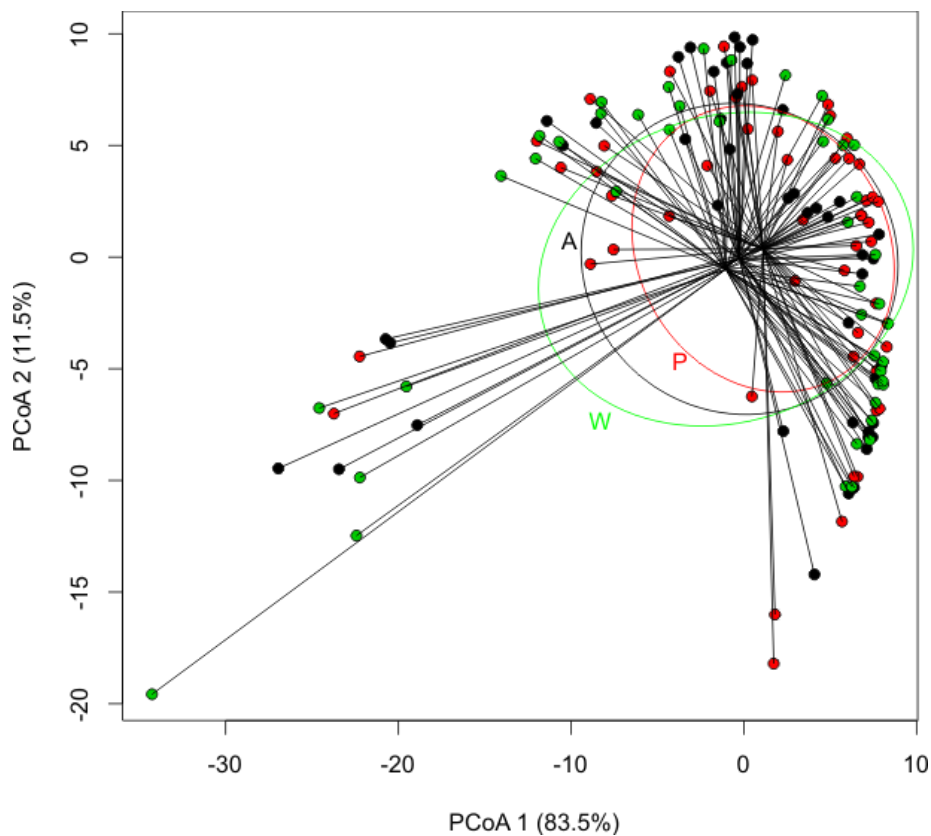


Figure 5.24 | PCoA through Euclidean distance of all PROHEMI faecal SCFA metabonomic profiles based on study period – the first two principal co-ordinates have been plotted with the percentage of variation explained shown. Pre-feeding samples are shown in **red**, Active samples are shown in **black** and Washout samples are shown in **green**.

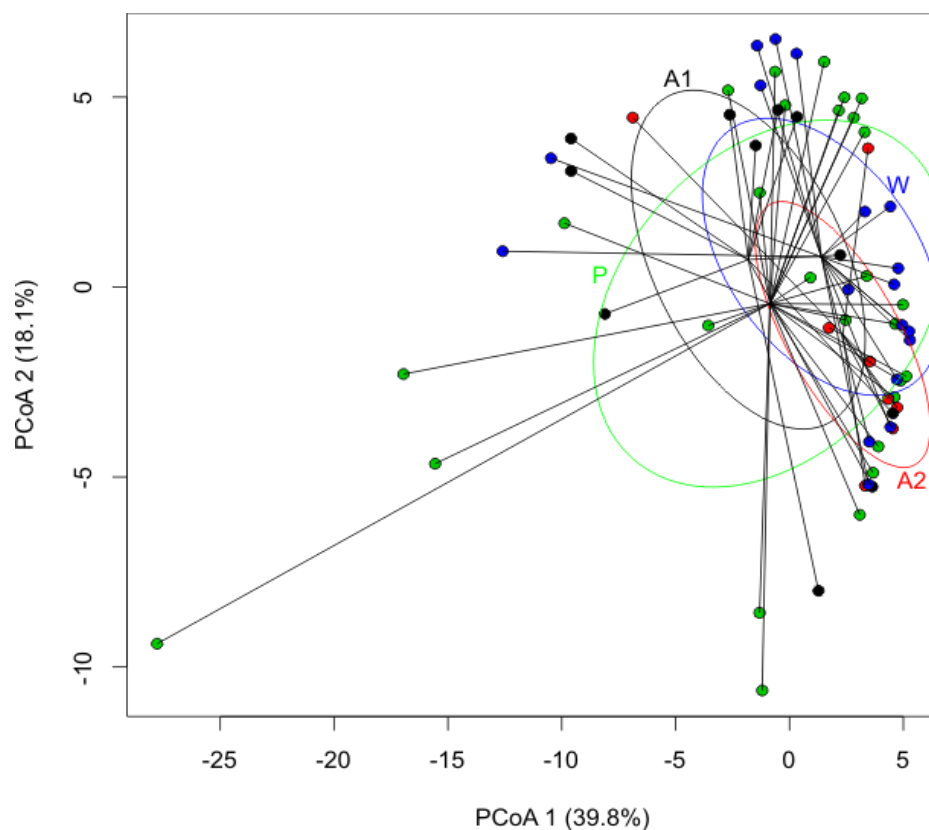


Figure 5.25| PCoA through Euclidean distance of Cardiff PROHEMI faecal SCFA metabonomic profiles based on study period – the first two principal co-ordinates have been plotted with the percentage of variation explained shown. Pre-feeding samples are shown in **green**, Active group 1 samples are shown in **black**, Active group 2 samples in **red** and Washout samples are shown in **blue**.

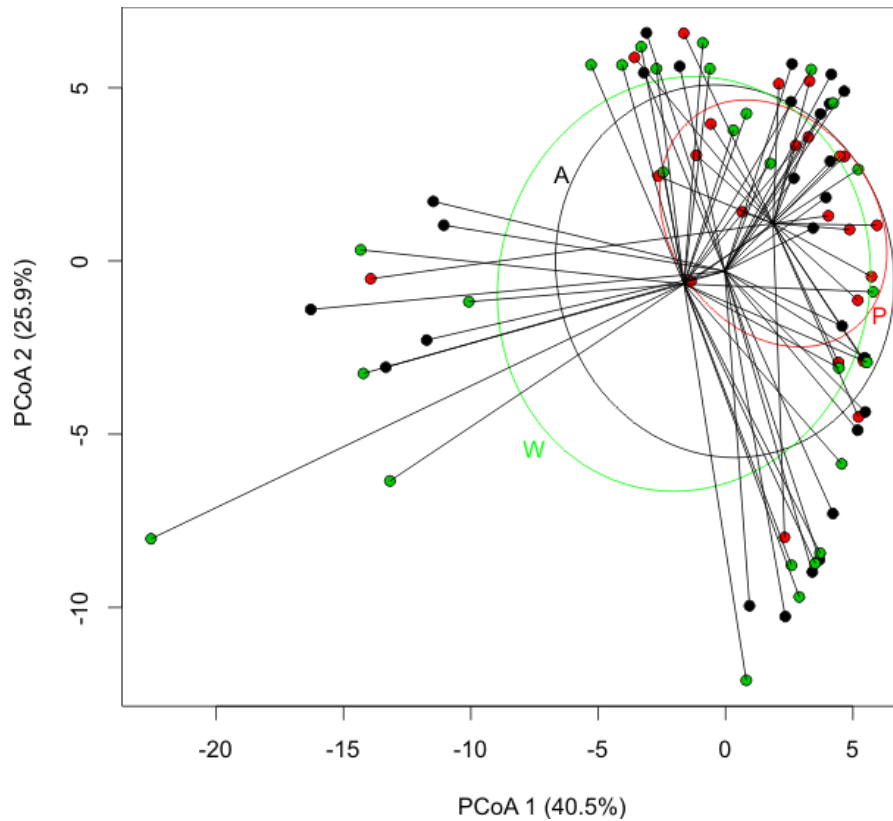


Figure 5.26| PCoA through Euclidean distance of Sheffield faecal SCFA metabonomic profiles based on study period – the first two principal co-ordinates have been plotted with the percentage of variation explained shown. Pre-feeding samples are shown in red, Active samples are shown in black and Washout samples are shown in green.

The SCFA metabonomic profiles of PROHEMI samples were also analysed by study centre through cluster analysis and PCoA. Cluster analysis of the samples showed a degree of clustering based upon study centre (**Fig. 5.27**). However, unlike the cluster analysis of gross faecal metabonomic profiles (**Fig. 5.21**), there appeared to be a greater degree of sample interspersing.

PCoA analysis of SCFA metabonomic profiles based upon study centre appeared to show separate clustering (**Fig. 5.28**). However, unlike PCoA analysis of the gross faecal metabonomic profiles of PROHEMI participants by study centre (**Fig. 5.22**), there was a larger degree of overlap between the two clusters. This overlap is the probably reason for the observed greater degree of Cardiff/Port Talbot and Sheffield sample interspersing observed in **Fig.5.27**. The results suggest that there is a geographical difference in the SCFA metabonomic profiles of participants, with Cardiff/Port Talbot and Sheffield samples showing a degree of separate clustering. However, it must be noted that the separate clustering observed in analysis of SCFA metabonomic profiles of PROHEMI participants is not as distinct as the separation observed in their respective gross metabonomic profiles.



Figure 5.27 | Cluster analysis of PROHEMI faecal SCFA metabonomic profiles based on study centre – the study period is represented by a ring on the outside of the dendrogram. Samples from the Cardiff study centre are blank while samples from the Sheffield study centre are **black**.

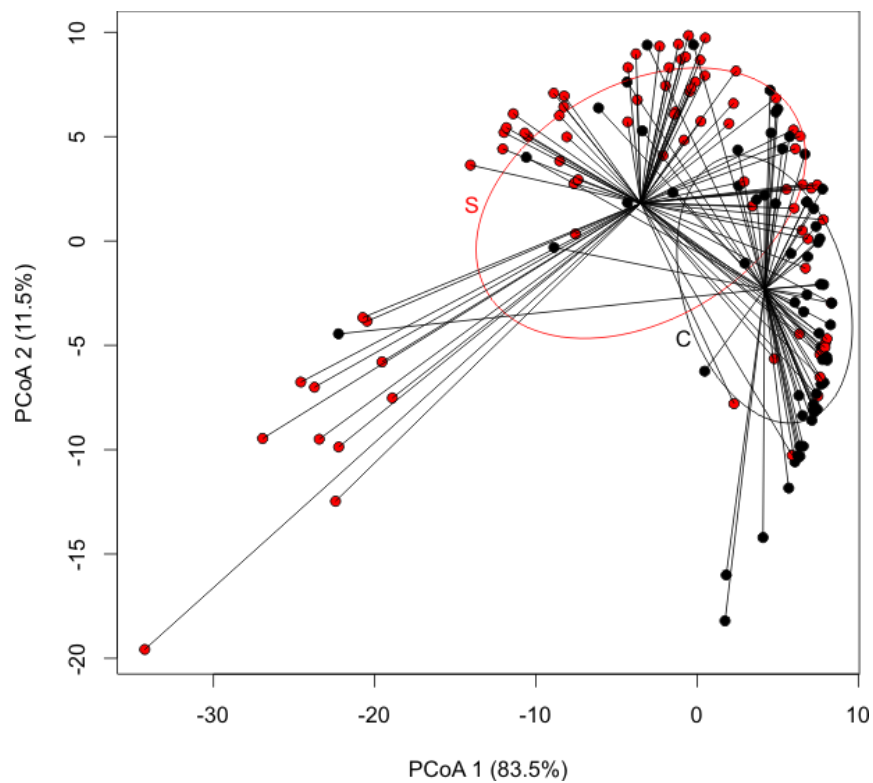


Figure 5.28 | PCoA through Euclidean distance of all PROHEMI faecal SCFA metabonomic profiles based on study centre – the first two principal co-ordinates have been plotted with the percentage of variation explained shown. Sheffield samples are shown in **red** and Cardiff samples are shown in **black**.

5.4 Discussion

5.4.1 Illumina MiSeq NGS of PROHMI faecal DNA

There appears to be a need for research into the effect of probiotic administration on the gut bacterial community and its effect on the metabonomic profiles of individuals needs to be carried out. I have shown that long-term probiotic administration does very little with respect to the alteration of the gut bacterial community of healthy individuals. In addition, gross metabonomic profiles and profiles associated with SCFA are also unaffected.

Analysis through Mothur showed there was a high degree of variability in terms of sequence counts for a given sample. This is due to methodological issues prior to and during the sequencing run. Normalisation, through sub-sampling to 1370 and 10197 sequences, was carried out in order to avoid sequencing bias (Schloss *et al.* 2011). Sub-sampling to the lowest possible number of sequences would have severely lowered the number of sequences in all samples to 33 sequences. The dataset would have been severely reduced with a loss of 278996 sequences from the highest sample. Sub-sampling to 1370 sequences and 10197 sequences removed 3 and 42 samples respectively from subsequent analysis. While this was not ideal, the samples were from all study periods and centres. Therefore, it is unlikely that bias was introduced from sub-sampling.

Inverse Simpson diversity indices show that there was no significant difference in the diversity of samples following sub-sampling and no significant difference between study periods. However, there was a significant difference between the two study centres, with Sheffield samples showing a decreased level of diversity in non-normalised and both normalised datasets. It has recently been shown that the α -diversity of the faecal microbiota of infants differs as they age with the highest diversity seen at 2 years and the lowest diversity seen at 4 months (Avershina *et al.* 2013). Differences in α -diversity have not only been observed across different ages. In a recent study, the distal gut microbiome diversity of healthy Bangladeshi children was compared to healthy children from the US. Bangladeshi children were shown to have a significantly ($p < 0.001$) higher α -diversity than US children (Lin *et al.* 2013). My results highlight a geographical difference in the α -diversity of the distal gut microbiota of healthy male subjects within the UK and should be researched further. There appears to be a discrepancy in knowledge regarding inter-centre differences in gut

bacterial diversity. Published studies seem to focus on a single study centre alone and when comparisons are carried out they are often comparisons of disparate locations. To my knowledge there are no comparisons between locations in the UK in terms of differences in the diversity of the gut bacterial community of individuals. Therefore, I cannot say whether this is in line with other research in this area or not.

β -diversity measurements were carried out using Mothur (Schloss *et al.* 2009). This open-source software utilises Jaccard similarity coefficient indices in order to determine the dissimilarity between communities in question (Schloss *et al.* 2009). The technique was developed and applied by Jaccard in order to describe the diversity of plants in the Alps (Jaccard 1912). Effectively, the technique takes the presence/absence of an OTU into account and not its abundance. Conversely however, the Yue and Clayton θ similarity coefficient analysis takes the abundance of a given OTU into account. Analysis through these two measurements showed no clustering due to probiotic administration. The results of PCoA on these two measures showed that all samples clustered together, regardless of study period, for each sub-sample. Furthermore, when the two study centres were analysed separately all study periods still clustered together.

UniFrac distance analysis, a comparatively new technique, has been applied in order to analyse complex microbial communities (Lozupone and Knight 2005). In a similar manner to Jaccard similarity coefficient indices analysis, the unweighted method of UniFrac does not take in to account the abundance of a given OTU. This form of the metric measures the presence/absence of a given OTU instead. It is therefore ideal in order to compare differing microbial communities as the OTU composition is likely to be different. However, the weighted method takes the abundance of a given OTU into account, in a similar fashion to the Yue and Clayton θ similarity coefficient analysis. Analysis through both unweighted and weighted UniFrac distance analysis emulated the results obtained from Jaccard similarity coefficient indices and Yue and Clayton θ similarity coefficient analysis. Ordination through nMDS of weighted and unweighted UniFrac distances showed no effect of probiotic administration as all samples clustered together, irrespective of study period. In addition, samples clustered together regardless of study period once both study centres were analysed separately. All four β -diversity measurements used suggested that probiotic administration did not affect the presence/absence or abundance of OTUs.

The data were analysed by study centre also and while probiotic administration showed no effect on β -diversity, there were observable differences between the two study centres. PCoA of Jaccard similarity coefficient indices and Yue and Clayton θ similarity coefficients showed separate clustering of samples from the two study centres. There was an observable overlap between samples from the two study centres however, in general separate clustering was observed. Ordination of weighted and unweighted UniFrac distances through nMDS showed similar results for both sub-samples. PROHEMI samples appeared to cluster separately due to study centre with some sample overlap between the two study centres. The results suggest a geographical difference between the presence/absence and abundance of OTUs.

Sequence data were analysed at the family and phylum level also. Analysis of the 1370 sub-sample showed a significant increase in the mean proportion of *Proteobacteria* between the Active and Pre-feeding periods. However, this was not shown in the 10197 sub-sample. This result suggests that the difference seen is probably due to a bias introduced from sub-sampling as opposed to a real biological effect. There were no other observed significant differences at the phylum level between the study periods.

Phylum level analysis of the sequence data showed a significant difference between the two study centres in both sub-samples. Sheffield samples were shown to have a higher mean proportion of *Bacteroidetes* while Cardiff/Port Talbot samples had a higher mean proportion of *Firmicutes*. Significant differences were also observable at the family level. However; in contrast to the phylum level analysis both sub-samples showed differences in the families exceeding significance. It is interesting to note that sub-sampling affected the number of bacterial families which exceeded significance with 9 families and 14 families reaching significance in the 1370 sub-sample and the 10197 sub-sample respectively. The observed difference between the two sub-samples is likely to be due to a sub-sampling artefact. Families which exceeded significance in the 1370 sub-sample also exceeded significance in the 10197 sub-sample. The evaluation of different sub-sampling methods has previously been researched and was shown to impact upon the sequence dataset in question (Carcner *et al.* 2011).

Analysis at the phylum leads us to believe that Cardiff/Port Talbot samples have a higher mean proportion of *Firmicutes* than Sheffield. However, family level analysis shows that not all mean proportions of the *Firmicutes* are significantly higher. Similarly, phylum level analysis leads us to believe that Sheffield samples have a higher mean proportion of *Bacteroidetes* than Cardiff/Port Talbot samples. However, family level analysis once again shows that not all mean proportions of *Bacteroidetes* are significantly higher.

My results suggest that probiotic administration does not impact upon the bacterial community of healthy individuals. It has recently been shown that the ratio of *Bacteroidetes* to *Firmicutes* was significantly ($p < 0.05$) increased following administration of *L. salivarius* (10^{10} CFU/day) for 12 weeks in obese adolescents (Larsen *et al.* 2013). Research into the effect of the administration of *B. longum* in combination with an inulin-based prebiotic in older healthy individuals has recently been carried out (Macfarlane *et al.* 2013). In this cohort, the consumption of 2×10^{11} *B. longum* with 6 g of the prebiotic twice daily significantly increased *Bifidobacteria*, *Actinobacteria* and *Firmicutes* ($p < 0.0001$, $p < 0.0004$ and $p < 0.0001$ respectively). This recent research suggests that probiotic administration can impact the composition of the gut microbiota. However, one study cohort was not healthy, as the participants were obese (Larsen *et al.* 2013), and the age of participants from the other study group ranged from 65-90 (Macfarlane *et al.* 2013). These two study cohorts are not indicative of the general population who take probiotic supplements daily. In accordance with my results, it has recently been shown that probiotic yoghurt consumption did not impact the gut microbiota of healthy subjects. Filteau and colleagues showed that the daily consumption of *L. acidophilus* LA-5 (10^9 CFU/day) with increasing doses of *B. animalis* ssp. *lactis* BB-12 (10^9 and 10^{10} CFU/day) and 40 mg/day green tea extract for 4 weeks did not affect the distal gut microbiota of healthy individuals. Vanhoutte and colleagues have also shown that the consumption of *S. boulardii* (2.5×10^9 viable cells/day) for 4 weeks did not impact upon the DGGE profiles of healthy volunteers. Therefore, my research is in accordance with these studies; it seems probiotic administration, in healthy individuals, does not impact upon the distal gut microbiota. The reasons behind this observation are unclear. However, there is a possibility that the probiotic organisms used in the PROHEMI study do not possess the ability to modulate the gut bacterial community of individuals. It has previously been shown that the feeding of *B. longum* 46 and *B. longum* 2C (total of 10^9 CFU/mL) for 6 months gave rise to a bifidogenic response (Lahtinen *et al.* 2009). The organisms used were different to the organisms used in the PROHEMI study and the observed differences were in an elderly cohort. This gives rise to another possible reason for

the lack of distal gut bacterial modulation observed. PROHEMI participants were healthy males and were not elderly. Perhaps modulation cannot be achieved in this healthy cohort as the distal gut microbiota is established and not under dysbiosis. It is also possible that not all of the probiotic mixture given to PROHEMI participants reaches the large intestine. While previous research has shown that the *L. acidophilus* strains used in the PROHEMI can be detected in the faecal material of healthy individuals following feeding (Mahenthiralingam *et al.* 2009), the same has yet to be shown for the bifidobacteria used in the study. The bifidobacteria used in the PROHEMI study may possess bifidogenic properties however; they may not reach the large intestine in sufficient numbers to exert these effects.

Analysis by study centre suggests that there are geographical differences in the gut bacterial community of individuals across the UK. There is a significant difference between the proportional abundances of the two major distal gut phyla, the *Bacteroidetes* and *Firmicutes*, across the two study centres. Previous research by Gordon and colleagues using obese mice suggests that the abundance of *Firmicutes* are significantly ($p < 0.05$) higher in obese mice while the abundance of *Bacteroidetes* are significantly ($p < 0.05$) lower (Ley *et al.* 2005). This observation was strengthened by analysis of obese and lean human twins by Turnbaugh and colleagues (Turnbaugh *et al.* 2009). Once again there was a significant ($p = 0.003$) reduction in the abundance of *Bacteroidetes* in obese subjects. However, there was no significant difference in the abundance of *Firmicutes*. My research, using healthy individuals, suggests a difference in the ratio of *Bacteroidetes* and *Firmicutes* due to geographical difference. Geographical differences in the composition of the distal gut microbiota have previously been discussed. NGS of the V3-V4 16S *rRNA* genes from the faecal DNA of Bangladeshi and USA children showed differences in the relative abundances of OTUs corresponding to the *Firmicutes* and *Bacteroidetes* (Lin *et al.* 2013). Bangladeshi children showed a higher abundance of *Firmicutes* to *Bacteroidetes* (60% vs. 20% respectively) while children from the USA showed a more even abundance (46% vs. 43%). Interestingly, this observation was extended to Bangladeshi adults also, with a high abundance of *Firmicutes* (50%) and a very low abundance of *Bacteroidetes* (6%). The authors cannot give an exact reason for the observed difference. However, they suggest that differences in diet, genetics and environmental factors, including socioeconomic factors, may play a role.

With regards to the effect of diet on the microbiome, newly published research has shown diet can modulate the distal gut microbiome in healthy subjects (David *et al.* 2013). Healthy subjects either followed an animal-based diet or plant-based diet for 5 days. Comparisons were made to a pre-diet period and post-diet period, where participants were instructed to eat normally. α -diversity analysis of the data showed no significant difference of Shannon diversity indices attributable to either diet. However, β -diversity was significantly affected by the animal-based diet. Furthermore, it was shown that \log_2 fold changes of the *Firmicutes* were significantly affected by both diets while \log_2 fold changes of the *Bacteroidetes* were significantly affected by the animal diet. My results show a difference in the gut bacterial community of individuals from the UK. These differences are unlikely to be due to socioeconomic factors however, differences in diet and lifestyle are likely drivers for the observed differences in the ratio of *Bacteroidetes* to *Firmicutes* observed in the samples.

The differences in the abundances of *Firmicutes* in the PROHEMI study are not a trivial matter. Previous research has shown that members of the *Firmicutes* phylum, namely members of clostridial cluster XIVa, play a pivotal role in the production of butyrate (Barcenilla *et al.* 2000). Of the isolated butyrate producers 80% clustered within clostridial cluster XIVa. Barcenilla and colleagues highlighted that the most abundant group of isolates (42%) clustered with *E. rectale* and *Roseburia cecicola*. Previously published data suggests that diet can affect the abundance of clostridial cluster XIVa members. Duncan and colleagues have shown that a reduced dietary intake of carbohydrates is linked with a reduction in the abundance of *Roseburia* spp. and the *E. rectale* subgroup of clostridial cluster XIVa in obese subjects (Duncan *et al.* 2007). Furthermore, the research was strengthened as Duncan and colleagues showed a decrease in butyrate concentrations in the faecal material. Research by Walker and colleagues has also shown that a diet high in resistant starch significantly ($p < 0.001$) increases the clostridial cluster XIVa member *E. rectale* and *Ruminococcus bromii* in overweight males (Walker *et al.* 2011). It is therefore apparent that diet can impact upon members of the *Firmicutes*. With regards to my data there is a significantly ($p < 0.0001$) higher mean proportion of *Lachnospiraceae* and *Ruminococcaceae* in Cardiff/Port Talbot samples. Both of these families lie within clostridial XIVa group and suggest that diet may be the driver for the observed difference. This idea is further strengthened by the previously discussed newly published research by David and colleagues (David *et al.* 2013). Healthy participants following an animal-based diet showed a significant reduction in *E. rectale* and *Roseburia faecis*. However, an animal-based diet significantly increased members of the *Bacteroidetes* including the *Porphyromonadaceae*.

5.4.2 ¹H NMR metabonomic analysis of PROHEMI faecal waters

Metabonomic analysis of the PROHEMI dataset showed similar results to the NGS results from the study. There appeared to be no effect of probiotic supplementation on the gross metabonomic and SCFA metabonomic profiles of individuals. The data were analysed by period separately for the two study centres with individuals clustering together regardless of the study period. There have been studies which have used metabonomic profiling following probiotic administration in murine models. In an experimentally induced colitis mouse model the efficacy of *L. brevis* HY7401, *Lactobacillus* sp. HY7801 and *B. longum* HY8004 (combined concentration of 3×10^9 CFU/mL) has been assessed (Hong *et al.* 2010). Colitis, in this model, was induced by dextran sulphate sodium. Probiotic fed mice showed increased levels of SCFA than mice which did not receive probiotic treatment. Administration of the probiotic species *L. acidophilus* La5 and *B. lactis* BB12 (10^9 CFU/day) for 30 days in aged (16 months) mice has also been researched (Brasili *et al.* 2013). Differences in the faecal and urinary metabolites were observed between the two study groups. Research into the effect of feeding *L. paracasei* NCC2461 (10^8 /day) in a humanised mouse model has been carried out (Martin *et al.* 2008). Analysis of SCFAs in this model showed a significant ($p < 0.001$) decrease in acetate, a significant ($p < 0.001$) increase in isobutyrate, a significant ($p < 0.001$) increase in butyrate and a significant ($p < 0.01$) increase in isovalerate compared to the control group. Furthermore, ¹H NMR analysis also showed a significant ($p < 0.01$) decrease in acetate following probiotic consumption compared to the control group. My results suggest that there is no effect of probiotic administration on faecal metabolites. However, the dataset needs to be analysed in more detail in order to ensure that specific changes are not being masked by metabolites which do not respond. In addition, branched SCFA were not included in my analysis and need to be considered in future analyses.

While probiotic administration did not seem to affect the gross metabonomic and SCFA metabonomic profiles of individuals, study centre differences were once again observed. Samples from Cardiff/Port Talbot and Sheffield clustered separately when gross faecal metabolites were analysed. Analysis of SCFA metabolites also showed a degree of separate clustering based upon study centre. However, there was a larger degree of sample overlap from the two study centres. The effect of diet on the distal gut microbiota has previously been discussed (Duncan *et al.* 2007; Walker *et al.* 2011). Furthermore, its impact upon the production of compounds such as SCFA has also been discussed. My data therefore suggest that there is a geographical difference in the metabolite profiles of individuals enrolled in the PROHEMI study. The results showed a difference between the gut bacterial community of individuals also and from previously published research there is a hint that these two observations are intrinsically linked. It is possible that differences in lifestyle or diet are driving changes in the distal gut bacterial community of individuals and in turn drives a difference in faecal metabolite profiles.

Further research needs to be carried out in order to ensure that the results are a true biological phenomenon and not merely methodological driven. With regards to the sequencing data, the effect of faecal material storage and the DNA extraction method followed on the variability of results has been discussed in **Chapter 6**.

5.5 Conclusions

The main conclusions for this chapter are as follows:

1. probiotic administration does not affect the distal gut bacterial community of healthy males;
2. there are difference in the distal gut bacterial community of healthy males from the two study centres;
3. NGS backs up the observations made following LHPCR analysis and validates the technique for use in analysing human faecal DNA;
4. there are no effects of probiotic administration upon the gross metabonomic and SCFA profiles of healthy individuals;
5. ^1H NMR analysis reveals a difference in the gross and SCFA metabonomic profiles of PROHEMI participants from the two studies centres.

6. THE EFFECT OF FREEZING FAECAL MATERIAL ON ITS BACTERIAL COMPOSITION

6.1 Introduction

The ability of the human gut microbiota to influence host health and well-being has become more apparent in recent years. Studies are highlighting the link between the host gut microbiota and the hypothalamic-pituitary-adrenal (HPA) axis (Ait-Belgnaoui *et al.* 2012), controversially with obesity (Ley *et al.* 2006b) and autism (Song *et al.* 2004). Konopka and Staley described the “great plate count anomaly” in nonphotosynthetic microorganisms from aquatic and terrestrial environments (Staley and Konopka 1985). The anomaly is equally applicable to the human gut, as the number of cultivable bacteria is significantly less than the total numbers of bacteria residing in the gut. The inability of culture dependent approaches to acquire all of the information has recently been highlighted. The impact of diet on the gut bacterial community of 14 overweight men was determined through 16S *rRNA* analysis (Walker *et al.* 2011). Of the 320 phylotypes identified in the study, only 33.4% showed 98% identity with cultured bacteria. The authors suggest that this is due to poor culture dependent coverage of less abundant bacterial groups. It was further suggested that the culture independent to dependent discrepancy arises through poor anaerobic isolation work; and not due to the inability of these organisms to be cultured.

Faecal material provides a non-invasive alternative for the collection of data on the distal colon. Therefore it has been the source of information for many studies from animals (Simpson *et al.* 2000) to humans (Eckburg *et al.* 2005). In order to obtain a snapshot of the distal gut microbiota and functions therein, many researchers extract DNA from faeces. The extraction method that should be followed has been debated, as the DNA obtained should be a true representation of the distal gut microbial community. That is to say that the method followed should not create biases in the data generated. Efficacy of commercially available faecal DNA extraction kits (McOrist *et al.* 2002), modified DNA extraction protocols using bead beating (Salonen *et al.* 2010), and modification of commercially available kits (Maukonen *et al.* 2012) have been evaluated. There is no clear consensus from the papers published over the last decade comparing extraction methods, which is the correct protocol to follow?

The confusion regarding which DNA extraction protocol to follow is further complicated by the confusion over the correct method for faecal storage prior to the highly variable results obtained from DNA extraction. Even in 2012 uncertainty remains (**Table. 6.1**). When DNA was extracted

from the faecal samples of 4 individuals (2 healthy controls and 2 irritable bowel syndrome patients) stored at 1, 4, 6, 8 and 24 h, samples clustered based on the individual and not due to the storage method or time stored (Carroll *et al.* 2012). In another piece of work it has also been shown that microbial diversity of faecal samples (n=4) is not significantly affected by storage at room temperature, up to 24 h. DNA was degraded and a significant change in the proportion of bacteria was observed after 2 weeks storage at room temperature (Cardona *et al.* 2012).

In order to overcome the effect of DNA degradation and loss of bacterial diversity that arises from storage at room temperature, faecal samples are often frozen. However, this in itself cannot guarantee the integrity of the DNA information obtained. It has been shown that the ratio of the two major phyla of the human gut, the *Firmicutes* and *Bacteroidetes*, is altered after faecal sample storage at -20°C for 53±5 days. There was a reduction in abundance of *Bacteroidetes* 16S *rRNA* genes obtained after extraction (Bahl *et al.* 2012). Faecal samples can also be stored at -80°C. It has been shown, in a similar manner to storage at room temperature up to 24 h, that samples cluster together based upon individual and not due to storage method or time. The weighted and unweighted UniFrac values of 16S *rRNA* gene sequences obtained from samples frozen at -80°C up to 6 months show significant similarity to their corresponding fresh samples (Carroll *et al.* 2012). Another layer of complexity is added when the thawing of frozen faecal samples is considered. It has been shown that thawing faecal samples from -80°C over an h or 3 h both cause DNA degradation. There is also a loss of bacterial 16S *rRNA* sequences from the *Bacteroides* genus (Cardona *et al.* 2012).

The confusion arising from varied results and different storage methods adds to the difficulty of carrying out inter study comparisons. It is also difficult to compare results from studies as different extraction methods are often followed. In collaborative studies, researchers may decide to overcome inter-centre variation by transporting faecal samples to one centre for DNA extraction. However, the implications of the effect of thawing frozen faecal samples shows that caution should be taken when transporting faecal samples from different locations. The extracted DNA may not provide a true representation of the community. While the stability of faecal microbial communities appear to be unaffected by storage at -80°C for 6 months, long-term storage at -20°C has not been fully researched. However, freezers at -20°C may be more readily available than -80°C freezers in developing countries. Therefore, there is a need for further research into the effect of storage at -20°C on the faecal bacterial community. It has been shown that the storage of faecal material at -20°C affects the ratio of *Firmicutes* to *Bacteroidetes*. However, it is unclear how long a sample may be stored at this temperature before the microbial

community is affected. Lyophilisation of faecal material from pigs prior to extraction has also been suggested as a method to increase the DNA recovery, as shown by restriction fragment polymorphism (Ruiz and Rubio 2009). However, this has not been applied to human faecal samples and there is a need for investigation through next generation sequencing.

The confusion in the literature highlights the need for further investigation and development of a standard operating procedure. There is a need to minimise the variation generated by methodological differences. Real biological effects may be masked or false biological effects generated through a methodological artefact.

Table 6.1|The effect of different storage conditions on DNA extraction results

Storage method	Duration	Effect	Reference
Freezing at -20°C	53 ± 5 days	Shift in the ratio of <i>Firmicutes</i> to <i>Bacteroidetes</i>	(Bahl <i>et al.</i> 2012)
Room temperature* (not defined)	3 h	No effect	
	24 h	DNA is degraded	
	2 weeks	Reduction in the mean proportion of sequences of uncultured bacteria from the <i>Bacteroides</i> , <i>Prevotellaceae</i> and <i>Bifidobacterium</i> taxa	
		DNA is degraded	(Cardona <i>et al.</i> 2012)
Time taken to thaw from -80°C before re-freezing at -80°C	1 h	Reduction in the mean proportion of sequences from an uncultured bacterium from the <i>Bacteroides</i> genus	
		DNA is degraded	
	3 h	Reduction in the mean proportion of sequences of uncultured bacteria from the <i>Bacteroides</i> , <i>Prevotellaceae</i> and <i>Bifidobacterium</i> taxa	
		DNA is degraded	
Room temperature** (approx. 25°C)	1, 4, 6, 8, 24 h	Clustering of faecal samples due to the individual and not storage method/time.	(Carroll <i>et al.</i> 2012)
	1 week		
Freezing at -80°C **	1, 2, 3, 4, 5, and 6 months	No significant differences in the bacterial group DNA after storage	

*when compared to the same sample frozen at -20°C

**all samples had DNA extracted after 30 min at room temperature prior to storage; this acted as a baseline for comparison

6.1.1 Chapter Aims

The aims of this chapter are as follows:

1. to determine whether the freezing of faecal material at -20°C prior to DNA extraction affects its bacterial composition;
2. if the bacterial composition of a faecal sample is affected by freezing at -20°C , I aim to determine how long a sample can be stored before it is detrimentally affected.

6.2 Methods and materials

6.2.1 454 FLX Titanium NGS for analysis on the effect of freezing faecal samples and the effect of homogenisation

Faecal samples from 9 individuals were processed and DNA extracted, as previously described (see **Chapter 2.3.1**). DNA was extracted from fresh samples and after samples had been frozen at -20°C for 24 h, 2 weeks, 3 months and 6 months. Extracted faecal DNA was subjected to 454 FLX Titanium PCR. The forward and reverse primers 338F (5'-ACTCCTACGGGAGGCAGCAG) and 926R (5'- CCGTCAATTCMTTTRAGT) were synthesised with 454 FLX Titanium adapters B (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG) and A (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG) respectively (Haas *et al.* 2011). The reverse primer was also synthesised with a unique sample barcode for downstream identification of sequences. Each sample was amplified in triplicate 50 µl reactions. Each reaction contained 50 ng of template DNA, 0.4 µM of total primers, 0.4 µM of mixed dNTPs, 2.5 U of NEB *Taq* DNA Polymerase (New England Biolabs® Inc. Ipswich, MA 01938-2723) and 1X accompanying standard reaction buffer (10mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.3 at 25°C). The thermal cycler (C1000™; BIO-RAD, Hertfordshire, UK) was programmed with an initial denaturing step of 94°C for 3 min, 20 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 3 min with a final extension step of 72°C for 3 min. Products were visualised by gel electrophoresis. The triplicate reactions were pooled and concentrated by ethanol precipitation, as previously described (see **Chapter 2.4**). The concentrated products were purified using the QIAquick PCR purification kit (QIAGEN, Crawley, West Sussex, UK) and correct sized products were selected by gel electrophoresis. The gel bands were purified using the QIAquick gel extraction kit (QIAGEN, Crawley, West Sussex, UK). Emulsion PCR and sequencing were carried out according to the manufacturer's protocol.

6.2.2 Bioinformatic analysis of NGS results

Mothur, an open-source programme, was used for the majority of the analysis (Schloss *et al.* 2009). The .sff file generated from 454 pyrosequencing was opened in Mothur. This generated two files, the sequence file in FASTA format and a quality file. Using the RDP's pyrosequencing pipeline, the sequence and quality files were uploaded to the pipeline initial process utility (available at: <http://rdp.cme.msu.edu/>). The utility also required the forward and reverse primer sequences as well as the unique sample barcodes used. The minimum quality score was set to q20.

Sequences with a score were then obtained through download. Mothur requires a group file whereby sequences are associated with their respective samples. The group file was created using Mothur.

All relevant files were placed in the same directory as the Mothur executable file along with a current version of the RDP training set (training set 9) and the Silva 16S *rRNA* gene FASTA reference sequences (Pruesse *et al.* 2007). Analysis in Mothur was carried out through the use of custom batch files (**Appendix VI**) based upon a protocol developed by Schloss and colleagues (Schloss *et al.* 2011). Briefly, sequences were shortened to 300bp, in order to increase quality, and aligned to the Silva 16S *rRNA* gene FASTA sequences. Unique sequences were selected, in order to reduce computing power, and chimeras were removed. The OTU table was generated using a 97% (0.03) cut-off. Normalisation of the OTU table was achieved through sub-sampling of the sequence data to the lowest number of sequences present in a given sample e.g. PH25 3M contained 699 sequences. Therefore, all other samples were sub-sampled in order to contain 699 sequences. This step omitted PH27 2W and PH34 3M from further analysis, as the number of sequences within these samples was markedly lower than 699. Remaining sample sequences were taxonomically identified using the RDP training set. Computations including the Yue and Clayton θ similarity coefficient, weighted and unweighted UniFrac distances, inverse Simpson's diversity and Jaccard similarity coefficient indices were carried out in Mothur. Non-metric multi-dimensional scaling (nMDS) and principal co-ordinate analysis (PCoA), of some of these measures, was also carried out in Mothur. The axes generated from nMDS and PCoA were plotted using R statistical software (R-Core-Team 2012) through the use of a custom script (**Appendix II**). The script utilised labdsv (Roberts 2010), vegan (Oksanen *et al.* 2011) and calibrate (Graffelman 2012) packages. Normality, homogeneity of variance testing, one-way ANOVA and Kruskal-Wallis H tests were carried out on the data in SPSS (IBM, Portsmouth, UK).

Bacteroidetes OTU abundances were exported into SPSS (IBM, Portsmouth, UK) statistics software. The data were tested for normality and for homogeneity of variance also. One-way ANOVA and Tamhane's T2 post-hoc test was also carried out on the data.

Phylum data, unweighted and weighted UniFrac distances were imported into R statistical software (R-Core-Team 2012). AGNES clustering, using Ward's method, of normalised OTU abundances and UniFrac distances followed by export in Newick format was carried out in R statistical software. This was achieved through a custom script which utilised ape (Paradis *et al.* 2004), BiodiverstiyR (Kindt and Coe 2005) and cluster (Maechler *et al.* 2005) R packages (**Appendix II**). Once exported in Newick format, the dendrogram was manipulated using iTol software online (Letunic and Bork 2011). The ratio of *Bacteroidetes* to *Firmicutes* was also applied to the dendrogram in the form of a bar-chart.

6.3 Results

6.3.1 α - diversity of samples

Samples were compared based upon storage time and the individual also. The inverse Simpson diversity indices have been plotted based upon the participant (**Fig. 6.1**) and time stored at -20°C (**Fig. 6.2**), before and after normalisation. There was no significant difference in the diversity between participants before and after normalisation, as shown by one-way ANOVA [$F(8, 34) = 1.651, p=0.147$] and [$F(8, 34) = 1.680, p=0.139$] respectively. Analysis based upon storage time shows a similar pattern. One-way ANOVA shows no significant difference in diversity between the samples after varying lengths of storage at -20°C before and after normalisation, [$F(4, 38) = 0.71, p=0.590$] and [$F(4, 38) = 0.511, p=0.728$]. Therefore, the level of diversity is not significantly different after storage at -20°C up to 6 months. Furthermore, inter-individual diversity also appears to be relatively uniform.

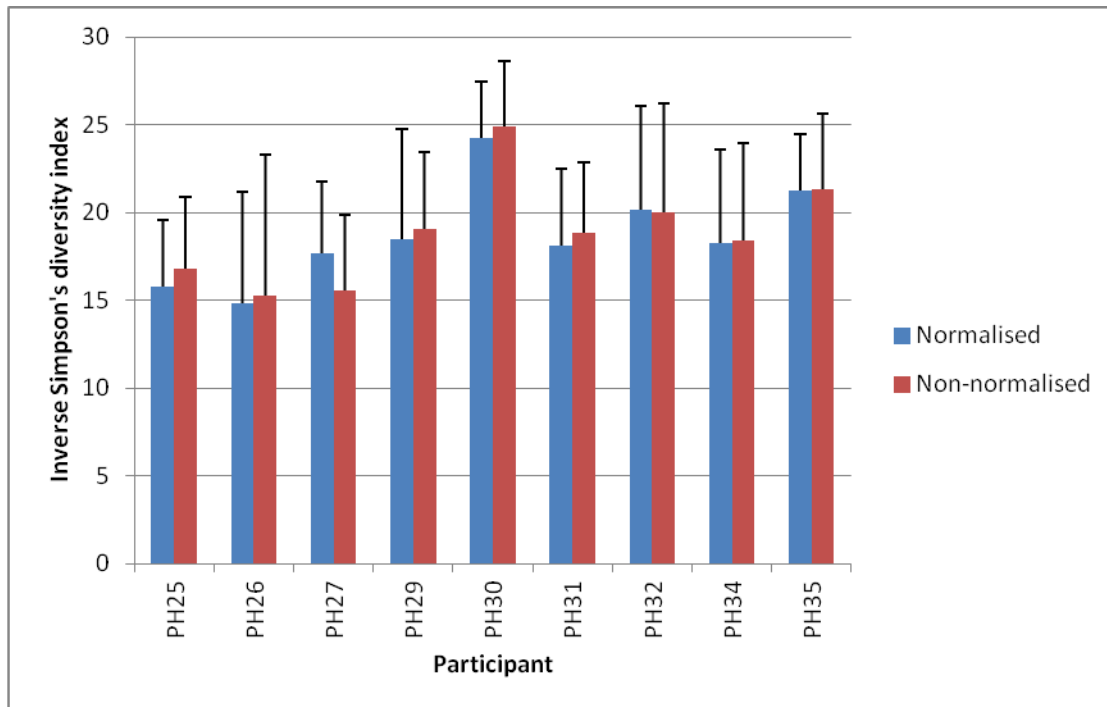


Figure 6.1| Inverse Simpson's diversity index of OTUs obtained from 454 pyrosequencing of DNA after different storage times – average inverse Simpson's diversity indices are shown from all storage times for each participant (n=9). Both normalised and non-normalised OTU datasets were subjected to the diversity index. Error bars show SD from the mean.

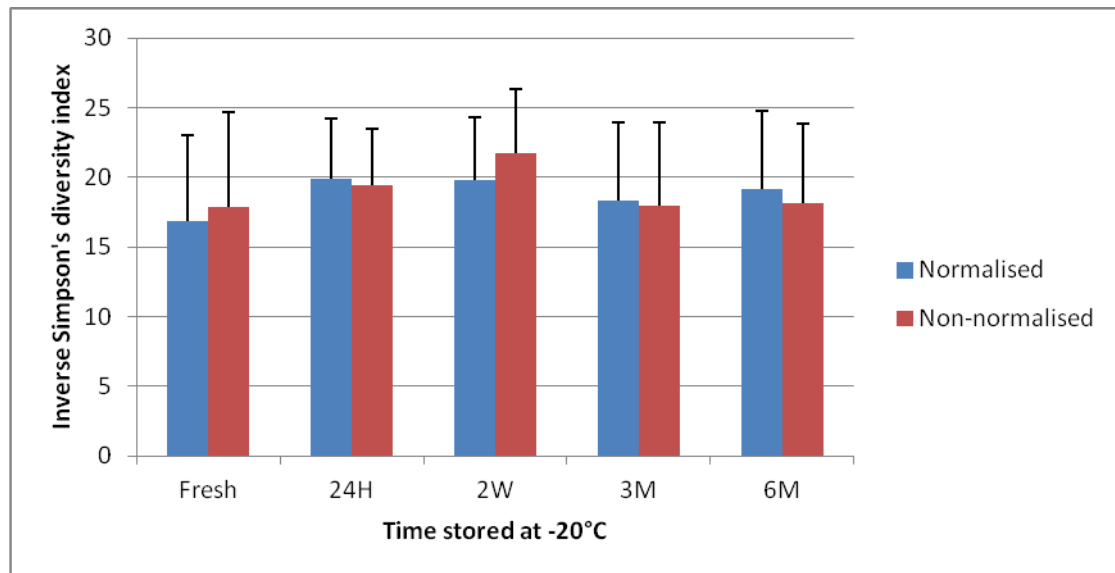


Figure 6.2| Inverse Simpson's diversity index of OTUs obtained from 454 pyrosequencing of DNA after different storage times – average inverse Simpson's diversity indices are shown for all storage time. Where 24H = 24 h, 2W = 2 weeks, 3M = 3 months and 6M = 6 months. Both normalised and non-normalised OTU datasets were subjected to the diversity index. Error bars show SD from the mean.

6.3.2 The effect of storage at -20°C on the bacterial community of a faecal sample

The similarity in community structure of samples following storage at -20°C for varying lengths of time has been analysed. To this end nMDS analysis of Jaccard similarity coefficient indices and the Yue and Clayton θ similarity coefficients of samples has been carried out. Samples from Fresh, 24H, 2W and 3M groups cluster together through Jaccard similarity coefficient index analysis. However, the 6M group shows a degree of separate clustering (**Fig. 6.3**). The results obtained through the Yue and Clayton θ similarity coefficient show a more dramatic change (**Fig. 6.4**). While Fresh, 24H and 2W groups cluster together the 3M group begins to cluster separately. The 6M group clusters completely separately from the Fresh, 24H and 2W groups. However, there is some overlap between the clusters generated from 3M and 6M.

UniFrac distance analysis was also carried out on the dataset. In a similar manner to the Jaccard similarity coefficient, PCoA of unweighted UniFrac distances of the samples show a degree of separate clustering following 6 months of storage at -20°C (**Fig. 6.5A**). The degree of separate clustering of 6M samples along with the samples which overlap with other storage periods can be seen through AGNES of the unweighted UniFrac distances (**Fig. 6.5B**). Cluster analysis of the unweighted UniFrac distances also shows that samples, with the exception of 6M samples, tend to cluster together based upon the individual and not due to time stored (**Fig. 6.5B**). Analysis through PCoA of weighted UniFrac distances shows separate clustering of 6M samples, in a similar fashion to the Yue and Clayton θ similarity coefficient (**Fig. 6.6A**). This cluster of 6M samples also appears tighter than its unweighted counterpart (**Fig. 6.5A**). Cluster analysis, through AGNES, of the weighted UniFrac distances shows that all but 2 of the 6M samples cluster together (**Fig. 6.6B**). Furthermore, unlike the unweighted iteration, weighted UniFrac distances of samples do not seem to cluster based upon the individual any longer. Instead, samples cluster with samples from different individuals at the same time-point and different time-points alike.

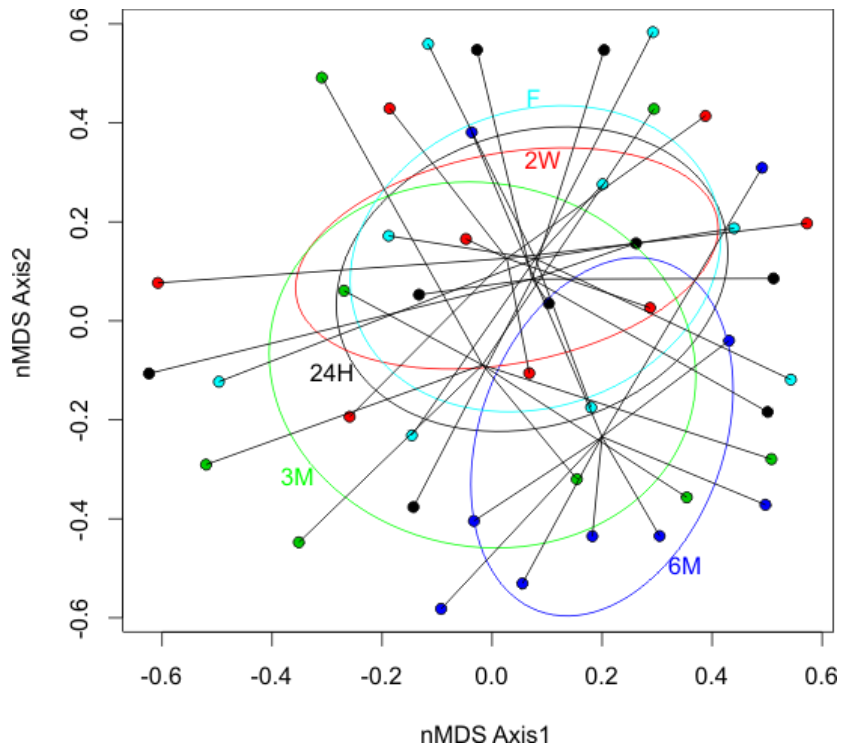


Figure 6.3| nMDS plot of the Jaccard similarity coefficient indices obtained from samples following storage at -20°C – samples have been coloured based upon their duration stored at -20°C, F = Fresh, 24H = 24 h, 2W = 2 weeks, 3M = 3 months and 6M = 6 months.

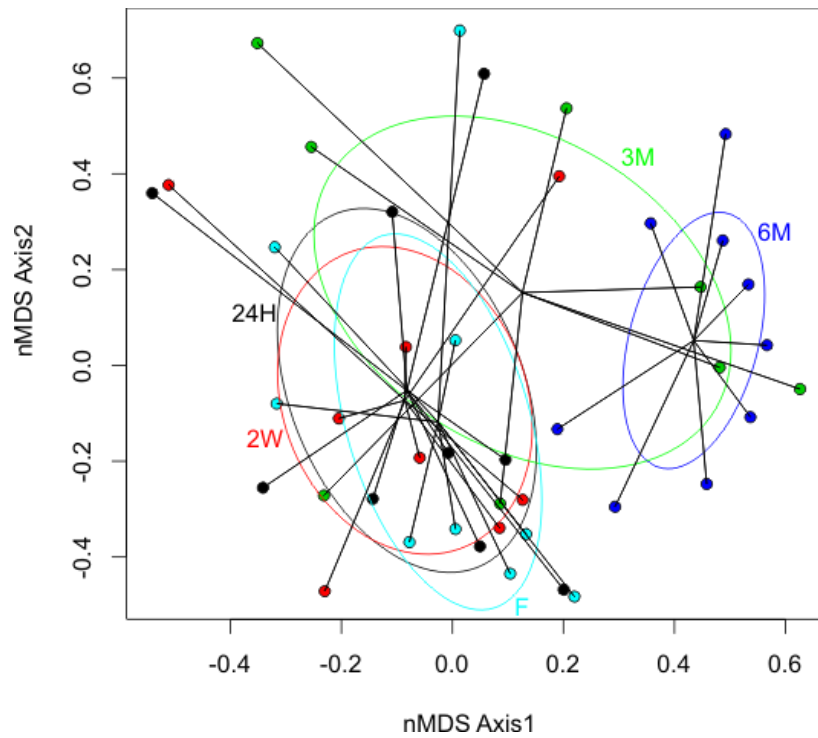


Figure 6.4| nMDS plot of the the Yue and Clayton θ similarity coefficients obtained from samples following storage at -20°C – samples have been coloured based upon their duration stored at -20°C , **F = Fresh, **24H** = 24 h, **2W** = 2 weeks, **3M** = 3 months and **6M** = 6 months.**

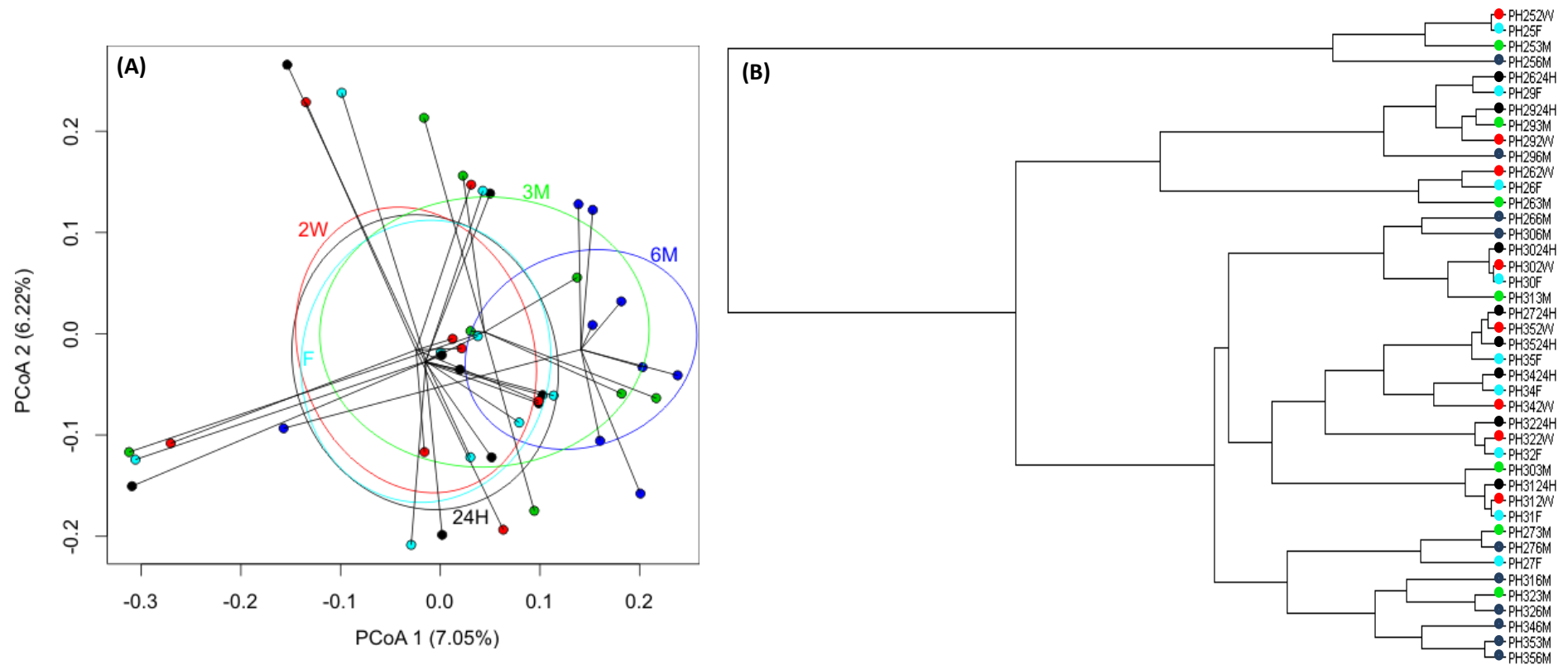


Figure 6.5] Unweighted UniFrac distance analysis of faecal DNA following storage at -20°C – (A) shows PCoA of the unweighted UniFrac distances where, the first and second components are plotted with the percentage of variation explained. Samples are colour coded, **F** = Fresh, **24H** = 24 h, **2W** = 2 weeks, **3M** = 3 months and **6M** = 6 months. (B) shows AGNES clustering of the unweighted UniFrac distances between samples. The samples are colour coded to match the PCoA plot.

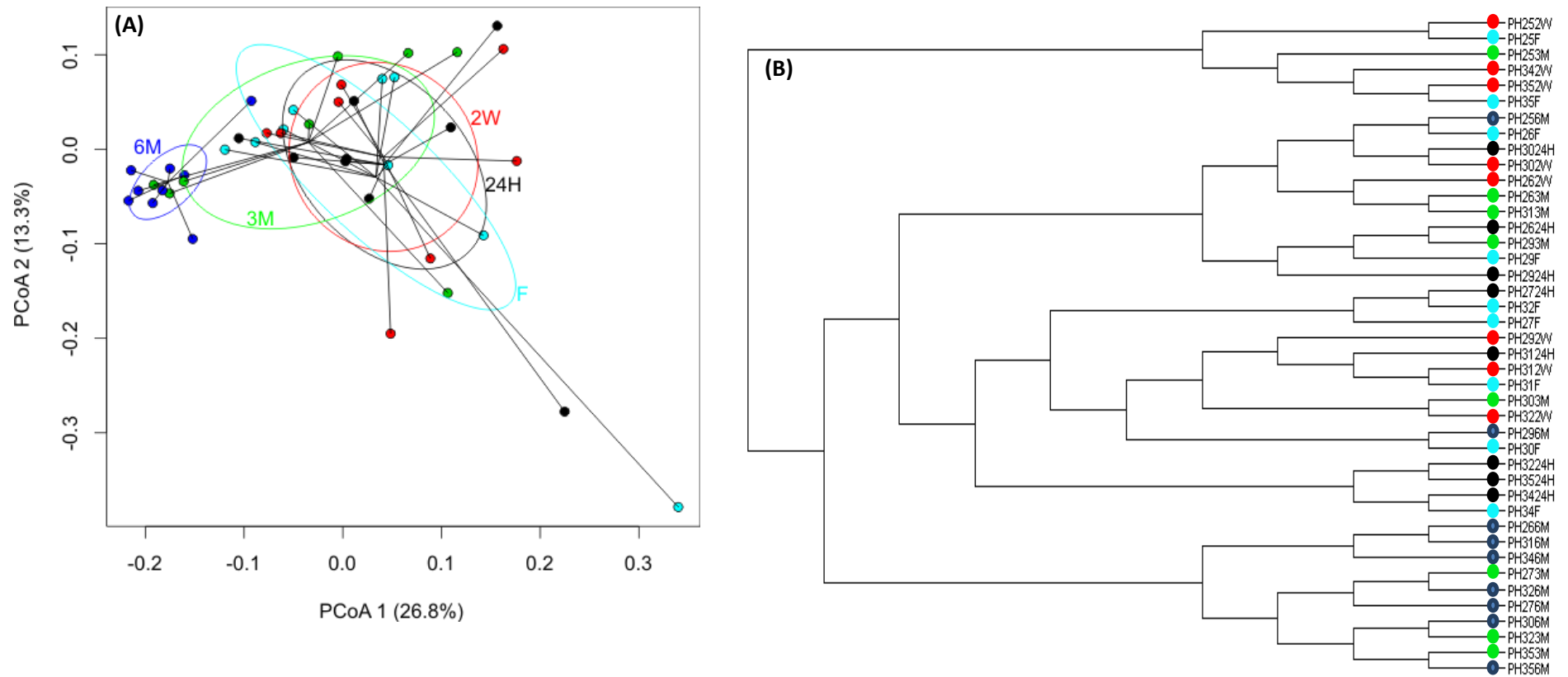


Figure 6.6] Weighted UniFrac distance analysis of faecal DNA following storage at -20°C – (A) shows PCoA of the weighted UniFrac distances where, the first and second components are plotted with the percentage of variation explained. Samples are colour coded, **F** = Fresh, **24H** = 24 h, **2W** = 2 weeks, **3M** = 3 months and **6M** = 6 months. (B) shows AGNES clustering of the weighted UniFrac distances between samples. The samples are colour coded to match the PCoA plot.

The proportional abundances of assigned OTUs at the phylum level have been plotted (**Fig. 6.7**). The majority of the samples contain a high abundance of *Firmicutes* and *Bacteroidetes* and the *Actinobacteria* are also abundant. The *Proteobacteria* are present but to a lesser extent. The ratio of *Bacteroidetes* to *Firmicutes* decreases as the storage time increases. This is further highlighted in a heatmap which clusters the most similar samples (**Fig. 6.8**). In the heatmap it is possible to see that 6 month (black) and the majority of 3 month (purple) samples cluster together. The reason for the clustering is due to the high number of OTUs in the *Firmicutes* phylum relative to the number of OTUs in the *Bacteroidetes* phylum. Cluster analysis through AGNES shows that the majority of samples after 6 months and 3 months of storage cluster together (**Fig. 6.9**). It is also possible to observe that the ratio of *Firmicutes* to *Bacteroidetes* in these samples is higher than in any of the other samples.

In order to inspect this difference further OTU counts for the *Bacteroidetes* phylum were collected, exported and plotted (**Fig. 6.10**). The average OTU counts for the *Bacteroidetes* at Fresh, 24 h, 2 weeks, 3 months and 6 months were 141.33, 155.33, 121.75, 65.25 and 16.33 respectively. The data were not normally distributed so a Log_{10} transformation was carried out. One – way ANOVA showed a highly significant difference between the mean of the *Bacteroidetes* counts [$F(4, 38) = 14.504, p < 0.001$]. Although after transformation the data were normal, there was no homogeneity of variance [Levene statistic (4, 38) = 4.588, $p = 0.004$]. Therefore, the Tamhane's T2 post-hoc test, which does not assume homogeneity of variance, was used. This post-hoc test showed that the average *Bacteroidetes* OTU counts for 6 months significantly differ from the counts obtained from Fresh, 24 h and 2 weeks storage at -20°C at the .05 significance level. There was no significant difference between Fresh, 24 h, 2 weeks and 3 months. Similarly there was no significant difference between the average *Bacteroidetes* OTU counts for 3 months and 6 months.

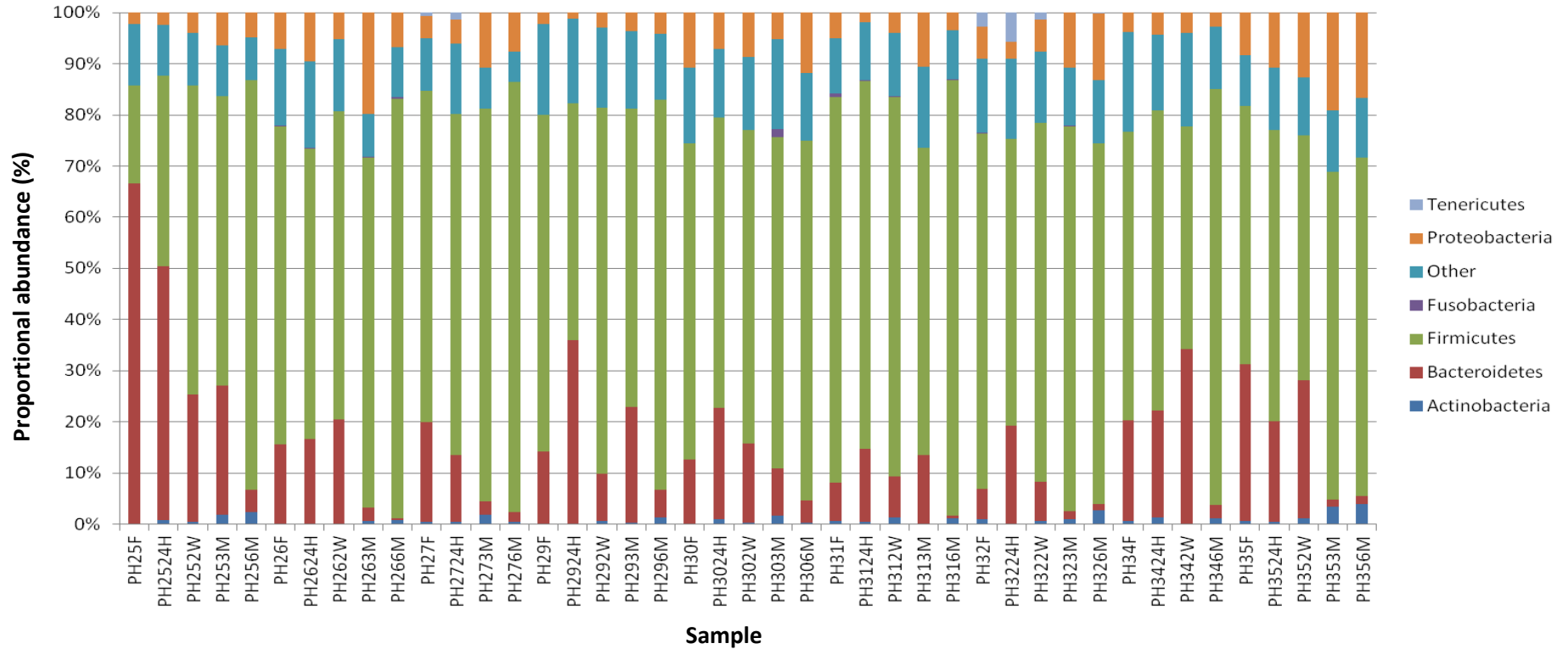


Figure 6.7| The effect of faecal material storage at -20°C on the bacterial community at the phylum level – proportional abundances of phyla are shown. Participant numbers are followed by the time stored at -20°C. F= fresh, 24H = 24 h, 2W = 2 weeks, 3M = 3 months and 6M = 6 months. Phyla are represented by colours and a key is shown.

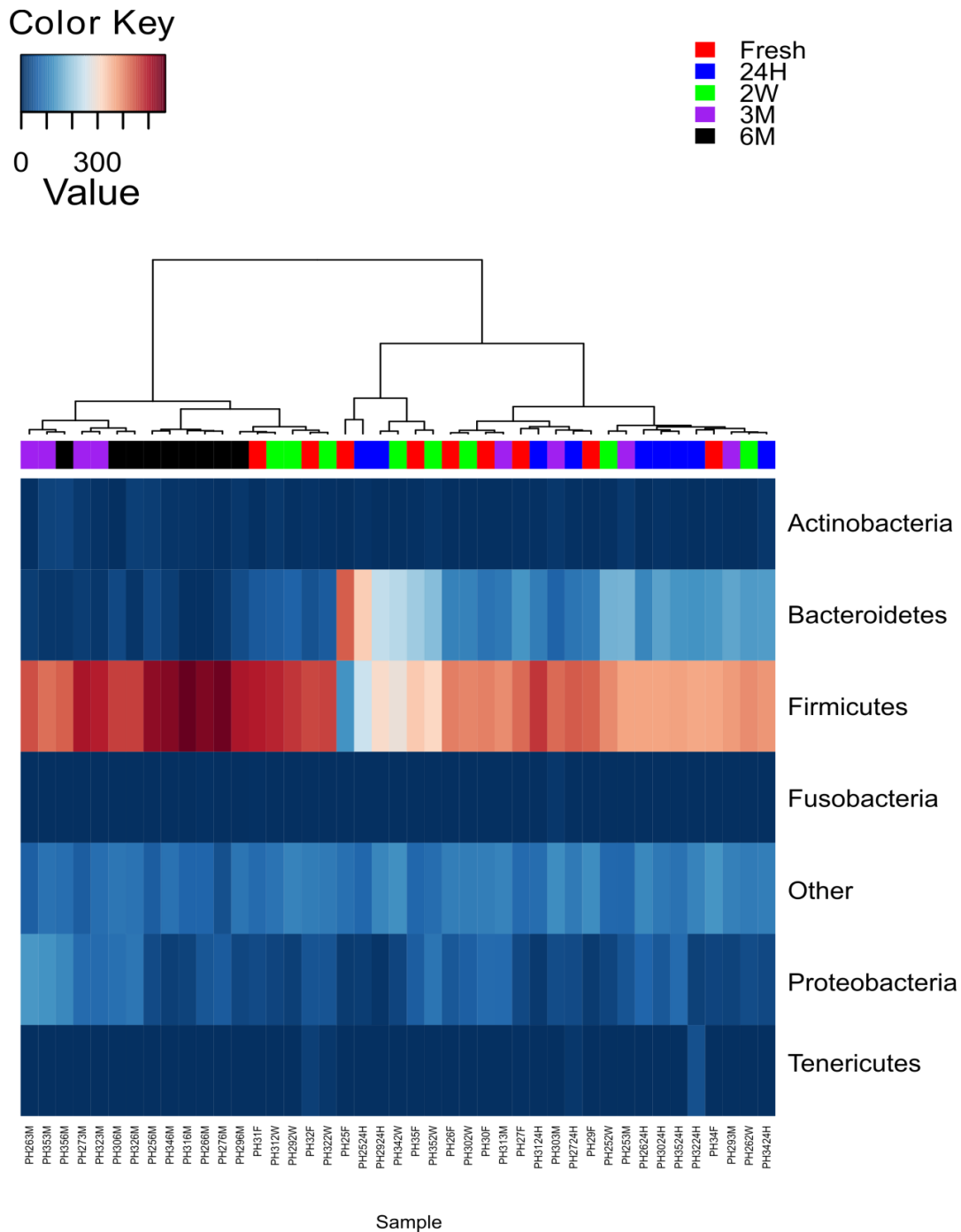


Figure 6.8|Heatmap to show the effect of faecal material storage at -20°C on the bacterial community at the phylum level – the heatmap shows absolute numbers of OTUs for a given phylum, these are represented by colour. The time stored at -20°C is also represented by colour in a horizontal bar at the top of the heatmap. Colour keys are shown and similar samples are clustered together.

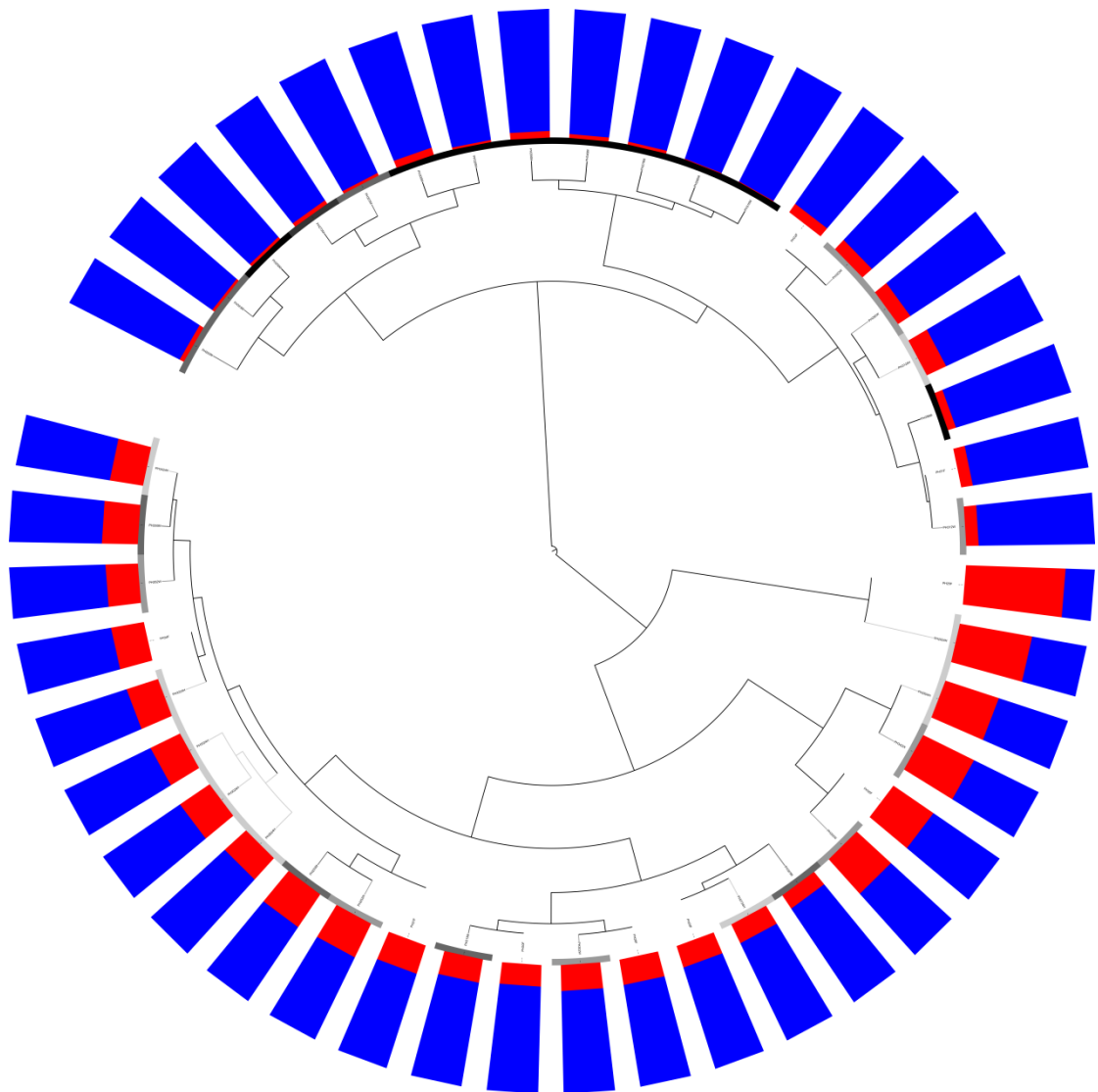


Figure 6.9|Cluster analysis to show the effect of storage at -20°C on the ratio of *Firmicutes* to *Bacteroidetes* – AGNES of OTUs at the phylum level was carried out. The inner circle shows the time stored at -20°C on a grayscale colour continuum. Blanks represent Fresh samples and the shade becomes darker to represent a longer period of storage with Black (the darkest) representing 6 months' storage. The proportional abundance of *Firmicutes* (blue) to *Bacteroidetes* (red) is also shown as a bar chart.

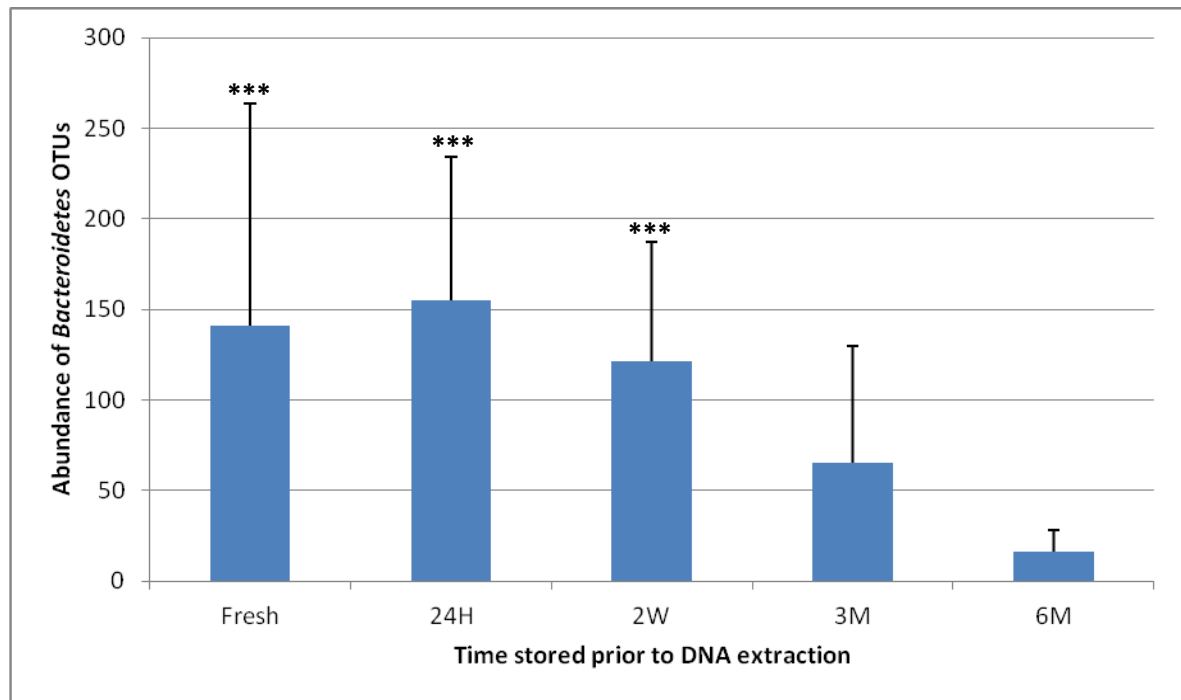


Figure 6.10 | The effect of storage at -20°C on the number of *Bacteroidetes* OTUs – the absolute number of *Bacteroidetes* OTUs are shown for each storage time; 24H = 24 h, 2W = 2 weeks, 3M = 3 months and 6M = 6 months. The error bars represent SD from the mean. ***denotes a highly significant difference ($p < 0.001$) between the given storage period and the 6 months storage period.

In order to determine whether a specific member of the *Bacteroidetes* phylum was affected by storage at -20°C or not, the dataset was analysed at the family level also. Box-plots of OTU abundance for the *Bacteroidetes* at the family level were therefore plotted (**Fig 6.11** and **Fig. 6.12**). The data were not normally distributed therefore, Kruskal – Wallis H tests were carried out. The ranked means were significantly different between the times stored at -20°C for *Bacteroidaceae* [$\chi^2 (4) = 20.130$, $p < 0.001$], *Rikenellaceae* [$\chi^2 (4) = 10.393$, $p = 0.034$], and *Porphyromonadaceae* [$\chi^2 (4) = 20.494$, $p < 0.001$]. Post-hoc analysis through pairwise comparisons shows that the mean ranks of *Bacteroidaceae*, *Rikenellaceae* and *Porphyromonadaceae* families were significantly higher at Fresh, 24 h and 2 weeks than 3 months and 6 months at the 0.05 level. There was no significant difference between the 3 months and 6 months' time-point in any of families and furthermore there was no significant difference between the ranked means of the *Prevotellaceae* family following storage at -20°C.

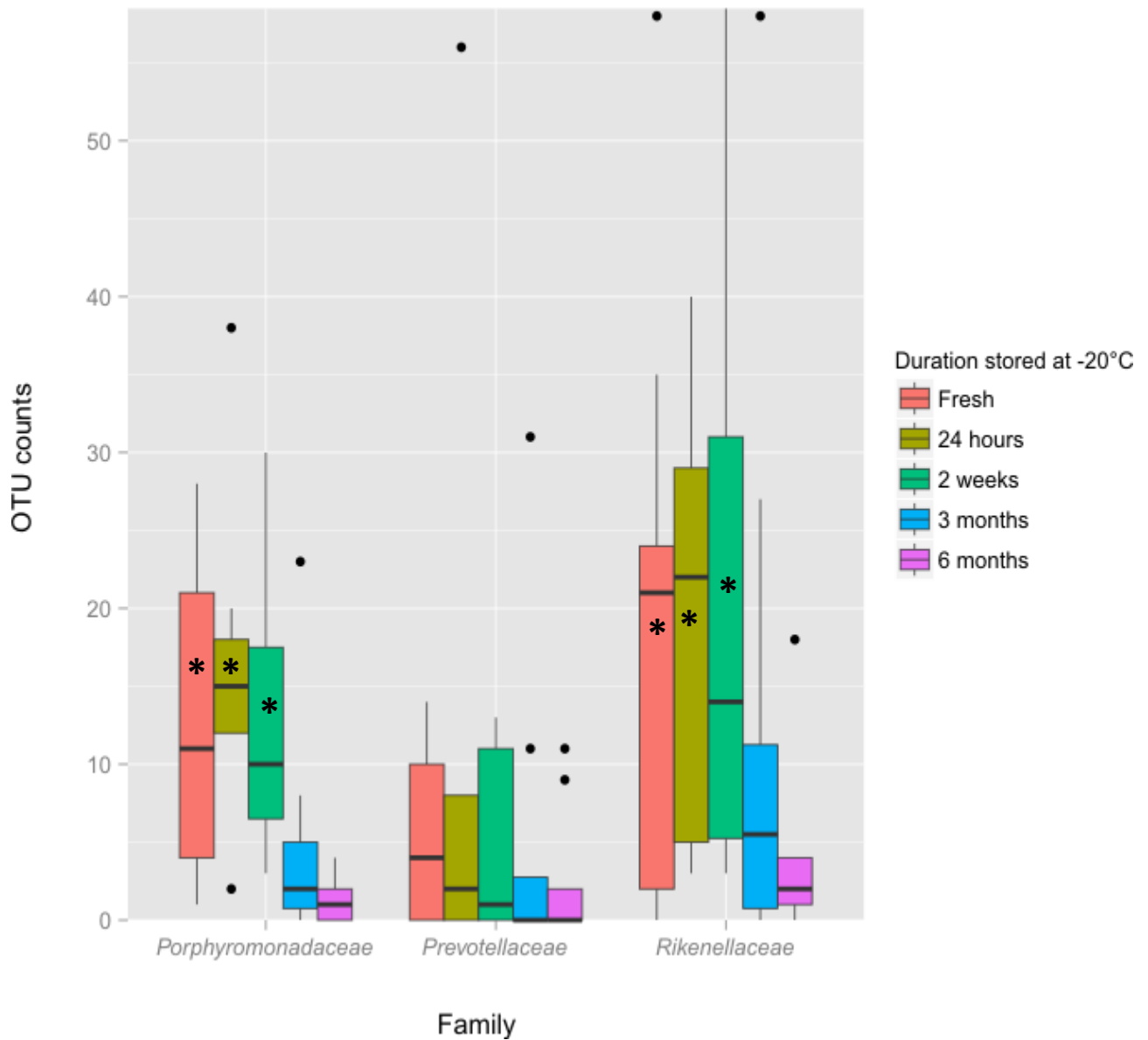


Figure 6.11| Box-plot showing the effect of storage at -20°C prior to DNA analysis on members of the *Bacteroidetes* phylum at the family level – the box-plots show the median, 1st and 3rd quartiles for 3 families at different storage periods, these are represented by colour and a key is shown. Outliers are represented by black dots. *denotes a significant difference between the rank mean of that given storage period and the ranked mean after 6 months of storage ($p < 0.05$).

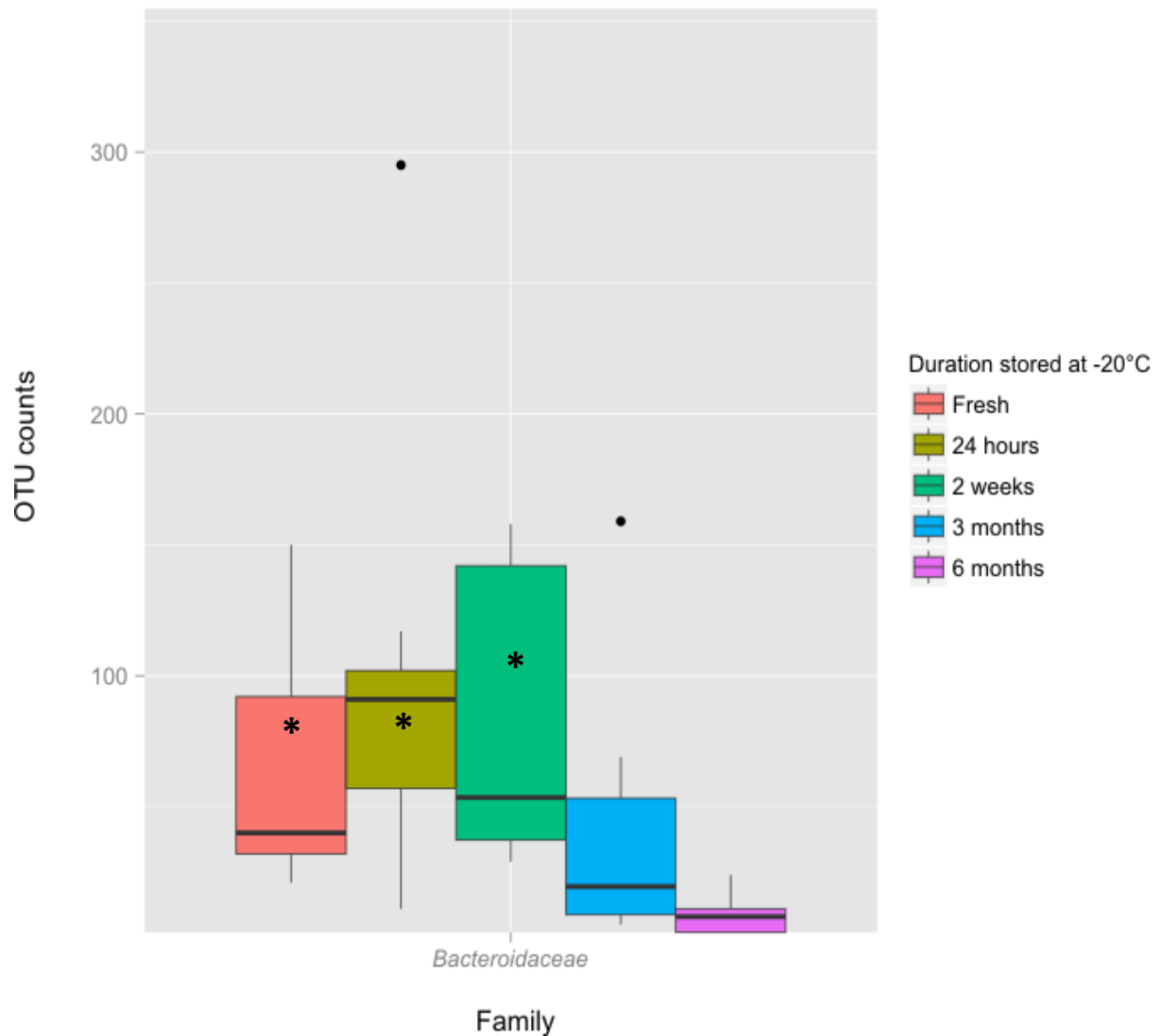


Figure 6.12| Box-plot showing the effect of storage at -20°C prior to DNA analysis on the *Bacteroidaceae* family of the *Bacteroidetes* phylum - the box-plots show the median, 1st and 3rd quartiles for the *Bacteroidaceae* family at different storage periods, these are represented by colour and a key is shown. Outliers are represented by black dots. *denotes a significant difference between the rank mean of that given storage period and the ranked mean after 6 months of storage ($p < 0.05$).

6.4 Discussion

The effect of storage upon the bacterial community of a faecal sample has been shown to be significant. Many researchers use faecal material as a proxy for the distal gut and the large intestine. It is therefore fundamentally important that this source of information represents the community truly. I have shown that the storage of this material at -20°C can affect the results.

The inverse Simpson diversity indices show that there are no significant differences in the diversity of the samples after storage. There is also no significant difference in the diversity of samples based upon the individual. By plotting the normalised and non-normalised diversity indices it is possible to see that sub-sampling has not decreased the diversity. This is desirable as sub-sampling should still represent the initial community. Sub-sampling is also an essential step in order to negate sequencing generated biases. The effect of storage on the diversity of the faecal bacterial community has previously been researched. In agreement with my observations, the diversity of the community as a whole was not affected by storage conditions (Cardona *et al.* 2012). It has also been shown that faecal samples may be kept at room temperature for 72 h before storage at -80°C , with no significant change to the community as a whole (Roesch *et al.* 2009).

The effect of storage at -20°C on the bacterial community of a sample becomes apparent when the β -diversity of the samples is analysed. nMDS analysis of Jaccard similarity coefficients generated from OTUs shows a moderate effect of storage on the community. This classical method was initially applied to describe the diversity of plants in the Alps (Jaccard 1912). Mothur utilises Jaccard's principle in order to describe the dissimilarity between communities (Schloss *et al.* 2009). This measure uses presence/absence of OTUs and does not take into account the abundance of a given OTU. This method shows moderate separate clustering of samples following 6 months of storage at -20°C . When the data are analysed through nMDS analysis of the Yue and Clayton θ similarity coefficients, a greater effect of storage is observed. Through this analysis it becomes apparent that samples cluster separately after 6 months of storage at -20°C . These samples show a degree of clustering with samples after 3 months of storage at -20°C . Similarity coefficients generated through the Yue and Clayton θ method (Schloss *et al.* 2009) take the abundance of a given OTU into account. Therefore, it is possible to surmise that the presence or absence of OTUs are not greatly affected by storage. However, the abundance of specific OTUs are affected by storage and furthermore, drive separate clustering of samples.

UniFrac distance analysis is a relatively new comparative method which has been applied to complex microbial communities (Lozupone and Knight 2005). The method determines the phylogenetic distance between samples or communities through the use of a phylogenetic tree. The unweighted version of the metric was applied in order to compare microbial communities from different types marine environments (Lozupone and Knight 2005). The unweighted metric does not take into account the abundance of a given OTU when comparing samples. Therefore, it is a useful technique in order to compare samples from different communities. The application of the unweighted form of this measure to my dataset shows that samples tend to cluster together based upon the individual and not time stored, with the exception of the 6M samples. This suggests that individuals have their own community of OTUs which drives this individual based clustering. With regards to the clustering of 6M samples, it appears that the storage of the faecal material negates inter-individual differences in gut bacterial community and is probably due to the loss of *Bacteroidetes* OTUs. Samples from other time-points generally do not cluster together however, the 6M samples do cluster together. The weighted version of UniFrac does take into account the abundance of OTUs (Lozupone *et al.* 2007). When this measure is applied to the dataset inter-individual variation becomes less apparent. Samples from different individuals cluster with the same time-points from other individuals and different time-points also. Once again, a large cluster of 6M samples can be observed with some 3M samples too. Therefore, I can conclude freezing does not greatly affect the presence/absence of OTUs up until 6 months as samples from individuals cluster together. However, freezing even for short periods seems to detrimentally affect the abundance of OTUs and through this decreases inter-individual variation. However, the abundances are not greatly affected until 6M of storage at -20°C.

Taxonomic assignment of OTUs at the phylum level show that the bacterial community of faecal samples resembles what has been previously shown (Ley *et al.* 2006a). In the fresh samples, the two major phyla are the *Firmicutes* and *Bacteroidetes* with *Actinobacteria* and *Proteobacteria* also present. In addition, the proportion of *Bacteroidetes* decreases as time stored at -20°C increases. This decrease in *Bacteroidetes* OTU abundance is responsible for the clustering observed in the nMDS plot of the Yue and Clayton θ similarity coefficients and in the PCoA and AGNES clustering of weighted UniFrac distances. Analysis, through the generation of a heatmap, shows that samples after storage for 3 months which cluster with samples after 6 months of storage do so due to a similar number of *Firmicutes* and *Bacteroidetes* OTUs. Analysis of the number of *Bacteroidetes* OTUs alone shows a highly significant reduction after 6 months of storage at -20°C. There was no significant difference between the 3 month storage period and 6 month storage period. This suggests that the 3 month period is a transition point between Fresh, 24 h, 2 weeks and 6 months. Although there is a decrease in the numbers of *Bacteroidetes* OTUs, they do not completely disappear. This further explains why separate clustering was not observed through Jaccard similarity coefficient and unweighted UniFrac distance analysis. *Bacteroidetes* OTUs are still present and therefore these measures do not show separate clustering of samples from the 6 month storage period. The Yue and Clayton θ similarity coefficient and weighted UniFrac distances do take into account the abundance of OTUs. Therefore, separate clustering at 6 months is observed due to the reduction in the number of *Bacteroidetes* OTUs

My results suggest that storage does not affect the bacterial diversity of a faecal sample as a whole. However, storage at -20°C does affect the *Bacteroidetes* phylum, a major phylum in the human gut. Although a significant difference in *Bacteroidetes* OTU abundance was not observed until 6 months of storage, there was a marked reduction after 3 months of storage. Due to there being no significant difference between the *Bacteroidetes* OTU abundance at 3 months and 6 months, it is advisable not to store faecal samples at -20°C for longer than 2 weeks prior to DNA extraction. It has previously been shown that storage at -20°C for 53 ± 5 days affects the ratio of *Firmicutes* to *Bacteroidetes* (Bahl *et al.* 2012). This period falls between the sampling points of 2 weeks to 3 months in my study where observable differences in the *Bacteroidetes* abundance become apparent. It is difficult to assign a reason for this decrease. It has been shown that the *Bacteroidetes* phylum is generally unaffected by freeze thawing in Arctic tundra soil (Männistö *et al.* 2009). It is therefore unlikely that freeze thawing of the faecal sample is directly causing a decrease in the *Bacteroidetes*. Members of this genus have been shown to degrade complex polysaccharides, including chitin (McBride *et al.* 2009). The commensal *Bacteroidetes* *thetaiotaomicron* has been shown to produce enzymes which degrade complex polysaccharides also (Xu *et al.* 2003). It has also been suggested that *Porphyromonas gingivalis*, a member of the *Bacteroidetes* phylum, lacks the protein secretion machinery which is common to other Gram negative bacteria (Sato *et al.* 2010). Many members of the *Bacteroidetes* genus contain the outer membrane protein porT. The researchers highlight that there are no orthologs of the *porT* gene in *Bacteroides fragilis* or *B. thetaiotaomicron*. However, the secretion machinery of *Bacteroidetes* may be affected by freezing and therefore export of the many carbohydrate degrading enzymes these bacteria produce cannot take place. My results show that the *Bacteroidaceae* and *Porphyromonadaceae* families decrease following storage at -20°C. Therefore, it could be possible that members of these families are degraded, due to their plethora of complex carbohydrate degrading enzymes. The subsequent DNA release may then be degraded from enzymes present within the faecal sample.

The effect of storage, combined with the already discussed variable results of DNA extraction on bacterial community community, gives the researcher much to consider. Indeed the effect of storage must be considered while designing experiments. It would appear that there is a finite window for the extraction of DNA to obtain a true representation of the faecal bacterial community. Roesch and colleagues have highlighted concerns that the faecal bacterial community needs to be a true representation of the distal gut (Roesch *et al.* 2009). It is especially important as much research focuses on the effect of our gut microbiota on the onset of diseases. New research suggests a link between our gut microbiota and the development of obesity. Researchers are especially interested in the proportion of *Firmicutes* to *Bacteroidetes* (Ley *et al.* 2006b; Turnbaugh *et al.* 2009). The results of Ley and colleagues' work have not been emulated in another study (Fleissner *et al.* 2010). My research shows that storage can affect the proportion of *Firmicutes* to *Bacteroidetes*. This raises questions as to how samples from the aforementioned studies were treated and stored. It may be prudent to ensure that observed differences in the ratio of *Firmicutes* to *Bacteroidetes* of already published work are true biological effects and not methodologically driven.

6.5 Conclusions

The main conclusions for this chapter are as follows:

1. freezing faecal material at -20°C gives rise to a significant reduction in the *Bacteroidetes* phylum;
2. significant reductions in the *Bacteroidetes* phylum occurs between 2 weeks and 3 months and it is therefore advisable not to store faecal material for longer than 2 weeks prior to DNA extraction.

7. GENERAL DISCUSSION, PERSPECTIVES AND FUTURE RESEARCH

7.1 Conclusions

The effect of long-term probiotic administration in healthy male individuals has been researched in the PROHEMI study, a pilot feeding study. Participants' distal gut bacterial composition, gut functions, genotoxicity and metabonomic profiles of their faecal waters have been researched through the use of a suite of techniques. In addition samples from Cardiff/Port Talbot and Sheffield have been compared against one and other.

Recent research has hinted that the method of faecal material storage and subsequent DNA extraction can give rise to variable results. With regards to faecal material, it has been shown that storage can detrimentally impact upon the ratio of *Bacteroidetes* to *Firmicutes*, the two major phyla in the human gut. Therefore a 6 month study was undertaken in order to strengthen the body of knowledge in this area.

The main conclusions of the studies now follow:

7.1.1 Probiotic administration does not affect the distal gut bacterial community of healthy male individuals (Chapters 4 and 5)

Through the community fingerprinting technique LHPCR it was shown that probiotic administration does not appear to affect the gross bacterial community of the distal gut in healthy individuals. Ordination techniques showed samples clustered together regardless of study period.

NGS through Illumina-Solexa technology strengthened this finding. Sequencing of the 16S *rRNA* V4 region of PROHEMI faecal DNA showed no change attributable to probiotic administration. Ordination techniques once again did not show separate clustering of samples due to probiotic supplementation. In addition, phylum analysis showed no significant difference between study periods in terms of the abundance of detected phyla.

Meta-analysis of the literature leads us to believe that modulation of human diet in turn will give rise to a change in the gut bacterial community of an individual {Walker, 2011 #813}. The efficacy of prebiotic feeding has also been carried out with phylum specific responses to given prebiotics shown {Gibson, 1999 #912}{Langlands, 2004 #913}. However, research into the effect of long-term probiotic supplementation upon the distal gut microbiota seems to

be lacking. Results from the PROHEMI study indicate that long-term probiotic supplementation does not affect the distal gut bacterial community of healthy individuals.

7.1.2 Probiotic administration differentially affects functions provided by the commensal microbiota of healthy male individuals (Chapter 3)

Probiotic administration appears to leave some functions provided by our distal gut microbiota unchanged while affecting others. Bacterial numbers expressing protease activity was reduced during probiotic supplementation in both study centres. The numbers of bacteria expressing β -glucuronidase activity in both Cardiff/Port Talbot samples and Sheffield samples increased during supplementation. β -galactosidase activity in Sheffield samples also increased during probiotic supplementation. Cholesterol degrading activity and esterase/lipase activity were low across all study periods and unaffected by probiotic supplementation.

Many studies focus upon metagenomic strategies in order to interrogate the functions provided by our resident gut microbiota {Gill, 2006 #892}{Qin, 2010 #51}. However, my research utilised functional media and direct culture from faecal material. Through functional screening of the faecal microbiota differential responses in gut functions have been shown during probiotic treatment. This study gave rise to an agar screening method for protease activity which avoids false positives from β -galactosidase producing bacteria {Morris, 2012 #295}. Bacterial proteases have been implicated in the onset of intestinal inflammation {Steck, 2011 #839} and in the development of IBS and IBD {Steck, 2012 #836}. Probiotic supplementation may provide a means to decrease the expression of gut bacterial proteases and reduce intestinal inflammation. While there is a potential benefit in probiotic supplementation for the reduction of bacteria expressing protease activity, the increase in number of bacteria expressing β -glucuronidase activity is alarming. β -glucuronidases are responsible for the reversal of glucuronidation, uncoupling inactive toxic compounds so they become active once more. The results from this study suggest that further research needs to be carried out into the effect of probiotic supplementation on β -glucuronidase activity in the gut. These findings pose questions as to whether probiotic supplementation should be given to individuals receiving drug therapy as drug toxicity may be increased.

7.1.3 Probiotic administration does not affect the genotoxicity and metabonomic profiles of faecal waters of healthy male individuals (Chapters 3 and 5)

Research into the effect of probiotic consumption on the reduction of carcinogen genotoxicity has previously been carried out. Meta-analysis by Burns and Rowland has shown that probiotic supplementation can reduce the genotoxic effect of carcinogens *in vivo* and *in vitro* {Burns, 2000 #916}. More recent research by Burns and Rowland has shown that the genotoxic potential of faecal water of a healthy individual was decreased in HT-29 cell comet assay {Burns, 2004 #151}. In this study, probiotic LAB bacteria were incubated with HT-29 cells directly prior to administration of faecal water. While this study showed the anti-genotoxic potential of probiotic bacteria, their effects following passage through the human GIT needs to be researched.

With this in mind, the aim was to determine whether long-term probiotic administration gives rise to anti-genotoxic effects. My research showed that the levels of genotoxicity across all study periods remained relatively uniform. Genotoxicity levels neither increased nor decreased upon induction of probiotic supplementation. The protocol followed for genotoxicity testing served as a pilot experiment. The results showed significant differences in the faecal water of individuals. It would therefore have been interesting to analyse PROHEIMI participants individually throughout the course of the pilot feeding study in order to determine whether there are responders and non-responders to probiotic treatment.

Metabonomic analysis has been applied in order to screen faecal water for metabolites indicative of disease {Marchesi, 2007 #205}{Odunsi, 2005 #897}. The technique has also been applied in order to determine whether LAB modify the metabonomic profiles of an experimental colitis mouse model {Hong, 2010 #898}. I aimed to determine whether probiotic supplementation in healthy individuals affects their metabonomic profiles. Metabonomic analysis of PROHEIMI participants' faecal waters showed no effect on the gross and SCFA metabonomic profiles following probiotic supplementation. Ordination techniques showed samples clustering together, irrespective of the study period.

7.1.4 There are differences in the distal gut bacterial community, functions and metabonomic profiles of individuals from the two study centres (Chapters 3, 4 and 5)

The PROHEMI study has given rise to an interesting observation as there appears to be differences between the two study centres in many respects. Community profiling, through LHPCR, showed a difference in the gross bacterial community of individuals. In addition, NGS through Illumina-Solexa technology also showed differences between the two study centres in terms of distal gut bacterial community. Metabonomic analysis also showed differences between the faecal waters of Cardiff/Port Talbot and Sheffield samples. The research has inadvertently given rise to results which suggest geographical differences in the gut bacterial community, functions and metabonomic profiles of individuals in the UK. Many studies focus upon continental differences in the gut bacterial community of individuals {Lin, 2013 #900}{De Filippo, 2010 #767} and differences in the gut bacterial community and metabonomic profiles of single cohorts, such as the elderly {Claesson, 2011 #76}{Claesson, 2012 #296}. To my knowledge there has been no seminal comparison between individuals from the UK, as researchers seem to focus upon a single study centre within the UK. Further research needs to be carried out in order to confirm this observation. However, my research hints that there are indeed geographical differences within the UK. The exact reason for this observation remains unclear. However, lifestyle and diet may play a pivotal role in the observed difference. As previously discussed, modifying the diet of over-weight men exerted differences upon the abundance of member of the *Firmicutes* {Walker, 2011 #813}. Another reason for the observed difference may be the water drunk by individuals on the PROHEMI study. There appears to be no published research into the effect of different water supplies upon the distal gut bacterial community of individuals. However, water in Wales is supplied by Dŵr Cymru while water in Sheffield is supplied by Yorkshire Water and this may give rise to the observed differences.

7.1.5 Freezing faecal material prior to DNA extraction detrimentally affects its bacterial composition

There is a growing body of evidence that leads us to believe that the freezing of faecal material at -20°C affects its bacterial composition. Bahl and colleagues highlighted that the ratio of *Bacteroidetes* to *Firmicutes* is affected by storage at -20°C {Bahl, 2012 #358}. In my 6 month study I aimed to determine if this observation was true and if so how long could a sample be stored before this ratio was affected. The results show that between 2 weeks and 3 months, the *Bacteroidetes* become severely depleted in the faecal samples. From this observation it is recommended to carry out DNA extractions immediately from fresh samples. If samples need to be stored, they should not be stored longer than 2 weeks at -20°C. Freezing samples also adds further complications as it has been shown that thawing of faecal samples can also affect its bacterial composition {Cardona, 2012 #422}. In order to avoid detrimentally affecting the bacterial composition of faecal material researchers may opt to freeze samples at -80°C. However, these freezers may not be available to those in other countries where sampling is taking place. These results have implications for those who aim to complete sampling prior to DNA extraction. Indeed, the effect of storage upon the bacterial community of a faecal sample may have implications already published studies. There is a growing body of evidence implicating the ratio of *Bacteroidetes* to *Firmicutes* in disorders such as obesity {Ley, 2005 #202}. However, questions need to be asked as to the method of storage of this faecal material. Are these observations truly biological or merely an artefact of the sampling methodology followed? Searching the NCBI PubMed database (<http://www.ncbi.nlm.nih.gov/pubmed>) with the following search terms “(((bacterial) AND faecal) AND DNA) AND extraction” gave 106 publications over the past decade. This stands to highlight the importance of bacterial DNA extraction in the field of biology. Researchers need to be aware of the effect of storage upon the bacterial community of faecal material. Inferred distal gut bacterial community community in humans, and animals also, can be affected by something as fundamental as the storage of this source of information.

7.2 Knowledge exchange from my research to Cultech (industrial partner)

Funding for the PROHEMI study came from an EU initiative, KESS, with funding from the ESF. This scholarship aims to develop links between SMEs, from convergence areas in Wales, and higher education institutions. PhD or Masters students have the opportunity to work with a company in their research area and to see how research is translated into viable product or services. The company receives research to bolster their products or services and have the opportunity to acquire research techniques and skills applicable for future research projects.

7.2.1 Community profiling of faecal DNA through LHPCR

The successful application of LHPCR in the PROHEMI study resulted in the adoption of the technique by Cultech. This method has been applied to screen faecal DNA obtained from babies following probiotic administration. Following this technique, samples of interest were subjected to NGS.

7.2.2 Functional media for faecal material and pure bacterial cultures screening

During the course of the PROHEMI study agar plates designed to screen for functions of interest were developed. One week was spent at the company in order to show how these plates were made. Subsequently, these plates have been used to screen other probiotic organisms the company has interest in and may be used for future research projects.

7.2.3 Multivariate statistical methods

The majority of statistical analysis carried out at Cultech was not multivariate. This was probably due to their previous preference and expertise in culture dependent methods, which rarely need multivariate analysis. However, as the company began to use more culture independent techniques, such as LHPCR and NGS, multivariate data analysis was needed. Using R statistical software {R-Core-Team, 2012 #210} custom scripts were developed in conjunction with Cultech in order to address their needs. Furthermore, time was spent showing how to use R, relevant packages and which techniques should be used in order to analyse their data.

7.2.4 Research into the effect of Cultech's probiotic supplement in healthy individuals

The project did not show many effects the company's probiotic supplement in healthy individuals. However, the research gives Cultech an advantage as their scientific portfolio has been bolstered by a long-term pilot feeding study. Regardless of the outcome, there is a benefit to the company from this research. The pilot study can give Cultech data to work with in order to calculate power and sample sizes for future feeding studies in healthy males. In addition, the EFSA has applied pressure upon producers of probiotics in recent years. Through its Nutrition and Health Claims Regulation (Regulation (EC) No 1924/2006) the EFSA aims to protect consumers from products with false health benefit claims or claims which simply do not have scientific evidence. It is therefore imperative for companies such as Cultech to carry out research into their products in order to ensure that they may sell without legislation impacting upon their sales.

7.3 General discussion

7.3.1 The value of the PROHEMI dataset

Many studies focus on the dysbiosis of the human gut microbiota in infirm and the effects from such imbalances. However, this pilot study has provided a useful data source on many parameters associated with healthy humans. Probiotic intervention aside, the PROHEMI study dataset has provided a wealth of information on healthy human males, by following them for a year. The baseline period served to provide information on the characteristics of the human gut and was continued throughout the intervention and washout periods. The application of other culture independent techniques, such as functional metagenomics, to this dataset could shed light onto functions expressed within the healthy human gut. Extracted DNA and faecal waters from the PROHEMI study can be stored for use with such techniques in the future.

7.3.2 Probiotic supplementation in healthy individuals

Probiotic intervention does not appear to impact upon the bacterial community of the distal gut in healthy human males. It therefore stands to reason that this community is relatively stable and resistant to modulation through probiotic feeding. During the intervention period, a reduction in protease expression in cultured bacteria was observed. Therefore, it is possible to surmise the probiotic organisms used in the PROHEMI study can modulate functions within the human gut without modifying the gross bacterial community. This seems advantageous as any detrimental effects exerted by the probiotic may be reversed upon cessation of its consumption.

Metabonomic profiles of PROHEMI participants were also unaffected by probiotic supplementation. Recent research has shown that probiotic supplementation with the VSL#3 preparation in diet-induced obese mice increased butyrate production {Yadav, 2013 #919}. This study shows probiotics have the potential to modulate gut metabolites. However, this study utilised a murine model which with an induced condition. Metabonomic analysis of samples from the PROHEMI showed no effect on gross metabonomic profiles or the profiles of SCFAs. It may be possible that in a similar fashion to gut bacterial modulation, probiotics do not exert effects in healthy hosts with an established gut microbiota.

7.3.3 Geographical differences in the gut microbiota, gut functions and metabonomic profiles of faecal waters

The PROHEMI study has given rise to an interesting observation; there appears to be a geographical difference in the gross bacterial community of individuals in the UK. Furthermore, there is also a difference in gut functions and the metabonomic profiles of PROHEMI participants from the two study centres. The differences observed suggest that this is a real biological phenomenon and not an artefact of lab differences in sample preparation. To my knowledge, there has not been a comparison of this kind between healthy individuals in the UK. Most research seems to focus on a single study centre; this is more than likely in effort to overcome study centre differences in results. However, a fundamental difference in the gut bacterial community, functions and metabonomic profiles has been overlooked. The concept of personalised medicine aims to provide personalised healthcare for a given individual based upon the genes that that person expresses. The importance of the gut microbiome in this field is becoming increasingly understood. Expression of bacterial enzymes and the metabolism of compounds by commensal gut bacteria may play a role in drug therapy. Differences in the gut bacterial community of individuals will differentially affect the metabolism of compounds in the gut. It therefore follows that individuals from Cardiff/Port Talbot and Sheffield could require completely different personalised healthcare programmes due to their difference in gut bacterial community.

7.3.5 The effect of freezing faecal material prior to DNA extraction

The observed loss of *Bacteroidetes* following the freezing of faecal samples at -20°C is of concern. This phylum is one of the most abundant in the human gut. However, my research has shown that storage of faecal material for 6 months at -20°C significantly decreases the abundance of this phylum. If researchers stored faecal samples at -20°C for this length of time or greater, the sample would no longer be a true representation of the distal gut community. The difference in the ratio of *Bacteroidetes* to *Firmicutes* was highlighted in my community fingerprint study; where Sheffield samples stored at -20°C for some time no longer resembled their initial LHPCR profiles (**Fig. 3.17**). The observation is of particular concern to those who have previously published data on the ratio of *Bacteroidetes* to *Firmicutes*. Researchers may not have been aware of the effect of storage upon the faecal microbial community. Samples may have been stored for some time prior to DNA extraction and analysis. If so observed differences in the ratio of *Bacteroidetes* to *Firmicutes* may not be a true biological difference but instead methodologically driven. Research into the effect of storage and its effects needs to be carried out as the use of faecal material as a proxy for the distal gut bacterial community is widespread. Storage method and DNA extraction method needs to be seriously considered by researchers in order to avoid masking true biological differences in the distal gut communities of samples.

7.4 Future research directions

7.4.1 qPCR of specific bacterial groups

While NGS and community profiling showed no difference in the gross bacterial community of individuals following probiotic supplementation, qPCR would strengthen this finding. It would also be advantageous to use this technique in order to strengthen the finding of differences in the ratio of *Bacteroidetes* to *Firmicutes* between Cardiff/Port Talbot and Sheffield samples.

7.4.2 Further metabonomic analysis of the PROHEMI samples

Further analysis into the metabonomic profiles of PROHEMI samples needs to be carried out. More sophisticated methods may give rise to differences between samples from the study periods. Furthermore, with regards to the observed differences in the metabonomic profiles of the two study centres, it may be possible to determine which metabolites are driving the separate clustering. My analysis looked at short chain fatty acids, it would be useful to include branched chain fatty acids and other metabolites also in future analyses.

7.4.3 Genotoxicity testing of a greater number of PROHEMI sample faecal waters

Analysis of more PROHEMI samples would allow independent study centre analysis of the effect of probiotic consumption on the genotoxicity of faecal water. Due to costs for commercial kits, I was not able to carry this out. Furthermore, inquiring into the diet of individuals would be useful as individual differences may be attributable to the diets of these individuals.

7.3.4 Further research into geographical differences in gut bacterial community and metabonomic profiles of healthy individuals in the UK

Studies which research differences in the gut bacterial community of individuals seem to compare individuals from disparate locations. Following my research, it would be of value to strengthen the notion that there are differences in the gut bacterial community and metabonomic profiles of individuals from locations across the UK. Further research would also shed light onto the reason behind this observed difference.

8. REFERENCES

- Abrahamsson, T. R., Jakobsson, H. E., Andersson, A. F., Björkstén, B., Engstrand, L. and Jenmalm, M. C.** (2012). Low diversity of the gut microbiota in infants with atopic eczema. *Journal of Allergy and Clinical Immunology* **129**:434-440.e432.
- Ait-Belgnaoui, A., Durand, H., Cartier, C., Chaumaz, G., Eutamene, H., Ferrier, L., Houdeau, E., Fioramonti, J., Bueno, L. and Theodorou, V.** (2012). Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology* **37**:1885-1895.
- Alander, M., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T. and Von Wright, A.** (1997). Recovery of *Lactobacillus rhamnosus* GG from human colonic biopsies. *Letters in Applied Microbiology* **24**:361-364.
- Allen, S. J., Wareham, K., Bradley, C., Harris, W., Dhar, A., Brown, H., Foden, A., Cheung, W. Y., Gravenor, M. B., Plummer, S., Phillips, C. J. and Mack, D.** (2012). A multicentre randomised controlled trial evaluating lactobacilli and bifidobacteria in the prevention of antibiotic-associated diarrhoea in older people admitted to hospital: the PLACIDE study protocol. *BMC Infectious diseases* **12**:108.
- Ames, B. N., McCann, J. and Yamasaki, E.** (1975). Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects* **31**:347-363.
- Antonopoulos, D. A., Huse, S. M., Morrison, H. G., Schmidt, T. M., Sogin, M. L. and Young, V. B.** (2009). Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infection and Immunity*. **77**:2367-2375.
- Arulampalam, V., Greicius, G. and Pettersson, S.** (2006). The long and winding road to gut homeostasis. *Current opinion in gastroenterology* **22**:349-353.
- Asano, Y., Hiramoto, T., Nishino, R., Aiba, Y., Kimura, T., Yoshihara, K., Koga, Y. and Sudo, N.** (2012). Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. *American Journal of Physiology - Gastrointestinal and Liver Physiology*.
- Avershina, E., Storro, O., Oien, T., Johnsen, R., Pope, P. and Rudi, K.** (2013). Major faecal microbiota shifts in composition and diversity with age in a geographically restricted cohort of mothers and their children. *FEMS Microbiology Ecology*.
- Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., Semenkovich, C. F. and Gordon, J. I.** (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America* **101**:15718-15723.
- Bahl, M. I., Bergström, A. and Licht, T. R.** (2012). Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. *FEMS Microbiology Letters* **329**:193-197.
- Balassiano, I. T., Yates, E. A., Domingues, R. M. C. P. and Ferreira, E. O.** (2012). *Clostridium difficile*: A problem of concern in developed countries and still a mystery in Latin America. *Journal of Medical Microbiology* **61**:169-179.
- Ballance, W. A., Dahms, B. B., Shenker, N. and Kliegman, R. M.** (1990). Pathology of neonatal necrotizing enterocolitis; A ten-year experience. *Journal of Pediatrics* **117**:S6-S13.

- Banks, P. A. and Freeman, M. L.** (2006). Practice guidelines in acute pancreatitis. *The American Journal of Gastroenterology* **101**:2379-2400.
- Barcenilla, A., Pryde, S. E., Martin, J. C., Duncan, S. H., Stewart, C. S., Henderson, C. and Flint, H. J.** (2000). Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Applied and Environmental Microbiology* **66**:1654-1661.
- Bartlett, J. G., Chang, T. E. W., Gurwith, M., Gorbach, S. L. and Onderdonk, A. B.** (1978). Antibiotic-associated pseudomembranous colitis due to toxin-producing Clostridia. *New England Journal of Medicine* **298**:531-534.
- Beckonert, O., Keun, H. C., Ebbels, T. M., Bundy, J., Holmes, E., Lindon, J. C. and Nicholson, J. K.** (2007). Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature protocols* **2**:2692-2703.
- Bentley, D. R., Balasubramanian, S., Swerdlow, H. P., Smith, G. P., Milton, J., Brown, C. G., Hall, K. P., Evers, D. J., Barnes, C. L., Bignell, H. R., Boutell, J. M., Bryant, J., Carter, R. J., Keira Cheetham, R., Cox, A. J., Ellis, D. J., Flatbush, M. R., Gormley, N. A., Humphray, S. J., Irving, L. J., Karbelashvili, M. S., Kirk, S. M., Li, H., Liu, X., Maisinger, K. S., Murray, L. J., Obradovic, B., Ost, T., Parkinson, M. L., Pratt, M. R., Rasolonjatovo, I. M., Reed, M. T., Rigatti, R., Rodighiero, C., Ross, M. T., Sabot, A., Sankar, S. V., Scally, A., Schroth, G. P., Smith, M. E., Smith, V. P., Spiridou, A., Torrance, P. E., Tzonev, S. S., Vermaas, E. H., Walter, K., Wu, X., Zhang, L., Alam, M. D., Anastasi, C., Aniebo, I. C., Bailey, D. M., Bancarz, I. R., Banerjee, S., Barbour, S. G., Baybayan, P. A., Benoit, V. A., Benson, K. F., Bevis, C., Black, P. J., Boodhun, A., Brennan, J. S., Bridgham, J. A., Brown, R. C., Brown, A. A., Buermann, D. H., Bundu, A. A., Burrows, J. C., Carter, N. P., Castillo, N., Chiara, E. C. M., Chang, S., Neil Cooley, R., Crake, N. R., Dada, O. O., Diakoumakos, K. D., Dominguez-Fernandez, B., Earnshaw, D. J., Egbujor, U. C., Elmore, D. W., Etchin, S. S., Ewan, M. R., Fedurco, M., Fraser, L. J., Fuentes Fajardo, K. V., Scott Furey, W., George, D., Gietzen, K. J., Goddard, C. P., Golda, G. S., Granieri, P. A., Green, D. E., Gustafson, D. L., Hansen, N. F., Harnish, K., Haudenschild, C. D., Heyer, N. I., Hims, M. M., Ho, J. T., Horgan, A. M., Hoschler, K., Hurwitz, S., Ivanov, D. V., Johnson, M. Q., James, T., Huw Jones, T. A., Kang, G. D., Kerelska, T. H., Kersey, A. D., Khrebtukova, I., Kindwall, A. P., Kingsbury, Z., Kokko-Gonzales, P. I., Kumar, A., Laurent, M. A., Lawley, C. T., Lee, S. E., Lee, X., Liao, A. K., Loch, J. A., Lok, M., Luo, S., Mammen, R. M., Martin, J. W., McCauley, P. G., McNitt, P., Mehta, P., Moon, K. W., Mullens, J. W., Newington, T., Ning, Z., Ling Ng, B., Novo, S. M., O'Neill, M. J., Osborne, M. A., Osnowski, A., Ostadan, O., Paraschos, L. L., Pickering, L., Pike, A. C., Pike, A. C., Chris Pinkard, D., Pliskin, D. P., Podhasky, J., Quijano, V. J., Raczky, C., Rae, V. H., Rawlings, S. R., Chiva Rodriguez, A., Roe, P. M., Rogers, J., Rogert Bacigalupo, M. C., Romanov, N., Romieu, A., Roth, R. K., Rourke, N. J., Ruediger, S. T., Rusman, E., Sanches-Kuiper, R. M., Schenker, M. R., Seoane, J. M., Shaw, R. J., Shiver, M. K., Short, S. W., Sizto, N. L., Sluis, J. P., Smith, M. A., Ernest Sohna Sohna, J., Spence, E. J., Stevens, K., Sutton, N., Szajkowski, L., Tregidgo, C. L., Turcatti, G., Vandevondele, S., Verhovskiy, Y., Virk, S. M., Wakelin, S., Walcott, G. C., Wang, J., Worsley, G. J., Yan, J., Yau, L., Zuerlein, M., Rogers, J., Mullikin, J. C., Hurler, M. E., McCooke, N. J., West, J. S., Oaks, F. L., Lundberg, P. L., Klenerman, D., Durbin, R. and Smith, A. J.** (2008). Accurate whole human genome sequencing using reversible terminator chemistry. *Nature* **456**:53-59.
- Besselink, M. G., van Santvoort, H. C., Buskens, E., Boermeester, M. A., van Goor, H., Timmerman, H. M., Nieuwenhuijs, V. B., Bollen, T. L., van Ramshorst, B., Witterman, B. J., Rosman, C., Ploeg, R. J., Brink, M. A., Schaapherder, A. F., Dejong, C. H., Wahab, P. J., van Laarhoven, C. J., van der Harst, E., van Eijck, C. H., Cuesta, M. A., Akkermans, L. M. and Gooszen, H. G.** (2008). Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. *The Lancet* **371**:651-659.

- Bin-Nun, A., Bromiker, R., Wilschanski, M., Kaplan, M., Rudensky, B., Caplan, M. and Hammerman, C.** (2005). Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. *Journal of Pediatrics* **147**:192-196.
- Bjerketorp, J., Ng Tze Chiang, A., Hjort, K., Rosenquist, M., Liu, W.-T. and Jansson, J. K.** (2008). Rapid lab-on-a-chip profiling of human gut bacteria. *Journal of Microbiological Methods* **72**:82-90.
- Brasili, E., Mengheri, E., Tomassini, A., Capuani, G., Roselli, M., Finamore, A., Sciubba, F., Marini, F. and Miccheli, A.** (2013). *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* BB12 induce different age-related metabolic profiles revealed by 1H-NMR spectroscopy in urine and feces of mice1-3. *Journal of Nutrition* **143**:1549-1557.
- Brezski, R. J. and Jordan, R. E.** (2010). Cleavage of IgGs by proteases associated with invasive diseases: an evasion tactic against host immunity? *mAbs* **2**:212-220.
- Brigidi, P., Vitali, B., Swennen, E., Bazzocchi, G. and Matteuzzi, D.** (2001). Effects of probiotic administration upon the composition and enzymatic activity of human fecal microbiota in patients with irritable bowel syndrome or functional diarrhea. *Research in Microbiology* **152**:735-741.
- British Heart Foundation.** (2013). Cardiovascular disease [Online]. British Heart Foundation. Available at: <http://www.bhf.org.uk/heart-health/conditions/cardiovascular-disease.aspx> [Accessed: 16/09/2013].
- Brusetti, L., Borin, S., Mora, D., Rizzi, A., Raddadi, N., Sorlini, C. and Daffonchio, D.** (2006). Usefulness of length heterogeneity-PCR for monitoring lactic acid bacteria succession during maize ensiling. *FEMS Microbiology Ecology* **56**:154-164.
- Burkholder, P. R. and McVeigh, I.** (1942). Synthesis of vitamins by intestinal bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **28**:285-289.
- Burns, A. J. and Rowland, I. R.** (2004). Antigenotoxicity of probiotics and prebiotics on faecal water-induced DNA damage in human colon adenocarcinoma cells. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **551**:233-243.
- Cannon, B. and Nedergaard, J. A. N.** (2004). Brown adipose tissue: Function and physiological significance. *Physiological Reviews* **84**:277-359.
- Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G. and Knight, R.** (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal* **6**:1621-1624.
- Carcer, D. A., Denman, S. E., McSweeney, C. and Morrison, M.** (2011). Evaluation of subsampling-based normalization strategies for tagged high-throughput sequencing data sets from gut microbiomes. *Applied and Environmental Microbiology* **77**:8795-8798.
- Cardona, S., Eck, A., Cassellas, M., Gallart, M., Alastrue, C., Dore, J., Azpiroz, F., Roca, J., Guarner, F. and Manichanh, C.** (2012). Storage conditions of intestinal microbiota matter in metagenomic analysis. *BMC Microbiology* **12**:158.
- Carriere, F., Barrowman, J. A., Verger, R. and Laugier, R.** (1993). Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology* **105**:876-888.
- Carroll, I. M., Ringel-Kulka, T., Siddle, J. P., Klaenhammer, T. R. and Ringel, Y.** (2012). Characterization of the fecal microbiota using high-throughput sequencing reveals a stable microbial community during storage. *PLoS ONE* **7**:e46953.

- Cenac, N., Andrews, C. N., Holzhausen, M., Chapman, K., Cottrell, G., Andrade-Gordon, P., Steinhoff, M., Barbara, G., Beck, P., Bunnett, N. W., Sharkey, K. A., Ferraz, J. G., Shaffer, E. and Vergnolle, N. (2007). Role for protease activity in visceral pain in irritable bowel syndrome. *Journal of Clinical Investigation* **117**:636-647.
- Chakravorty, S., Helb, D., Burday, M., Connell, N. and Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Journal of Microbiological Methods* **69**:330-339.
- Christensen, H. R., Larsen, C. N., Kæstel, P., Rosholm, L. B., Sternberg, C., Michaelsen, K. F. and Frøkiær, H. (2006). Immunomodulating potential of supplementation with probiotics: A dose-response study in healthy young adults. *FEMS Immunology and Medical Microbiology* **47**:380-390.
- Chung, H., Pamp, S. J., Hill, J. A., Surana, N. K., Edelman, S. M., Troy, E. B., Reading, N. C., Villablanca, E. J., Wang, S., Mora, J. R., Umesaki, Y., Mathis, D., Benoist, C., Relman, D. A. and Kasper, D. L. (2012). Gut immune maturation depends on colonization with a host-specific microbiota. *Cell* **149**:1578-1593.
- Collins, A. R. (2004). The comet assay for DNA damage and repair: principles, applications, and limitations. *Molecular Biotechnology* **26**:249-261.
- Corr, S. C., Hill, C. and Gahan, C. G. M. (2009). Chapter 1 Understanding the Mechanisms by Which Probiotics Inhibit Gastrointestinal Pathogens. In: Steve, L.T. (ed.) *Advances in Food and Nutrition Research*. Vol. Volume 56. Academic Press, pp. 1-15.
- Corr, S. C., Li, Y., Riedel, C. U., O'Toole, P. W., Hill, C. and Gahan, C. G. M. (2007). Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. *Proceedings of the National Academy of Sciences of the United States of America* **104**:7617-7621.
- Crosby, L. D. and Criddle, C. S. (2003). Understanding bias in microbial community analysis techniques due to *rrn* operon copy number heterogeneity. *Biotechniques* **34**:790-794.
- Cui, Q., Lewis, I. A., Hegeman, A. D., Anderson, M. E., Li, J., Schulte, C. F., Westler, W. M., Eghbalnia, H. R., Sussman, M. R. and Markley, J. L. (2008). Metabolite identification via the Madison Metabolomics Consortium Database. *Nature Biotechnology* **26**:162-164.
- Culligan, E. P., Marchesi, J. R., Hill, C. and Sleator, R. D. (2012). Mining the human gut microbiome for novel stress resistance genes. *Gut Microbes* **3**:394-397.
- David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J. and Turnbaugh, P. J. (2013). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**:559-563.
- De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., Collini, S., Pieraccini, G. and Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences* **107**:14691-14696.
- de Lorenzo, V. (2005). Problems with metagenomic screening. *Nature Biotechnology* **23**:1045-1045.
- De Man, J. C., Rogosa, M. and Sharpe, M. E. (1960). A medium for the cultivation of Lactobacilli. *Journal of Applied Microbiology* **23**:130-135.
- De Moreno De LeBlanc, A. and Perdígón, G. (2005). Reduction of β -glucuronidase and nitroreductase activity by yoghurt in a murine colon cancer model. *Biocell* **29**:15-24.

- de Sablet, T., Chassard, C., Bernalier-Donadille, A., Vareille, M., Gobert, A. P. and Martin, C. (2009). Human microbiota-secreted factors inhibit shiga toxin synthesis by enterohemorrhagic *Escherichia coli* O157:H7. *Infection and Immunity* **77**:783-790.
- de Vrese, M., Stegelmann, A., Richter, B., Fenselau, S., Laue, C. and Schrezenmeir, J. (2001). Probiotics—compensation for lactase insufficiency. *The American Journal of Clinical Nutrition* **73**:421s-429s.
- Deshpande, G., Rao, S., Patole, S. and Bulsara, M. (2010). Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. *Pediatrics* **125**:921-930.
- Dethlefsen, L., Huse, S., Sogin, M. L. and Relman, D. A. (2008). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biology* **6**:e280.
- Dicksved, J., Flöistrup, H., Bergström, A., Rosenquist, M., Pershagen, G., Scheynius, A., Roos, S., Alm, J. S., Engstrand, L., Braun-Fahrländer, C., Von Mutius, E. and Jansson, J. K. (2007). Molecular fingerprinting of the fecal microbiota of children raised according to different lifestyles. *Applied and Environmental Microbiology* **73**:2284-2289.
- Duncan, S. H., Belenguer, A., Holtrop, G., Johnstone, A. M., Flint, H. J. and Lobley, G. E. (2007). Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Applied and Environmental Microbiology* **73**:1073-1078.
- Duncan, S. H., Lobley, G. E., Holtrop, G., Ince, J., Johnstone, A. M., Louis, P. and Flint, H. J. (2008). Human colonic microbiota associated with diet, obesity and weight loss. *International Journal of Obesity* **32**:1720-1724.
- Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. and Relman, D. A. (2005). Diversity of the Human Intestinal Microbial Flora. *Science* **308**:1635-1638.
- Ellis, C. N., Drake, L. A., Prendergast, M. M., Abramovits, W., Boguniewicz, M., Daniel, C. R., Lebwohl, M., Stevens, S. R., Whitaker-Worth, D. L., Cheng, J. W. and Tong, K. B. (2002). Cost of atopic dermatitis and eczema in the United States. *Journal of the American Academy of Dermatology* **46**:361-370.
- Ellis, R. J., Morgan, P., Weightman, A. J. and Fry, J. C. (2003). Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Applied and Environmental Microbiology* **69**:3223-3230.
- Englyst, H. N., Hay, S. and Macfarlane, G. T. (1987). Polysaccharide breakdown by mixed populations of human faecal bacteria. *FEMS Microbiology Letters* **45**:163-171.
- Eusebio, A., Tacao, M., Chaves, S., Tenreiro, R. and Almeida-Vara, E. (2011). Molecular assessment of microbiota structure and dynamics along mixed olive oil and winery wastewaters biotreatment. *Biodegradation* **22**:773-795.
- Evans, J. M., Morris, L. S. and Marchesi, J. (2013). The gut microbiome: The role of a virtual organ in the endocrinology of the host. *Journal of Endocrinology*.
- Fairbairn, D. W., Olive, P. L. and O'Neill, K. L. (1995). The comet assay: A comprehensive review. *Mutation Research - Reviews in Genetic Toxicology* **339**:37-59.
- Finegold, S. M. (2008). Therapy and epidemiology of autism-clostridial spores as key elements. *Medical Hypotheses* **70**:508-511.

- Fischer, S. G. and Lerman, L. S.** (1983). DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proceedings of the National Academy of Sciences of the United States of America* **80**:1579-1583.
- Fisher, M. M. and Triplett, E. W.** (1999). Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Applied and Environmental Microbiology*. **65**:4630-4636.
- Fleissner, C. K., Huebel, N., Abd El-Bary, M. M., Loh, G., Klaus, S. and Blaut, M.** (2010). Absence of intestinal microbiota does not protect mice from diet-induced obesity. *British Journal of Nutrition* **104**:919-929.
- Fogh, J., Fogh, J. M. and Orfeo, T.** (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *Journal of the National Cancer Institute* **59**:221-226.
- Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J., Munoz-Quezada, S. and Gil, A.** (2013). Sources, isolation, characterisation and evaluation of probiotics. *British Journal of Nutrition* **109 Suppl 2**:S35-50.
- Frias-Lopez, J., Shi, Y., Tyson, G. W., Coleman, M. L., Schuster, S. C., Chisholm, S. W. and DeLong, E. F.** (2008). Microbial community gene expression in ocean surface waters. *Proceedings of the National Academy of Sciences of the United States of America* **105**:3805-3810.
- Fujimori, S., Tatsuguchi, A., Gudis, K., Kishida, T., Mitsui, K., Ehara, A., Kobayashi, T., Sekita, Y., Seo, T. and Sakamoto, C.** (2007). High dose probiotic and prebiotic cotherapy for remission induction of active Crohn's disease. *Journal of Gastroenterology and Hepatology* **22**:1199-1204.
- Fung, T. T., Rimm, E. B., Spiegelman, D., Rifai, N., Tofler, G. H., Willett, W. C. and Hu, F. B.** (2001). Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk. *American Journal of Clinical Nutrition* **73**:61-67.
- Furrie, E., Macfarlane, S., Kennedy, A., Cummings, J. H., Walsh, S. V., O'Neil, D. A. and Macfarlane, G. T.** (2005). Synbiotic therapy (*Bifidobacterium longum*/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: A randomised controlled pilot trial. *Gut* **54**:242-249.
- Gaujoux, R. and Seoighe, C.** (2010). A flexible R package for nonnegative matrix factorization. *BMC Bioinformatics* **11**:367.
- Gehring, U., Bolte, G., Borte, M., Bischof, W., Fahlbusch, B., Wichmann, H. E. and Heinrich, J.** (2001). Exposure to endotoxin decreases the risk of atopic eczema in infancy: a cohort study. *Journal of Allergy and Clinical Immunology* **108**:847-854.
- Gibson, G. R. and Roberfroid, M. B.** (1995). Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *Journal of Nutrition* **125**:1401-1412.
- Gillevet, P., Sikaroodi, M., Keshavarzian, A. and Mutlu, E. A.** (2010). Quantitative Assessment of the Human Gut Microbiome Using Multitag Pyrosequencing. *Chemistry & Biodiversity* **7**:1065-1075.
- Gionchetti, P., Rizzello, F., Helwig, U., Venturi, A., Lammers, K. M., Brigidi, P., Vitali, B., Poggioli, G., Miglioli, M. and Campieri, M.** (2003). Prophylaxis of pouchitis onset with probiotic therapy: A double-blind, placebo-controlled trial. *Gastroenterology* **124**:1202-1209.

- Glinghammar, B., Venturi, M., Rowland, I. R. and Rafter, J. J.** (1997). Shift from a dairy product-rich to a dairy product-free diet: influence on cytotoxicity and genotoxicity of fecal water--potential risk factors for colon cancer. *The American Journal of Clinical Nutrition* **66**:1277-1282.
- Glöckner, F. O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A. and Amann, R.** (2000). Comparative 16S *rRNA* analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of *Actinobacteria*. *Applied and Environmental Microbiology* **66**:5053-5065.
- Graffelman, J.** (2012). calibrate: Calibration of Scatterplot and Biplot Axes. *R package version 1.7.1*.
- Guarner, F., Ellen Sanders, M., Gibson, G., Klaenhammer, T., Cabana, M., Scott, K., Reid, G., Delzenne, N. M., Fahey, G. C. and Hill, C.** (2011). Probiotic and prebiotic claims in Europe: seeking a clear roadmap. *British Journal of Nutrition* **106**:1765-1767.
- Guslandi, M., Giollo, P. and Testoni, P. A.** (2003). A pilot trial of *Saccharomyces boulardii* in ulcerative colitis. *European Journal of Gastroenterology and Hepatology* **15**:697-698.
- Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., Ciulla, D., Tabbaa, D., Highlander, S. K., Sodergren, E., Methé, B., DeSantis, T. Z., The Human Microbiome, C., Petrosino, J. F., Knight, R. and Birren, B. W.** (2011). Chimeric 16S *rRNA* sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Research* **21**:494-504.
- Hall, T. A.** (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**:95 - 98.
- Heaton, K.** (1999). The Bristol stool form scale. *Understanding your bowels*. London: Family Doctor Publications in association with the British Medical Association.
- Hennequin, C., Kauffmann-Lacroix, C., Jobert, A., Viard, J. P., Ricour, C., Jacquemin, J. L. and Berche, P.** (2000). Possible role of catheters in *Saccharomyces boulardii* Fungemia. *European Journal of Clinical Microbiology and Infectious Diseases* **19**:16-20.
- Henry, M. C. W. and Moss, R. L.** (2009). Necrotizing enterocolitis. *Annual Review of Medicine* **60**:111-124.
- Hickson, M., D'Souza, A. L., Muthu, N., Rogers, T. R., Want, S., Rajkumar, C. and Bulpitt, C. J.** (2007). Use of probiotic *Lactobacillus* preparation to prevent diarrhoea associated with antibiotics: Randomised double blind placebo controlled trial. *British Medical Journal* **335**:80-83.
- Hong, Y.-S., Ahn, Y.-T., Park, J.-C., Lee, J.-H., Lee, H., Huh, C.-S., Kim, D.-H., Ryu, D. and Hwang, G.-S.** (2010). ¹H NMR-based metabonomic assessment of probiotic effects in a colitis mouse model. *Archives of Pharmacal Research* **33**:1091-1101.
- Hughes, K. L.** (1972). Recent knowledge of the strict anaerobes of the gut. *Australian veterinary journal* **48**:508-514.
- Hughes, R., Magee, E. A. and Bingham, S.** (2000). Protein degradation in the large intestine: relevance to colorectal cancer. *Current Issues in Intestinal Microbiology* **1**:51-58.
- Huisman, O. and D'Ari, R.** (1981). An inducible DNA replication-cell division coupling mechanism in *E. coli*. *Nature* **290**:797-799.

- Humblot, C., Murkovic, M., Rigottier-Gois, L., Bensaada, M., Bouclet, A., Andrieux, C., Anba, J. and Rabot, S.** (2007). β -Glucuronidase in human intestinal microbiota is necessary for the colonic genotoxicity of the food-borne carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline in rats. *Carcinogenesis* **28**:2419-2425.
- Jaccard, P.** (1912). The distribution of the flora in the alpine zone. *New Phytologist* **11**:37-50.
- Jaeger, K. E., Ransac, S., Dijkstra, B. W., Colson, C., Van Heuvel, M. and Misset, O.** (1994). Bacterial lipases. *FEMS Microbiology Reviews* **15**:29-63.
- Janda, J. M. and Abbott, S. L.** (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology* **45**:2761-2764.
- Janssen, P. H.** (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and Environmental Microbiology* **72**:1719-1728.
- Jernberg, C., Lofmark, S., Edlund, C. and Jansson, J. K.** (2007). Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME Journal* **1**:56-66.
- Jeun, J., Kim, S., Cho, S. Y., Jun, H. j., Park, H. J., Seo, J. G., Chung, M. J. and Lee, S. J.** (2010). Hypocholesterolemic effects of *Lactobacillus plantarum* KCTC3928 by increased bile acid excretion in C57BL/6 mice. *Nutrition* **26**:321-330.
- Jia, X. and Xiao, W.** (2004). Assessing DNA damage using a reporter gene system. In: Yan, Z. and Caldwell, G.W. (eds.) *Optimization in Drug Discovery*. Humana Press, pp. 315-323.
- Jia, X., Zhu, Y. and Xiao, W.** (2002). A stable and sensitive genotoxic testing system based on DNA damage induced gene expression in *Saccharomyces cerevisiae*. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **519**:83-92.
- Jones, B. V., Begley, M., Hill, C., Gahan, C. G. and Marchesi, J. R.** (2008). Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proceedings of the National Academy of Sciences of the United States of America* **105**:13580-13585.
- Jones, B. V., Sun, F. and Marchesi, J. R.** (2007a). Using skimmed milk agar to functionally screen a gut metagenomic library for proteases may lead to false positives. *Letters in Applied Microbiology* **45**:418-420.
- Kajander, K., Hatakka, K., Poussa, T., Färkkilä, M. and Korpela, R.** (2005). A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: A controlled 6-month intervention. *Alimentary Pharmacology and Therapeutics* **22**:387-394.
- Kim, H. J., Camilleri, M., McKinzie, S., Lempke, M. B., Burton, D. D., Thomforde, G. M. and Zinsmeister, A. R.** (2003). A randomized controlled trial of a probiotic, VSL#3, on gut transit and symptoms in diarrhoea-predominant irritable bowel syndrome. *Alimentary Pharmacology and Therapeutics* **17**:895-904.
- Kim, H. J., Vazquez Roque, M. I., Camilleri, M., Stephens, D., Burton, D. D., Baxter, K., Thomforde, G. and Zinsmeister, A. R.** (2005). A randomized controlled trial of a probiotic combination VSL# 3 and placebo in irritable bowel syndrome with bloating. *Neurogastroenterology and Motility* **17**:687-696.
- Kindt, R. and Coe, R.** (2005). Tree diversity analysis A manual and software for common statistical methods for ecological and biodiversity studies. Nairobi: World Agroforestry Centre (ICRAF).

- Kirk, J. L., Beaudette, L. A., Hart, M., Moutoglis, P., Klironomos, J. N., Lee, H. and Trevors, J. T. (2004). Methods of studying soil microbial diversity. *Journal of Microbiological Methods* **58**:169-188.
- Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., Angenent, L. T. and Ley, R. E. (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences* **108**:4578-4585.
- Kopp, M. V., Hennemuth, I., Heinzmann, A. and Urbanek, R. (2008). Randomized, double-blind, placebo-controlled trial of probiotics for primary prevention: No clinical effects of *Lactobacillus* GG supplementation. *Pediatrics* **121**:e850-e856.
- Kruis, W., Frič, P., Pokrotnieks, J., Lukáš, M., Fixa, B., Kaščák, M., Kamm, M. A., Weismueller, J., Beglinger, C., Stolte, M., Wolff, C. and Schulze, J. (2004). Maintaining remission of ulcerative colitis with the probiotic *Escherichia coli* Nissle 1917 is as effective as with standard mesalazine. *Gut* **53**:1617-1623.
- Kuisma, J., Mentula, S., Jarvinen, H., Kahri, A., Saxelin, M. and Farkkila, M. (2003). Effect of *Lactobacillus rhamnosus* GG on ileal pouch inflammation and microbial flora. *Alimentary Pharmacology and Therapeutics* **17**:509-515.
- Kukkonen, K., Savilahti, E., Haahtela, T., Juntunen-Backman, K., Korpela, R., Poussa, T., Tuure, T. and Kuitunen, M. (2007). Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: A randomized, double-blind, placebo-controlled trial. *Journal of Allergy and Clinical Immunology* **119**:192-198.
- Kunz, A. N., Noel, J. M. and Fairchok, M. P. (2004). Two cases of *Lactobacillus* bacteremia during probiotic treatment of short gut syndrome. *Journal of Pediatric Gastroenterology and Nutrition* **38**:457-458.
- Lahtinen, S. J., Tammela, L., Korpela, J., Parhiala, R., Ahokoski, H., Mykkanen, H. and Salminen, S. J. (2009). Probiotics modulate the *Bifidobacterium* microbiota of elderly nursing home residents. *Age (Dordr)* **31**:59-66.
- Land, M. H., Rouster-Stevens, K., Woods, C. R., Cannon, M. L., Cnota, J. and Shetty, A. K. (2005). *Lactobacillus* sepsis associated with probiotic therapy. *Pediatrics* **115**:178-181.
- Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., Clemente, J. C., Burkepille, D. E., Vega Thurber, R. L., Knight, R., Beiko, R. G. and Huttenhower, C. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology* **31**:814-821.
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**:2947-2948.
- Larsen, C. N., Nielsen, S., Kæstel, P., Brockmann, E., Bennedsen, M., Christensen, H. R., Eskesen, D. C., Jacobsen, B. L. and Michaelsen, K. F. (2006). Dose-response study of probiotic bacteria *Bifidobacterium animalis* subsp *lactis* BB-12 and *Lactobacillus paracasei* subsp *paracasei* CRL-341 in healthy young adults. *European Journal of Clinical Nutrition* **60**:1284-1293.
- Larsen, N., Vogensen, F. K., Gøbel, R. J., Michaelsen, K. F., Forssten, S. D., Lahtinen, S. J. and Jakobsen, M. (2013). Effect of *Lactobacillus salivarius* Ls-33 on fecal microbiota in obese adolescents. *Clinical Nutrition* **32**:935-940.

- Larsen, N., Vogensen, F. K., van den Berg, F. W. J., Nielsen, D. S., Andreasen, A. S., Pedersen, B. K., Al-Soud, W. A., Sørensen, S. J., Hansen, L. H. and Jakobsen, M. (2010). Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLoS ONE* **5**:e9085.
- Larue, R., Yu, Z., Parisi, V. A., Egan, A. R. and Morrison, M. (2005). Novel microbial diversity adherent to plant biomass in the herbivore gastrointestinal tract, as revealed by ribosomal intergenic spacer analysis and rrs gene sequencing. *Environmental Microbiology* **7**:530-543.
- Latiano, A., Palmieri, O., Pastorelli, L., Vecchi, M., Pizarro, T. T., Bossa, F., Merla, G., Augello, B., Latiano, T., Corritore, G., Settesoldi, A., Valvano, M. R., D'Incà, R., Stronati, L., Annese, V. and Andriulli, A. (2013). Associations between genetic polymorphisms in *IL-33*, *IL1R1* and risk for inflammatory bowel disease. *PLoS ONE* **8**:e62144.
- Lederberg, J. (2000). Infectious History. *Science* **288**:287-293.
- Lee, J., Seto, D. and Bielory, L. (2008). Meta-analysis of clinical trials of probiotics for prevention and treatment of pediatric atopic dermatitis. *Journal of Allergy and Clinical Immunology* **121**:116-121.e111.
- Letunic, I. and Bork, P. (2011). Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Research* **39**:W475-W478.
- Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. and Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America* **102**:11070-11075.
- Ley, R. E., Peterson, D. A. and Gordon, J. I. (2006a). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* **124**:837-848.
- Ley, R. E., Turnbaugh, P. J., Klein, S. and Gordon, J. I. (2006b). Microbial ecology: Human gut microbes associated with obesity. *Nature* **444**:1022-1023.
- Lherm, T., Monet, C., Nougère, B., Soulier, M., Larbi, D., Le Gall, C., Caen, D. and Malbrunot, C. (2002). Seven cases of fungemia with *Saccharomyces boulardii* in critically ill patients. *Intensive Care Medicine* **28**:797-801.
- Lin, A., Bik, E. M., Costello, E. K., Dethlefsen, L., Haque, R., Relman, D. A. and Singh, U. (2013). Distinct distal gut microbiome diversity and composition in healthy children from Bangladesh and the United States. *PLoS ONE* **8**:e53838.
- Lin, H. C., Su, B. H., Chen, A. C., Lin, T. W., Tsai, C. H., Yeh, T. F. and Oh, W. (2005). Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. *Pediatrics* **115**:1-4.
- Longstreth, G. F., Thompson, W. G., Chey, W. D., Houghton, L. A., Mearin, F. and Spiller, R. C. (2006). Functional bowel disorders. *Gastroenterology* **130**:1480-1491.
- Lozupone, C. A., Hamady, M., Kelley, S. T. and Knight, R. (2007). Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology* **73**:1576-1585.
- Lozupone, C. and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**:8228-8235.
- Macfarlane, S., Cleary, S., Bahrami, B., Reynolds, N. and Macfarlane, G. T. (2013). Synbiotic consumption changes the metabolism and composition of the gut microbiota in older people and modifies inflammatory processes: A randomised, double-blind, placebo-controlled crossover study. *Alimentary Pharmacology and Therapeutics* **38**:804-816.

- Macho Fernandez, E., Pot, B. and Grangette, C.** (2011). Beneficial effect of probiotics in IBD: Are peptidoglycan and NOD2 the molecular key effectors? *Gut Microbes* **2**:280-286.
- Maechler, M., Rousseeuw, P., Struyf, A. and Hubert, M.** (2005). Cluster analysis basics and extensions.
- Mahenthiralingam, E., Marchbank, A., Drevinek, P., Garaiova, I. and Plummer, S.** (2009). Use of colony-based bacterial strain typing for tracking the fate of *Lactobacillus* strains during human consumption. *BMC Microbiology* **9**:251.
- Mändar, R. and Mikelsaar, M.** (1996). Transmission of mother's microflora to the newborn at birth. *Neonatology* **69**:30-35.
- Männistö, M. K., Tirola, M. and Häggblom, M. M.** (2009). Effect of freeze-thaw cycles on bacterial communities of Arctic tundra soil. *Microbial Ecology* **58**:621-631.
- Marchesi, J. R., Holmes, E., Khan, F., Kochhar, S., Scanlan, P., Shanahan, F., Wilson, I. D. and Wang, Y.** (2007). Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. *Journal of Proteome Research* **6**:546-551.
- Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., Berka, J., Braverman, M. S., Chen, Y.-J., Chen, Z., Dewell, S. B., Du, L., Fierro, J. M., Gomes, X. V., Godwin, B. C., He, W., Helgesen, S., Ho, C. H., Irzyk, G. P., Jando, S. C., Alenquer, M. L. I., Jarvie, T. P., Jirage, K. B., Kim, J.-B., Knight, J. R., Lanza, J. R., Leamon, J. H., Lefkowitz, S. M., Lei, M., Li, J., Lohman, K. L., Lu, H., Makhijani, V. B., McDade, K. E., McKenna, M. P., Myers, E. W., Nickerson, E., Nobile, J. R., Plant, R., Puc, B. P., Ronan, M. T., Roth, G. T., Sarkis, G. J., Simons, J. F., Simpson, J. W., Srinivasan, M., Tartaro, K. R., Tomasz, A., Vogt, K. A., Volkmer, G. A., Wang, S. H., Wang, Y., Weiner, M. P., Yu, P., Begley, R. F. and Rothberg, J. M.** (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**:376-380.
- Mariat, D., Firmesse, O., Levenez, F., Guimaraes, V. D., Sokol, H., Dore, J., Corthier, G. and Furet, J. P.** (2009). The *Firmicutes/Bacteroidetes* ratio of the human microbiota changes with age. *BMC Microbiology* **9**:123.
- Marteau, P., Lémann, M., Seksik, P., Laharie, D., Colombel, J. F., Bouhnik, Y., Cadiot, G., Soulé, J. C., Bourreille, A., Metman, E., Lerebours, E., Carbonnel, F., Dupas, J. L., Veyrac, M., Coffin, B., Moreau, J., Abitbol, V., Blum-Sperisen, S. and Mary, J. Y.** (2006). Ineffectiveness of *Lactobacillus johnsonii* LA1 for prophylaxis of postoperative recurrence in Crohn's disease: A randomised, double blind, placebo controlled GETAID trial. *Gut* **55**:842-847.
- Marteau, P., Pochart, P., Flourié, B., Pellier, P., Santos, L., Desjeux, J. F. and Rambaud, J. C.** (1990). Effect of chronic ingestion of a fermented dairy product containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on metabolic activities of the colonic flora in humans. *The American Journal of Clinical Nutrition* **52**:685-688.
- Martin, F. P., Wang, Y., Sprenger, N., Yap, I. K., Lundstedt, T., Lek, P., Rezzi, S., Ramadan, Z., van Bladeren, P., Fay, L. B., Kochhar, S., Lindon, J. C., Holmes, E. and Nicholson, J. K.** (2008). Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. *Molecular Systems Biology* **4**:157.
- Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D. and Verstraete, W.** (2008). How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environmental Microbiology* **10**:1571 - 1581.

- Maukonen, J., Simões, C. and Saarela, M.** (2012). The currently used commercial DNA-extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples. *FEMS Microbiology Ecology* **79**:697-708.
- McBride, M. J., Xie, G., Martens, E. C., Lapidus, A., Henrissat, B., Rhodes, R. G., Goltsman, E., Wang, W., Xu, J., Hunnicutt, D. W., Staroscik, A. M., Hoover, T. R., Cheng, Y.-Q. and Stein, J. L.** (2009). Novel features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. *Applied and Environmental Microbiology* **75**:6864-6875.
- McFarland, L. V.** (2006). Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of *Clostridium difficile* disease. *American Journal of Gastroenterology* **101**:812-822.
- McGuire, B. B., Brannigan, A. E. and O'Connell, P. R.** (2007). Ileal pouch–anal anastomosis. *British Journal of Surgery* **94**:812-823.
- McOrist, A. L., Jackson, M. and Bird, A. R.** (2002). A comparison of five methods for extraction of bacterial DNA from human faecal samples. *Journal of Microbiological Methods* **50**:131-139.
- Miller, J. H.** (1972). Assay of β -galactosidase. *Experiments in Molecular Genetics*. New York: Cold Spring Harbor, pp. p322-355.
- Mimura, T., Rizzello, F., Helwig, U., Poggioli, G., Schreiber, S., Talbot, I. C., Nicholls, R. J., Gionchetti, P., Campieri, M. and Kamm, M. A.** (2004). Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* **53**:108-114.
- Mizusawa, S., Court, D. and Gottesman, S.** (1983). Transcription of the *sulA* gene and repression by LexA. *J Mol Biol* **171**:337-343.
- Møller, P. L., Jørgensen, F., Hansen, O. C., Madsen, S. M. and Stougaard, P.** (2001). Intra- and extracellular β -galactosidases from *Bifidobacterium bifidum* and *B. infantis*: Molecular cloning, heterologous expression, and comparative characterization. *Applied and Environmental Microbiology* **67**:2276-2283.
- Morris, L. S., Evans, J. and Marchesi, J. R.** (2012). A robust plate assay for detection of extracellular microbial protease activity in metagenomic screens and pure cultures. *Journal of Microbiological Methods* **91**:144-146.
- Murashima, K., Chen, C.-L., Kosugi, A., Tamaru, Y., Doi, R. H. and Wong, S.-L.** (2002). Heterologous production of *Clostridium cellulovorans* engB, using protease-deficient *Bacillus subtilis*, and preparation of active recombinant cellulosomes. *Journal of Bacteriology* **184**:76-81.
- Mutlu, E. A., Gillevet, P. M., Rangwala, H., Sikaroodi, M., Naqvi, A., Engen, P. A., Kwasny, M., Lau, C. K. and Keshavarzian, A.** (2012). Colonic microbiome is altered in alcoholism. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **302**:G966-G978.
- Muyzer, G., de Waal, E. C. and Uitterlinden, A. G.** (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**:695-700.
- Neuwirth, E.** (2007). RColorBrewer: ColorBrewer palettes. R package version 1.0-2.

- Ng, S. C., Bernstein, C. N., Vatn, M. H., Lakatos, P. L., Loftus, E. V., Tysk, C., O'Morain, C., Moum, B., Colombel, J.-F., on behalf of the, E. and Natural History Task Force of the International Organization of Inflammatory Bowel, D. (2013). Geographical variability and environmental risk factors in inflammatory bowel disease. *Gut* **62**:630-649.
- Nicholson, J. K., Lindon, J. C. and Holmes, E. (1999). 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **29**:1181-1189.
- Nizet, V. (2006). Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *Current Issues in Molecular Biology* **8**:11-26.
- Normann, E., Fahlén, A., Engstrand, L. and Lilja, H. E. (2013). Intestinal microbial profiles in extremely preterm infants with and without necrotizing enterocolitis. *Acta Paediatrica, International Journal of Paediatrics* **102**:129-136.
- Oberreuther-Moschner, D. L., Jahreis, G., Rechkemmer, G. and Pool-Zobel, B. L. (2004). Dietary intervention with the probiotics *Lactobacillus acidophilus* 145 and *Bifidobacterium longum* 913 modulates the potential of human faecal water to induce damage in HT29 clone 19A cells. *British Journal of Nutrition* **91**:925-932.
- Oda, Y., Nakamura, S.-i., Oki, I., Kato, T. and Shinagawa, H. (1985). Evaluation of the new system (umu-test) for the detection of environmental mutagens and carcinogens. *Mutation Research/Environmental Mutagenesis and Related Subjects* **147**:219-229.
- Odunsi, K., Wollman, R. M., Ambrosone, C. B., Hutson, A., McCann, S. E., Tammela, J., Geisler, J. P., Miller, G., Sellers, T., Cliby, W., Qian, F., Keitz, B., Intengan, M., Lele, S. and Alderfer, J. L. (2005). Detection of epithelial ovarian cancer using ¹H-NMR-based metabonomics. *International Journal of Cancer* **113**:782-788.
- O'Hara, A. M. and Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Reports* **7**:688-693.
- Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H. and Wagner, H. (2011). vegan: Community ecology package. *R package version 1.17-6*.
- Olivares, M., Díaz-Ropero, M. P., Gómez, N., Lara-Villoslada, F., Sierra, S., Maldonado, J. A., Martín, R., Rodríguez, J. M. and Xaus, J. (2006). The consumption of two new probiotic strains, *Lactobacillus gasseri* CECT 5714 and *Lactobacillus coryniformis* CECT 5711, boosts the immune system of healthy humans. *International Microbiology* **9**:47-52.
- O'Mahony, L., McCarthy, J., Kelly, P., Hurley, G., Luo, F., Chen, K., O'Sullivan, G. C., Kiely, B., Collins, J. K., Shanahan, F. and Quigley, E. M. M. (2005). *Lactobacillus* and *Bifidobacterium* in irritable bowel syndrome: Symptom responses and relationship to cytokine profiles. *Gastroenterology* **128**:541-551.
- Omar, J. M., Chan, Y. M., Jones, M. L., Prakash, S. and Jones, P. J. H. (2013). *Lactobacillus fermentum* and *Lactobacillus amylovorus* as probiotics alter body adiposity and gut microflora in healthy persons. *Journal of Functional Foods* **5**:116-123.
- Oßwald, K., Becker, T. W., Grimm, M., Jahreis, G. and Pool-Zobel, B. L. (2000). Inter- and intra-individual variation of faecal water — genotoxicity in human colon cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **472**:59-70.
- Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A. and Brown, P. O. (2007). Development of the Human Infant Intestinal Microbiota. *PLoS Biology* **5**:e177.
- Paradis, E., Claude, J. and Strimmer, K. (2004). APE: Analyses of phylogenetics and evolution in R language version 2.7-1. *Bioinformatics* **20**:289-290.

- Park, M., Kwon, B., Shim, J., Huh, C. and Ji, G.** (2008). Heterologous expression of cholesterol oxidase in *Bifidobacterium longum* under the control of 16S rRNA gene promoter of bifidobacteria. *Biotechnology Letters* **30**:165-172.
- Parks, D. H. and Beiko, R. G.** (2010). Identifying biologically relevant differences between metagenomic communities. *Bioinformatics* **26**:715-721.
- Pecoits-Filho, R.** (2007). Dietary protein intake and kidney disease in western diet. pp. 102-112.
- Pereg, D., Kimhi, O., Tirosh, A., Orr, N., Kayouf, R. and Lishner, M.** (2005). The effect of fermented yogurt on the prevention of diarrhea in a healthy adult population. *American Journal of Infection Control* **33**:122-125.
- Pereira, D. I. A. and Gibson, G. R.** (2002). Cholesterol assimilation by lactic acid bacteria and Bifidobacteria isolated from the human gut. *Applied and Environmental Microbiology* **68**:4689-4693.
- Pinto, A. J. and Raskin, L.** (2012). PCR Biases Distort Bacterial and Archaeal Community Structure in Pyrosequencing Datasets. *PLoS ONE* **7**:e43093.
- Plummer, S., Weaver, M. A., Harris, J. C., Dee, P. and Huter, J.** (2004). *Clostridium difficile* pilot study: Effects of probiotic supplementation on the incidence of *C. difficile* diarrhoea. *International Microbiology* **7**:59-62.
- Podolsky, D. K.** (2002). Inflammatory bowel disease. *New England Journal of Medicine* **347**:417-429.
- Potempa, J. and Pike, R. N.** (2009). Corruption of Innate Immunity by Bacterial Proteases. *Journal of Innate Immunity* **1**:70-87.
- Prasad, J., Sazawal, S., Dhingra, U. and Gopal, P. K.** (2013). Detection of viable *Bifidobacterium lactis* HN019 (DR10™) in stools of children during a synbiotic dietary intervention trial. *International Dairy Journal* **30**:64-67.
- Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J. and Glöckner, F. O.** (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research* **35**:7188-7196.
- Qin, J. J., Li, Y. R., Cai, Z. M., Li, S. H., Zhu, J. F., Zhang, F., Liang, S. S., Zhang, W. W., Guan, Y. L., Shen, D. Q., Peng, Y. Q., Zhang, D. Y., Jie, Z. Y., Wu, W. X., Qin, Y. W., Xue, W. B., Li, J. H., Han, L. C., Lu, D. H., Wu, P. X., Dai, Y. L., Sun, X. J., Li, Z. S., Tang, A. F., Zhong, S. L., Li, X. P., Chen, W. N., Xu, R., Wang, M. B., Feng, Q., Gong, M. H., Yu, J., Zhang, Y. Y., Zhang, M., Hansen, T., Sanchez, G., Raes, J., Falony, G., Okuda, S., Almeida, M., LeChatelier, E., Renault, P., Pons, N., Batto, J. M., Zhang, Z. X., Chen, H., Yang, R. F., Zheng, W. M., Li, S. G., Yang, H. M., Wang, J., Ehrlich, S. D., Nielsen, R., Pedersen, O. and Kristiansen, K.** (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* **490**:55-60.
- Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.-M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H. B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Dore, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P., Ehrlich, S. D. and Wang, J.** (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**:59-65.

- Quigley, E. M. M.** (2003). Current concepts of the irritable bowel syndrome. *Scandinavian Journal of Gastroenterology, Supplement* **38**:1-8.
- Quillardet, P., Huisman, O., D'Ari, R. and Hofnung, M.** (1982). SOS chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K-12 to measure genotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* **79**:5971-5975.
- Ranjard, L., Poly, F., Lata, J. C., Mougel, C., Thioulouse, J. and Nazaret, S.** (2001). Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: Biological and methodological variability. *Applied and Environmental Microbiology* **67**:4479-4487.
- Rao, D. R., Chawan, C. B. and Pulusani, S. R.** (1981). Influence of milk and Thermophilus milk on plasma cholesterol levels and hepatic cholesterogenesis in rats. *Journal of Food Science* **46**:1339-1341.
- Rappe, M. S. and Giovannoni, S. J.** (2003). The uncultured microbial majority. *Annual Review of Microbiology* **57**:369-394.
- Rayes, N., Seehofer, D., Theruvath, T., Schiller, R. A., Langrehr, J. M., Jonas, S., Bengmark, S. and Neuhaus, P.** (2005). Supply of pre- and probiotics reduces bacterial infection rates after liver transplantation - A randomized, double-blind trial. *American Journal of Transplantation* **5**:125-130.
- R-Core-Team.** (2012). R: A language and environment for statistical computing. *R version 2.11.1*. Vienna: R foundation for statistical computing.
- Reddy, B. S., Mangat, S., Weisburger, J. H. and Wynder, E. L.** (1977). Effect of high-risk diets for colon carcinogenesis on intestinal mucosal and bacterial beta-glucuronidase activity in F344 rats. *Cancer Research* **37**:3533-3536.
- Reid, G., Gaudier, E., Guarner, F., Huffnagle, G. B., Macklaim, J. M., Munoz, A. M., Martini, M., Ringel-Kulka, T., Sartor, B., Unal, R., Verbeke, K. and Walter, J.** (2010). Responders and non-responders to probiotic interventions: How can we improve the odds? *Gut Microbes* **1**:200-204.
- Reifferscheid, G., Heil, J., Oda, Y. and Zahn, R. K.** (1991). A microplate version of the SOS/umu-test for rapid detection of genotoxins and genotoxic potentials of environmental samples. *Mutation Research/Environmental Mutagenesis and Related Subjects* **253**:215-222.
- Reinhardt, C., Bergentall, M., Greiner, T. U., Schaffner, F., Ostergren-Lunden, G., Petersen, L. C., Ruf, W. and Backhed, F.** (2012). Tissue factor and PAR1 promote microbiota-induced intestinal vascular remodelling. *Nature* **483**:627-631.
- Rerksuppaphol, S. and Rerksuppaphol, L.** (2012). Randomized controlled trial of probiotics to reduce common cold in schoolchildren. *Pediatrics International* **54**:682-687.
- Rieger, M. A., Parlesak, A., Pool-Zobel, B. L., Rechkemmer, G. and Bode, C.** (1999). A diet high in fat and meat but low in dietary fibre increases the genotoxic potential of 'faecal water'. *Carcinogenesis* **20**:2311-2316.
- Ritchie, N. J., Schutter, M. E., Dick, R. P. and Myrold, D. D.** (2000). Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. *Applied and Environmental Microbiology* **66**:1668-1675.
- Roberts, A. B., Wallace, B. D., Venkatesh, M. K., Mani, S. and Redinbo, M. R.** (2013). Molecular Insights into Microbial β -Glucuronidase Inhibition to Abrogate CPT-11 Toxicity. *Molecular Pharmacology*.
- Roberts, D. W.** (2010). labdsv: Ordination and Multivariate Analysis for Ecology. *R package version 1.4-1*.

- Rochet, V., Rigottier-Gois, L., Sutren, M., Kremetscki, M. N., Andrieux, C., Furet, J. P., Tailliez, P., Levenez, F., Mogenet, A., Bresson, J. L., Méance, S., Cayuela, C., Leplingard, A. and Doré, J. (2006). Effects of orally administered *Lactobacillus casei* DN-114001 on the composition or activities of the dominant faecal microbiota in healthy humans. *British Journal of Nutrition* **95**:421-429.
- Roesch, L. F. W., Fulthorpe, R. R., Riva, A., Casella, G., Hadwin, A. K. M., Kent, A. D., Daroub, S. H., Camargo, F. A. O., Farmerie, W. G. and Triplett, E. W. (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME Journal* **1**:283-290.
- Roesch, L. F., Casella, G., Simell, O., Krischer, J., Wasserfall, C. H., Schatz, D., Atkinson, M. A., Neu, J. and Triplett, E. W. (2009). Influence of fecal sample storage on bacterial community diversity. *The open microbiology journal* **3**:40-46.
- Roessler, A., Forstten, S. D., Glej, M., Ouwehand, A. C. and Jahreis, G. (2012). The effect of probiotics on faecal microbiota and genotoxic activity of faecal water in patients with atopic dermatitis: A randomized, placebo-controlled study. *Clinical Nutrition* **31**:22-29.
- Rogers, G. B., Hart, C. A., Mason, J. R., Hughes, M., Walshaw, M. J. and Bruce, K. D. (2003). Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S Ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. *Journal of Clinical Microbiology*. **41**:3548-3558.
- Romick-Rosendale, L. E., Goodpaster, A. M., Hanwright, P. J., Patel, N. B., Wheeler, E. T., Chona, D. L. and Kennedy, M. A. (2009). NMR-based metabonomics analysis of mouse urine and fecal extracts following oral treatment with the broad-spectrum antibiotic enrofloxacin (Baytril). *Magnetic Resonance in Chemistry* **47**:S36-S46.
- Rosenfeldt, V., Benfeldt, E., Nielsen, S. D., Michaelsen, K. F., Jeppesen, D. L., Valerius, N. H. and Paerregaard, A. (2003). Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. *Journal of Allergy and Clinical Immunology* **111**:389-395.
- Rothberg, J. M., Hinz, W., Rearick, T. M., Schultz, J., Mileski, W., Davey, M., Leamon, J. H., Johnson, K., Milgrew, M. J., Edwards, M., Hoon, J., Simons, J. F., Marran, D., Myers, J. W., Davidson, J. F., Branting, A., Nobile, J. R., Puc, B. P., Light, D., Clark, T. A., Huber, M., Branciforte, J. T., Stoner, I. B., Cawley, S. E., Lyons, M., Fu, Y., Homer, N., Sedova, M., Miao, X., Reed, B., Sabina, J., Feierstein, E., Schorn, M., Alanjary, M., Dimalanta, E., Dressman, D., Kasinskis, R., Sokolsky, T., Fidanza, J. A., Namsaraev, E., McKernan, K. J., Williams, A., Roth, G. T. and Bustillo, J. (2011). An integrated semiconductor device enabling non-optical genome sequencing. *Nature* **475**:348-352.
- Round, J. L. and Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology* **9**:313-323.
- Rousseaux, C., Thuru, X., Gelot, A., Barnich, N., Neut, C., Dubuquoy, L., Dubuquoy, C., Merour, E., Geboes, K., Chamillard, M., Ouwehand, A., Leyer, G., Carcano, D., Colombel, J. F., Ardid, D. and Desreumaux, P. (2007). *Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors. *Nature Medicine* **13**:35-37.
- Ruiz, R. and Rubio, L. A. (2009). Lyophilisation improves the extraction of PCR-quality community DNA from pig faecal samples. *Journal of the Science of Food and Agriculture* **89**:723-727.
- Rutgeerts, P., van Assche, G. and Vermeire, S. (2004). Optimizing anti-TNF treatment in inflammatory bowel disease. *Gastroenterology* **126**:1593-1610.

- Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1350-1354.
- Sakamoto, H., Yokota, H., Kibe, R., Sayama, Y. and Yuasa, A. (2002). Excretion of bisphenol A-glucuronide into the small intestine and deconjugation in the cecum of the rat. *Biochimica et Biophysica Acta - General Subjects* **1573**:171-176.
- Sařaga, M., Sobczak, M. and Fichna, J. (2013). Inhibition of proteases as a novel therapeutic strategy in the treatment of metabolic, inflammatory and functional diseases of the gastrointestinal tract. *Drug Discovery Today* **18**:708-715.
- Salonen, A., Nikkilä, J., Jalanka-Tuovinen, J., Immonen, O., Rajilić-Stojanović, M., Kekkonen, R. A., Palva, A. and de Vos, W. M. (2010). Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: Effective recovery of bacterial and archaeal DNA using mechanical cell lysis. *Journal of Microbiological Methods* **81**:127-134.
- Sandler, R. H., Finegold, S. M., Bolte, E. R., Buchanan, C. P., Maxwell, A. P., Väisänen, M.-L., Nelson, M. N. and Wexler, H. M. (2000). Short-Term Benefit From Oral Vancomycin Treatment of Regressive-Onset Autism. *Journal of Child Neurology* **15**:429-435.
- Sanger, F., Air, G. M., Barrell, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., Hutchison, C. A., Slocombe, P. M. and Smith, M. (1977). Nucleotide sequence of bacteriophage [phi]X174 DNA. *Nature* **265**:687-695.
- Sato, H., Frank, D. W., Hillard, C. J., Feix, J. B., Pankhaniya, R. R., Moriyama, K., Finck-Barbançon, V., Buchaklian, A., Lei, M., Long, R. M., Wiener-Kronish, J. and Sawa, T. (2003). The mechanism of action of the *Pseudomonas aeruginosa*-encoded type III cytotoxin, ExoU. *EMBO Journal* **22**:2959-2969.
- Sato, K., Naito, M., Yukitake, H., Hirakawa, H., Shoji, M., McBride, M. J., Rhodes, R. G. and Nakayama, K. (2010). A protein secretion system linked to bacteroidete gliding motility and pathogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **107**:276-281.
- Saulnier, D. M. A., Gibson, G. R. and Kolida, S. (2008). In vitro effects of selected synbiotics on the human faecal microbiota composition. *FEMS Microbiology Ecology* **66**:516-527.
- Scanlan, P. D., Shanahan, F., Clune, Y., Collins, J. K., O'Sullivan, G. C., O'Riordan, M., Holmes, E., Wang, Y. and Marchesi, J. R. (2008). Culture-independent analysis of the gut microbiota in colorectal cancer and polyposis. *Environmental Microbiology* **10**:789-798.
- Schloss, P. D., Gevers, D. and Westcott, S. L. (2011). Reducing the Effects of PCR Amplification and Sequencing Artifacts on 16S rRNA-Based Studies. *PLoS ONE* **6**:e27310.
- Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J. and Weber, C. F. (2009). Introducing mothur: Open-Source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**:7537-7541.
- Schmieder, R. (2008). ADAPT. San Diego: Department of Computer Science and Computational Science Research.
- Schofield, J. K., Fleming, D., Grindlay, D. and Williams, H. (2011). Skin conditions are the commonest new reason people present to general practitioners in England and Wales. *British Journal of Dermatology* **165**:1044-1050.

- Schrezenmeir, J. and de Vrese, M.** (2001). Probiotics, prebiotics, and synbiotics - Approaching a definition. *American Journal of Clinical Nutrition* **73**:361S-364S.
- Schultz, M., Timmer, A., Herfarth, H. H., Sartor, R. B., Vanderhoof, J. A. and Rath, H. C.** (2004). *Lactobacillus* GG in inducing and maintaining remission of Crohn's disease. *BMC Gastroenterology* **4**.
- Schwartz, A., Gruhl, B., Lobnitz, M., Michel, P., Radke, M. and Blaut, M.** (2003). Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. *Pediatric Research* **54**:393-399.
- Schwartz, A., Taras, D., Schafer, K., Beijer, S., Bos, N. A., Donus, C. and Hardt, P. D.** (2009). Microbiota and SCFA in lean and overweight healthy subjects. *Obesity* **18**:190-195.
- Sekirov, I. R., S. L. ; Antunes, L. C.; Finlay, B. B.** (2010). Gut microbiota in health and disease. *Physiological Reviews* **90**:859-904.
- Sekirov, I., Tam, N. M., Jogova, M., Robertson, M. L., Li, Y., Lupp, C. and Finlay, B. B.** (2008). Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. *Infection and Immunity*. **76**:4726-4736.
- Selinger, C. P., Bell, A., Cairns, A., Lockett, M., Sebastian, S. and Haslam, N.** (2013). Probiotic VSL#3 prevents antibiotic-associated diarrhoea in a double-blind, randomized, placebo-controlled clinical trial. *Journal of Hospital Infection*.
- Shinkai, T., Arioka, H., Kunikane, H., Eguchi, K., Sasaki, Y., Tamura, T., Ohe, Y., Oshita, F., Nishio, M., Karato, A., Okamoto, H., Nakashima, H., Ohmatsu, H., Shiraishi, J., Nomura, N. and Saijo, N.** (1994). Phase I clinical trial of irinotecan (CPT-11), 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin, and cisplatin in combination with fixed dose of vindesine in advanced non-small cell lung cancer. *Cancer Research* **54**:2636-2642.
- Shirkey, T. W., Siggers, R. H., Goldade, B. G., Marshall, J. K., Drew, M. D., Laarveld, B. and Van Kessel, A. G.** (2006). Effects of commensal bacteria on intestinal morphology and expression of proinflammatory cytokines in the gnotobiotic pig. *Experimental Biology and Medicine* **231**:1333-1345.
- Simpson, J. M., McCracken, V. J., Gaskins, H. R. and Mackie, R. I.** (2000). Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after Introduction of *Lactobacillus reuteri* strain MM53. *Applied and Environmental Microbiology* **66**:4705-4714.
- Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., Arrieta, J. M. and Herndl, G. J.** (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences* **103**:12115-12120.
- Solaiman, D. K. and Somkuti, G. A.** (1991). Expression of streptomycete cholesterol oxidase in *Escherichia coli*. *Journal of Industrial Microbiology and Biotechnology* **8**:253-258.
- Song, Y., Liu, C. and Finegold, S. M.** (2004). Real-time PCR quantitation of Clostridia in feces of autistic children. *Applied and Environmental Microbiology*. **70**:6459-6465.
- Staley, J. T. and Konopka, A.** (1985). Measurement of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annual Review of Microbiology* **39**:321-346.

- Steck, N., Hoffmann, M., Sava, I. G., Kim, S. C., Hahne, H., Tonkonogy, S. L., Mair, K., Krueger, D., Pruteanu, M., Shanahan, F., Vogelmann, R., Schemann, M., Kuster, B., Sartor, R. B. and Haller, D.** (2011). *Enterococcus faecalis* metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. *Gastroenterology* **141**:959-971.
- Steck, N., Mueller, K., Schemann, M. and Haller, D.** (2012). Bacterial proteases in IBD and IBS. *Gut* **61**:1610-1618.
- Strachan, D. P.** (1989). Hay fever, hygiene, and household size. *BMJ* **299**.
- Strachan, D. P.** (2000). Family size, infection and atopy: the first decade of the "hygiene hypothesis". *Thorax* **55 Suppl 1**:S2-10.
- Suzuki, M., Rappe, M. S. and Giovannoni, S. J.** (1998). Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. *Applied and Environmental Microbiology* **64**:4522-4529.
- Takasuna, K., Hagiwara, T., Hirohashi, M., Kato, M., Nomura, M., Nagai, E., Yokoi, T. and Kamataki, T.** (1996). Involvement of β -glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. *Cancer Research* **56**:3752-3757.
- Tannock, G. W., Munro, K., Harmsen, H. J. M., Welling, G. W., Smart, J. and Gopal, P. K.** (2000). Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Applied and Environmental Microbiology* **66**:2578-2588.
- Tarmy, E. M., Venitt, S. and Brookes, P.** (1973). Mutagenicity of the carcinogen 7 bromomethylbenz[a]anthracene: A quantitative study in repair deficient strains of *Escherichia coli*. *Mutation Research* **19**:153-166.
- Taylor, A. L., Dunstan, J. A. and Prescott, S. L.** (2007). Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: A randomized controlled trial. *Journal of Allergy and Clinical Immunology* **119**:184-191.
- The Human Microbiome Consortium.** (2012). Structure, function and diversity of the healthy human microbiome. *Nature* **486**:207-214.
- Thibault, H., Aubert-Jacquin, C. and Goulet, O.** (2004). Effects of long-term consumption of a fermented infant formula (with *Bifidobacterium breve* c50 and *Streptococcus thermophilus* 065) on acute diarrhea in healthy infants. *Journal of Pediatric Gastroenterology and Nutrition* **39**:147-152.
- Thompson, G. R. and Trexler, P. C.** (1971). Gastrointestinal structure and function in germ-free or gnotobiotic animals. *Gut* **12**:230-235.
- Thompson, W. G., Longstreth, G. F., Drossman, D. A., Heaton, K. W., Irvine, E. J. and Müller-Lissner, S. A.** (1999). Functional bowel disorders and functional abdominal pain. *Gut* **45**:II43-II47.
- Tirola, M. A., Suvilampi, J. E., Kulomaa, M. S. and Rintala, J. A.** (2003). Microbial diversity in a thermophilic aerobic biofilm process: analysis by length heterogeneity PCR (LH-PCR). *Water Research* **37**:2259-2268.

- Tuohy, K. M., Pinart-Gilberga, M., Jones, M., Hoyles, L., McCartney, A. L. and Gibson, G. R. (2007).** Survivability of a probiotic *Lactobacillus casei* in the gastrointestinal tract of healthy human volunteers and its impact on the faecal microflora. *Journal of Applied Microbiology* **102**:1026-1032.
- Tuohy, K. M., Probert, H. M., Smejkal, C. W. and Gibson, G. R. (2003).** Using probiotics and prebiotics to improve gut health. *Drug Discovery Today* **8**:692-700.
- Turnbaugh, P. J. and Gordon, J. I. (2009).** The core gut microbiome, energy balance and obesity. *Journal of Physiology* **587**:4153-4158.
- Turnbaugh, P. J., Hamady, M., Yatsunencko, T., Cantarel, B. L., Duncan, A., Ley, R. E., Sogin, M. L., Jones, W. J., Roe, B. A., Affourtit, J. P., Egholm, M., Henrissat, B., Heath, A. C., Knight, R. and Gordon, J. I. (2009).** A core gut microbiome in obese and lean twins. *Nature* **457**:480-484.
- Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R. and Gordon, J. I. (2007).** The human microbiome project. *Nature* **449**:804-810.
- Uccello, M., Malaguarnera, G., Basile, F., D'Agata, V., Malaguarnera, M., Bertino, G., Vacante, M., Drago, F. and Biondi, A. (2012).** Potential role of probiotics on colorectal cancer prevention. *BMC Surgery* **12**:S35.
- V. Wintzingerode, F., Göbel, U. B. and Stackebrandt, E. (1997).** Determination of microbial diversity in environmental samples: pitfalls of PCR-based *rRNA* analysis. *FEMS Microbiology Reviews* **21**:213-229.
- Vahabnezhad, E., Mochon, A. B., Wozniak, L. J. and Ziring, D. A. (2013).** *Lactobacillus* bacteremia associated with probiotic use in a pediatric patient with ulcerative colitis. *Journal of Clinical Gastroenterology* **47**:437-439.
- Vanhoutte, T., De Preter, V., De Brandt, E., Verbeke, K., Swings, J. and Huys, G. (2006).** Molecular monitoring of the fecal microbiota of healthy human subjects during administration of lactulose and *Saccharomyces boulardii*. *Applied and Environmental Microbiology* **72**:5990-5997.
- Venitt, S. and Bosworth, D. (1986).** Further studies on the detection of mutagenic and genotoxic activity in human faeces: aerobic and anaerobic fluctuation tests with *S. typhimurium* and *E. coli*, and the SOS Chromotest. *Mutagenesis* **1**:49-64.
- Verdenelli, M. C., Silvi, S., Cecchini, C., Orpianesi, C. and Cresci, A. (2011).** Influence of a combination of two potential probiotic strains, *Lactobacillus rhamnosus* IMC 501[®] and *Lactobacillus paracasei* IMC 502[®] on bowel habits of healthy adults. *Letters in Applied Microbiology* **52**:596-602.
- Vidlock, E. J. and Cremonini, F. (2012).** Meta-analysis: probiotics in antibiotic-associated diarrhoea. *Alimentary Pharmacology & Therapeutics* **35**:1355-1369.
- Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., Brown, D., Stares, M. D., Scott, P., Bergerat, A., Louis, P., McIntosh, F., Johnstone, A. M., Lobley, G. E., Parkhill, J. and Flint, H. J. (2011).** Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME Journal* **5**:220-230.
- Wang, Y., Hoenig, J. D., Malin, K. J., Qamar, S., Petrof, E. O., Sun, J., Antonopoulos, D. A., Chang, E. B. and Claud, E. C. (2009).** 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *ISME Journal* **3**:944-954.
- Warnes, G. R. (2010).** gplots: Various R programming tools for plotting data. *R package version 2.8.0*.

- Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J.** (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**:697-703.
- Weizman, Z., Asli, G. and Alsheikh, A.** (2005). Effect of a probiotic infant formula on infections in child care centers: Comparison of two probiotic agents. *Pediatrics* **115**:5-9.
- Whitman, W. B., Coleman, D. C. and Wiebe, W. J.** (1998). Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences* **95**:6578-6583.
- WHO.** (2002). Guidelines for the evaluation of probiotics in food. London, Ontario:
- Whorwell, P. J., Altringer, L., Morel, J., Bond, Y., Charbonneau, D., O'Mahony, L., Kiely, B., Shanahan, F. and Quigley, E. M. M.** (2006). Efficacy of an encapsulated probiotic *Bifidobacterium infantis* 35624 in women with irritable bowel syndrome. *American Journal of Gastroenterology* **101**:1581-1590.
- Wickham, H.** (2007). Reshaping data with the reshape package. *Journal of Statistical Software* **21**:1-20.
- Wickham, H.** (2009). ggplot2 Elegant Graphics for Data Analysis. VIII ed. Houston: Springer, p. 213.
- Williams, E. A., Stimpson, J., Wang, D., Plummer, S., Garaiova, I., Barker, M. E. and Corfe, B. M.** (2009). Clinical trial: a multistrain probiotic preparation significantly reduces symptoms of irritable bowel syndrome in a double-blind placebo-controlled study. *Alimentary Pharmacology and Therapeutics* **29**:97-103.
- Williams, H. C. and Grindlay, D. J. C.** (2010). What's new in atopic eczema? An analysis of systematic reviews published in 2007 and 2008. Part 1. Definitions, causes and consequences of eczema. *Clinical and Experimental Dermatology* **35**:12-15.
- Wiström, J., Norrby, S. R., Myhre, E. B., Eriksson, S., Granström, G., Lagergren, L., Englund, G., Nord, C. E. and Svenungsson, B.** (2001). Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. *Journal of Antimicrobial Chemotherapy* **47**:43-50.
- Woese, C. R.** (1987). Bacterial evolution. *Microbiological Reviews* **51**:221-271.
- Woese, C. R., Kandler, O. and Wheelis, M. L.** (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences* **87**:4576-4579.
- Woese, C. R., Magrum, L. J., Gupta, R., Siegel, R. B., Stahl, D. A., Kop, J., Crawford, N., Brosius, R., Gutell, R., Hogan, J. J. and Noller, H. F.** (1980). Secondary structure model for bacterial 16S ribosomal RNA: phylogenetic, enzymatic and chemical evidence. *Nucleic Acids Research* **8**:2275-2294.
- Xu, J., Bjursell, M. K., Himrod, J., Deng, S., Carmichael, L. K., Chiang, H. C., Hooper, L. V. and Gordon, J. I.** (2003). A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science* **299**:2074-2076.
- Yagur-Kroll, S., Bilic, B. and Belkin, S.** (2010). Strategies for enhancing bioluminescent bacterial sensor performance by promoter region manipulation. *Microbial Biotechnology* **3**:300-310.
- Yatsunencko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., Knight, R. and Gordon, J. I.** (2012). Human gut microbiome viewed across age and geography. *Nature* **486**:222-227.

- Yuasa, H., Hirobe, M., Tomei, S. and Watanabe, J.** (2000). Carrier-mediated transport of riboflavin in the rat colon. *Biopharmaceutics & Drug Disposition* **21**:77-82.
- Zhang, M., Liang, Y., Zhang, X., Xu, Y., Dai, H. and Xiao, W.** (2008). Deletion of yeast CWP genes enhances cell permeability to genotoxic agents. *Toxicological Sciences* **103**:68-76.
- Zoppi, G., Cinquetti, M., Benini, A., Bonamini, E. and Minelli, E. B.** (2001). Modulation of the intestinal ecosystem by probiotics and lactulose in children during treatment with ceftriaxone. *Current Therapeutic Research* **62**:418-435.

9. APPENDICES

Appendix I – Fragment sizes of 16S *rRNA* sequences obtained from the RDP (<http://rdp.cme.msu.edu/>) following an *in silico* LHPCR experiment

Generated fragment sizes for an *in silico* LHPCR experiment are available at:

<https://drive.google.com/file/d/0ByapqDVxrBNqWHFHUmZwbFpTazA/edit?usp=sharing>

Appendix II – Example R scripts used during the PROHEMI study

Example R scripts are available at:

<https://drive.google.com/file/d/0ByapqDVxrBNqNm1GeWdJR1c5MEU/edit?usp=sharing>

Lines with # symbols have been commented out and do not serve any function in R statistical software {R-Core-Team, 2012 #210}.

Appendix III – Stability file for Illumina MiSeq contig assembly from 2 paired end reads

The stability file utilised by Mothur in the 1st step in MiSeq data analysis is available at:

<https://drive.google.com/file/d/0ByapqDVxrBNqLTNITU9wOHR5LTA/edit?usp=sharing>

Appendix IV – Mothur batch files used in Mothur analysis of the MiSeq dataset

Mothur has the ability to run in batch mode where commands may be stored in a file and ran sequentially. Lines with # symbols are comments and are not executed by Mothur. Example batch files used for the analysis of the PROHEMI MiSeq dataset are available at:

<https://drive.google.com/file/d/0ByapqDVxrBNqbXk2TjNhU0t0ZUE/edit?usp=sharing>

Appendix V – Sequence counts for PROHEMI samples subjected to Illumina MiSeq NGS

The number of sequences obtained from Illumina MiSeq NGS for given sample is available at:

<https://drive.google.com/file/d/0ByapqDVxrBNqQUd5Y2lxY1NrQk0/edit?usp=sharing>

Appendix VI – Mothur batch files used in 454 analysis of DNA obtained from frozen faecal material

Mothur has the ability to run in batch mode where commands may be stored in a file and ran sequentially. Lines with # symbols are comments and are not executed by Mothur. Example batch files used for the analysis of the 454 dataset obtained from faecal DNA frozen at -20°C for up to 6 months are available at:

<https://drive.google.com/file/d/0ByapqDVxrBNqTWJ4NjF5ekh1OHc/edit?usp=sharing>

Appendix VII – Publications during my PhD studies:

Morris, L. S., Evans, J. and Marchesi, J. R. (2012). A robust plate assay for detection of extracellular microbial protease activity in metagenomic screens and pure cultures. *Journal of Microbiological Methods* 91:144-146.

Evans, J. M., Morris, L. S. and Marchesi, J. R (2013). The gut microbiome: The role of a virtual organ in the endocrinology of the host. *Journal of Endocrinology*



Note

A robust plate assay for detection of extracellular microbial protease activity in metagenomic screens and pure cultures

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ABSTRACT

A robust, efficient and cost-effective agar that utilises lactose free milk powder for identification of bacterial protease activity in pure cultures and metagenomic screens has been developed and tested on protease positive bacteria, selected strains and false protease positives isolated from a previously constructed metagenomic library.

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The deficiency of current culture techniques to isolate most microorganisms from some environments has led to the emergence and progression of functional metagenomics as a means for unearthing the vast and relatively untapped biological resources of the microbial world. Its ability to do so has been demonstrated with the discovery of novel and biotechnologically useful molecules (Uchiyama and Miyazaki, 2009) (Schloss and Handelsman, 2003) and clinically relevant antibiotic resistance genes (Allen et al.).

One function of interest here is microbial proteases as these are an important target for metagenomic screening due to their extensive possibility for use in industry (Rao et al., 1998) and also because of their role as virulence factors (Lantz, 1997; Maeda and Molla, 1989; Miyoshi and Shinoda, 2000). However there is a current deficit in metagenomic screens successfully isolating these enzymes. While there are a number of reasons for this scarcity, one problem that we aim to resolve with this research is the current issue of false positives arising from the use of skimmed milk agar as a standard means for screening metagenomic libraries for proteolytic activity. Previous research by Jones et al. (Jones et al., 2007) found that an initial 231 metagenomic clones deemed positive for protease activity by the formation of distinctive halos of clearing around colonies on standard skimmed milk agar (SSMA) were in fact, glycoside hydrolases which produced acetic acid from lactose fermentation and the pH drop was responsible for the phenotype, not protease activity. The demonstrated ineffectiveness of this agar has led us to question the validity of putative proteases detected using this method, for example Pailin et al. (2001) used SSMA to identify extracellular protease activity in

strains of the lactic acid bacteria; *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

Therefore it can be concluded that while SSMA could be appropriate for identifying lactose utilisation and the presence of galactosidases, it is not a sufficiently robust screening technique for protease activity and an alternative needs to be developed in order to save time and money. Here, we have developed a simple and easy way to prepare media which utilises lactose-free and fat-free skimmed milk powder as the substrate for detecting protease activity and allows discrimination against acid production since lactose hydrolysis is no longer an issue.

Bacillus subtilis MY2016 was used as a positive control for protease activity on all types of agar used in this study since it is known to secrete a number of extracellular proteases. These cultures were routinely grown on all agar at 30 °C. Strains of *Streptococcus thermophilus* 2483 and *Lactobacillus bulgaricus* 859 were obtained from the culture collection of University College cork, Department of Microbiology. Cultures were revived on MRS agar (Thermo Scientific, Oxoid) and incubated at 37 °C in an anaerobic chamber (Merck, Darmstadt, Germany) and examined after 48 hour incubation.

False protease positive glycoside hydrolase metagenomic clones were obtained from a previous metagenomic library (Jones et al., 2007) and were routinely revived from freezer stock on LB (Luria-bertani) agar supplemented with 12.5 µg/ml chloramphenicol. All cultures were routinely re-streaked every one–two days for the duration of this research.

SSMA was prepared as follows: 10% semi-skimmed milk solution was prepared in deionised water and autoclaved at 121 °C for 15 min. 1.5% (w/v) purified agar (Thermo Scientific) was also prepared in deionised water and autoclaved at 121 °C for 15 min. Upon sterilisation, both were kept at 55 °C in a water bath then the semi-skimmed milk solution

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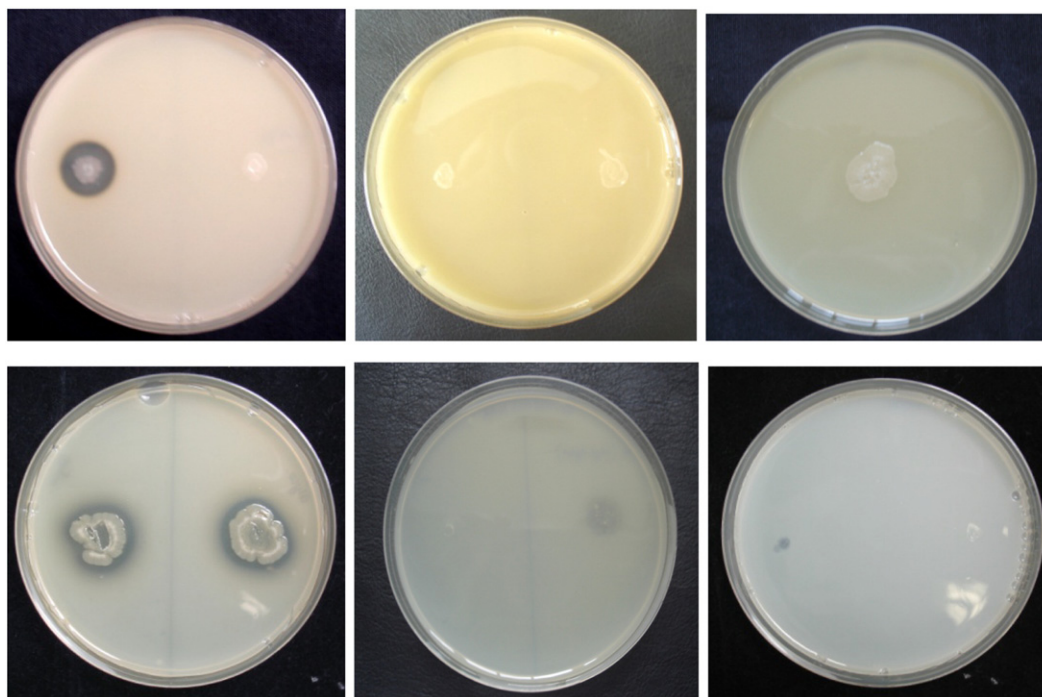


Fig. 1. Plate assays for protease activity. From top left; *B. subtilis* MY2016 clearing LF-SMA and a negative control, top middle; *S. thermophilus* 859 and *L. bulgaricus* 2483 failing to clear LF-SMA, top right; a glycoside hydrolase positive metagenomic clone failing to clear LF-SMA, bottom left; two glycoside hydrolase positive metagenomic clones clearing SMA, bottom middle; *S. thermophilus* 859 clearing SMA, bottom right; *L. bulgaricus* 2483 beginning to clear SMA. Cultures were incubated according to their appropriate conditions on all types of agar. Cultures were re-streaked every one to two days for the duration of this research to ensure the initial phenotype observed was replicable.

was added to the agar to give a final volume of 1% (w/v) semi-skimmed milk.

Lactose-free semi-skimmed milk powder was obtained from Valio (suomikauppa.fi) and agar was prepared in the same manner as described above to give a final concentration of 1% (w/v). After inoculation, cultures were incubated according to their appropriate conditions. Cultures were re-streaked every one to two days for the duration of this research to ensure the initial phenotype observed was replicable.

In order to prepare crude cell free extracts (CCFE) for protease assays, cells were grown overnight in appropriate media supplemented with the appropriate antibiotic. An aliquot (1%) of this starting culture was used to inoculate 50 ml of fresh media and grown to an optical density of 0.5 (600 nm). After this point cultures were left to grow for a further 3 h. Cells were harvested by centrifugation at 4000 rpm for 10 min. The pellet was re-suspended in 2 ml of Phosphate buffered saline (PBS) and each sample was bead beaten (0.1 mm diameter glass beads, 0.5 g) for 30 s and repeated a further 2 times with cooling on ice for 5 min between each beating. Samples were centrifuged at 20,000 ×g for 10 min and the resulting supernatant was taken as the cell free extract. An aliquot of the CCFE (100 µl) was added to 100 µl azocasein (5 mg/ml in 50 mM Tris-HCl) protease substrate and the mixture was incubated at 37 °C for 4 h, the reaction was terminated by the addition of 400 µl of 10% (w/v) trichloroacetic acid (TCA). Protein was precipitated by centrifugation at 12,000 ×g for 5 min and the resulting supernatant was transferred to a clean tube containing 700 µl of a 525 mM NaOH solution. The absorbance of the liberated azo-dye was measured using a spectrophotometer at 442 nm. Each reaction was carried out in triplicate.

It was found that the putative protease deficient strains used were capable of degrading skimmed milk agar but not lactose-free milk agar. Similarly the metagenomic clones had a corresponding outcome whereas for the bacterial strains known to be proteolytically active; a positive phenotype was observed on both types of media (Fig. 1). To affirm this, cultures were further subject to azocasein assay (Fig. 2)

where *B. subtilis* was shown to degrade the azocasein substrate and glycoside hydrolase metagenomic clones showed less than 20% activity.

From our study, Valio™ lactose-free milk agar was found to be an effective and robust agar for correctly identifying proteases by way of distinct zones of clearing around a bacterial colony. From the screens undertaken in this study no false positives arose with the use of this type of agar. We would suggest that this agar would be much more appropriate for future screening of metagenomic libraries for protease activity. The robustness of this agar will allow for more efficient characterisation of enzyme activity which can then lead to identification of clinically or industrially relevant proteases.

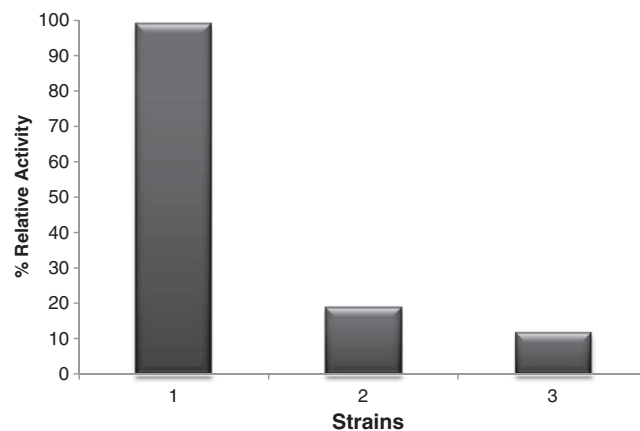


Fig. 2. Protease activity as measured by the release of acid-soluble substance from azocasein (5 mg/ml) in HCl buffered Tris to a pH of 8.0 incubated at 37 °C for 4 h. Results are shown as the mean value of the results that were in triplicate and are shown as a relative percentage of total protease activity. 1 = *B. subtilis* MY2016, 2 = Glycoside hydrolase clone, 3 = glycoside hydrolase clone.

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References

- Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., Handelsman, J., 2010. Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* 8, 251–259.
- Jones, B.V., Sun, F., Marchesi, J.R., 2007. Using skimmed milk agar to functionally screen a gut metagenomic library for proteases may lead to false positives. *Lett. Appl. Microbiol.* 45, 418–420.
- Lantz, M.S., 1997. Are bacterial proteases important virulence factors? *J. Periodontal Res.* 32, 126–132.
- Maeda, H., Molla, A., 1989. Pathogenic potentials of bacterial proteases. *Clin. Chim. Acta* 185, 357–367.
- Miyoshi, S.-I., Shinoda, S., 2000. Microbial metalloproteases and pathogenesis. *Microbes Infect.* 2, 91–98.
- Pailin, T., Kang, D.H., Schmidt, K., Fung, D.Y., 2001. Detection of extracellular bound proteinase in EPS-producing lactic acid bacteria cultures on skim milk agar. *Lett. Appl. Microbiol.* 33 (1), 45–49 (Jul).
- Rao, M.B., Tanksale, A.M., Ghatge, M.S., Deshpande, V.V., 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* 62, 597–635.
- Schloss, P.D., Handelsman, J., 2003. Biotechnological prospects from metagenomics. *Curr. Opin. Biotechnol.* 14, 303–310.
- Uchiyama, T., Miyazaki, K., 2009. Functional metagenomics for enzyme discovery: challenges to efficient screening. *Curr. Opin. Biotechnol.* 20, 616–622.

The gut microbiome: the role of a virtual organ in the endocrinology of the host

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Abstract

The human microbiome contains a vast array of microbes and genes that show greater complexity than the host's own karyome; the functions of many of these microbes are beneficial and show co-evolution with the host, while others are detrimental. The microbiota that colonises the gut is now being considered as a virtual organ or emergent system, with properties that need to be integrated into host biology and physiology. Unlike other organs, the functions that the gut microbiota plays in the host are as yet not fully understood and can be quite easily disrupted by antibiotics, diet or surgery. In this review, we look at some of the best-characterised functions that only the gut microbiota plays and how it interacts with the host's endocrine system and we try to make it clear that the 21st-century biology cannot afford to ignore this facet of biology, if it wants to fully understand what makes us human.

Key Words

- ▶ human microbiome
- ▶ cross-talk
- ▶ gut microbiome
- ▶ endocrine system

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Introduction

In the many years of studying the human body, it has become accepted that all the organs are known and well characterised in terms of their main functions. The whole discipline of anatomy has been focused on documenting the fine structure of these organs, while physiology and biochemistry have been determining their functions and specific chemical reactions. In the last 10 years, a significant revolution has been progressing, which started with the notion that for the best part of a hundred years we have been trying to describe how mammals function while ignoring one of the main organs in the body. However, this organ is not found or described in any conventional textbook and there are no clinical experts who can understand its functions and the pathologies that arise when it becomes diseased. This dearth of information is because this organ does not conform to the current

definition of being an organ, i.e. 'a fully differentiated and functional unit', and should probably best be thought of as a virtual organ (O'Hara & Shanahan 2006) since it is composed of microbes and all its functions are derived from these parts. In fact, it most probably should be considered as a system in the same way we look at our immune system, which is made of different cells, each having its own set of functions and roles. This paradigm shift is still undergoing some refinement, but it does beg the question 'what would biologists do if they were suddenly presented with a whole new organ/system?' Furthermore, why should we even think of it as an organ or system? To answer the last question first, we see this as a virtual organ/system due to the genetic and metabolic diversity that resides within it.

Microbiologists have coined a phrase for this system, the microbiome, which is defined as the 'the genetic

material (DNA) within a microbial community'; this can also be referred to as the metagenome of the microbiota. The largest contributors to the human microbiome are the bacteria present in the intestinal tract, which have been estimated to be in the range of 100 trillion cells, ten times more cells than there are human cells in the host's body. In terms of the total non-redundant genetic load found in the human gut microbiome, it is 150 times larger than the host's karyome ((Qin *et al.* 2010), which is between 20 000 and 26 000 genes (Pennisi 2003, Collins *et al.* 2004)), and each individual has ~540 000 bacterial genes in the gut (21–27×the human karyome). While the karyome is generally regarded as fixed with respect to the gene catalogue, and at this stage ignoring any epigenetic mechanisms, the gut microbiome is far more random and each individual will contain a unique collection of microbes that are easily altered (see below). Humans are born either sterile or colonised with a very basic microbiome, and analysis of the meconium has shown that any microbes that are present are not necessarily those that ultimately colonise the adult gut (Koenig *et al.* 2011). The microbiome that is obtained is partly inherited from the mother and partly from the exposure to the environment in the first 2 years of life. Hence, there is a degree of determinism (both by exposure to the mother and due to genetic selection (Tims *et al.* 2013)) and also a significant proportion of random colonisation. However, it is striking that after 2 years of age, all adults' colons will be predominantly colonised with members of two phyla, namely *Bacteroidetes*: Gram-negative, anaerobic, non-spore-forming bacteria, which are enriched with carbohydrate-degrading enzymes, and *Firmicutes*: Gram-positive, formerly called 'the low-GC bacteria', anaerobic, spore-forming bacteria, which ferment simple sugars to produce a variety of short-chain fatty acids (SCFAs), such as butyrate, acetate and propionate (Fischbach & Sonnenburg 2011). Between them, these phyla can constitute over 90% of the bacteria present in the large intestinal lumen (Turnbaugh *et al.* 2009, Qin *et al.* 2010, Claesson *et al.* 2011, HMPC *et al.* 2012) and mucosa (Eckburg *et al.* 2005, Chen *et al.* 2012, Harrell *et al.* 2012), and the proportion of each phylum in the colon ranges from being nearly 90% *Firmicutes*, at one end of the continuum, to 90% *Bacteroidetes*, at the other end. What dictates this distribution is unclear, and the consequences for the host are also unclear. Furthermore, our view of the intestinal environment is skewed towards the large intestine and is predominantly focused on faecal material, and even then we are very bacteria centric (Marchesi 2010). Bearing this biased view of the intestinal

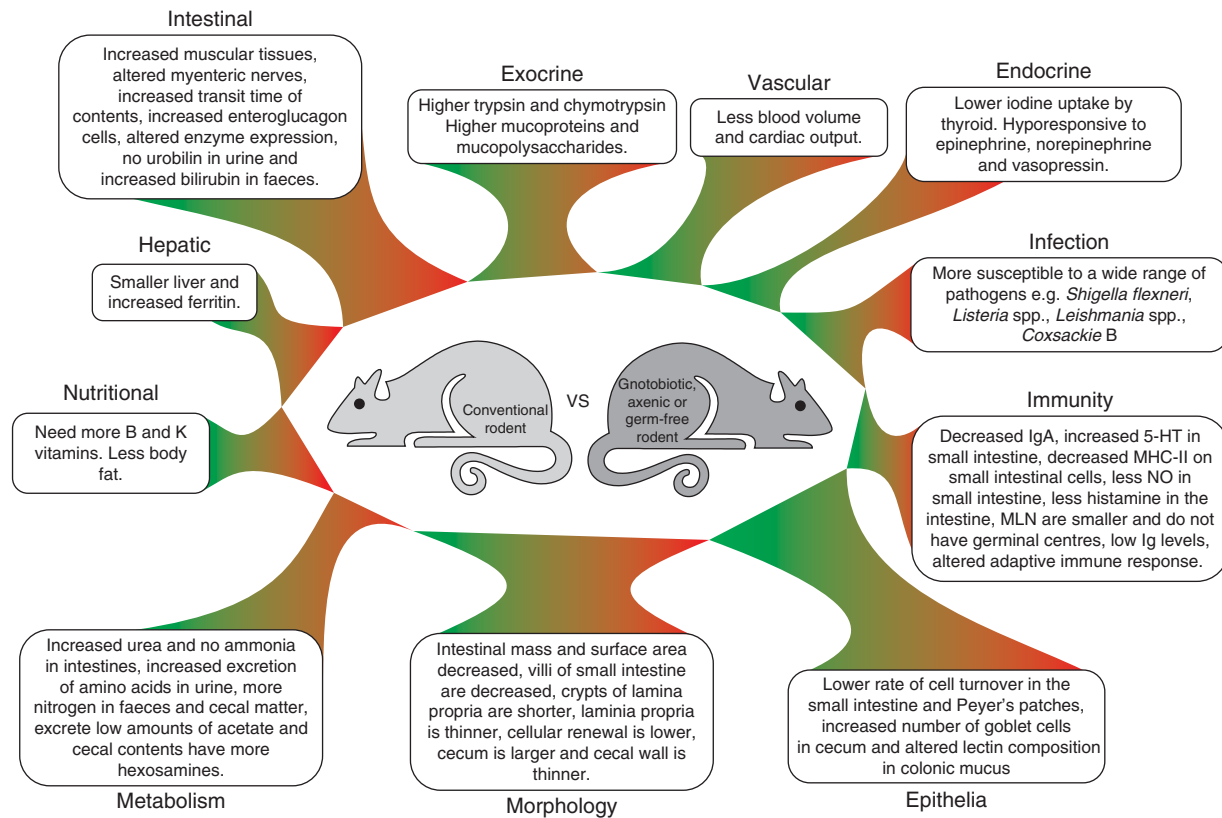
environment in mind, we have begun to determine what functions this collection of organisms plays in the host.

A virtual organ/system with functions of importance to the host

In the last 10 years, it has become increasingly apparent that the gut microbiota plays a significant role in host biology. We have a substantial body of evidence that these functions can be beneficial or detrimental to the host, and if they were assets, we would wish to maximise the former and somehow offset the latter. A significant portion of the evidence that supports a role for the gut microbiota in host development and function comes from studying sterile animals, especially rodents (Fig. 1). A recent comprehensive review of the use of such animals has shown how widespread the impact of not having a gut microbiota is (Smith *et al.* 2007). Some of the best studied of these bacterial functions include providing the host with energy in the form of SCFAs such as butyrate (Louis & Flint 2009) and propionate (Macfarlane & Macfarlane 2011), bile salt metabolism (Jones *et al.* 2008, Swann *et al.* 2011) and role in the brain–gut axis (Collins *et al.* 2012). Many ecological analyses of the colonic microbiota have shown that while it is relatively stable (Scanlan *et al.* 2006, Jalanka-Tuovinen *et al.* 2011, Kolmeder *et al.* 2012), it can be significantly perturbed by antibiotics (Dethlefsen *et al.* 2008, Jernberg *et al.* 2010), diet (Claesson *et al.* 2012, Ravussin *et al.* 2012) and surgery (Zhang *et al.* 2009, Li *et al.* 2011). Unlike a conventional organ, the functions of this virtual organ can be significantly altered or removed due to these environmental factors, which can result in disease in the host, e.g. *Clostridium difficile*-associated diarrhoea (Kachrimanidou & Malisiovas 2011), or a significant alteration of the host's metabolite profile (Yap *et al.* 2008), the significance of which still remains unclear. The variability in the gut microbiome and its functions are important in two respects: the functions that one inherits and acquires are driven mainly by a random process and once established they can be perturbed by interventions.

Bile metabolism and gut bacteria

Bile acids/salts are cholesterol-derived host metabolites that play a role in several host processes (Fig. 2). Their principal functions are to aid in fat adsorption and prevent small intestinal bacterial overgrowth. Both these functions can be explained by the fact that bile acids are surfactants (not detergents), with a hydrophilic taurine or glycine group covalently bound to a hydrophobic steroid

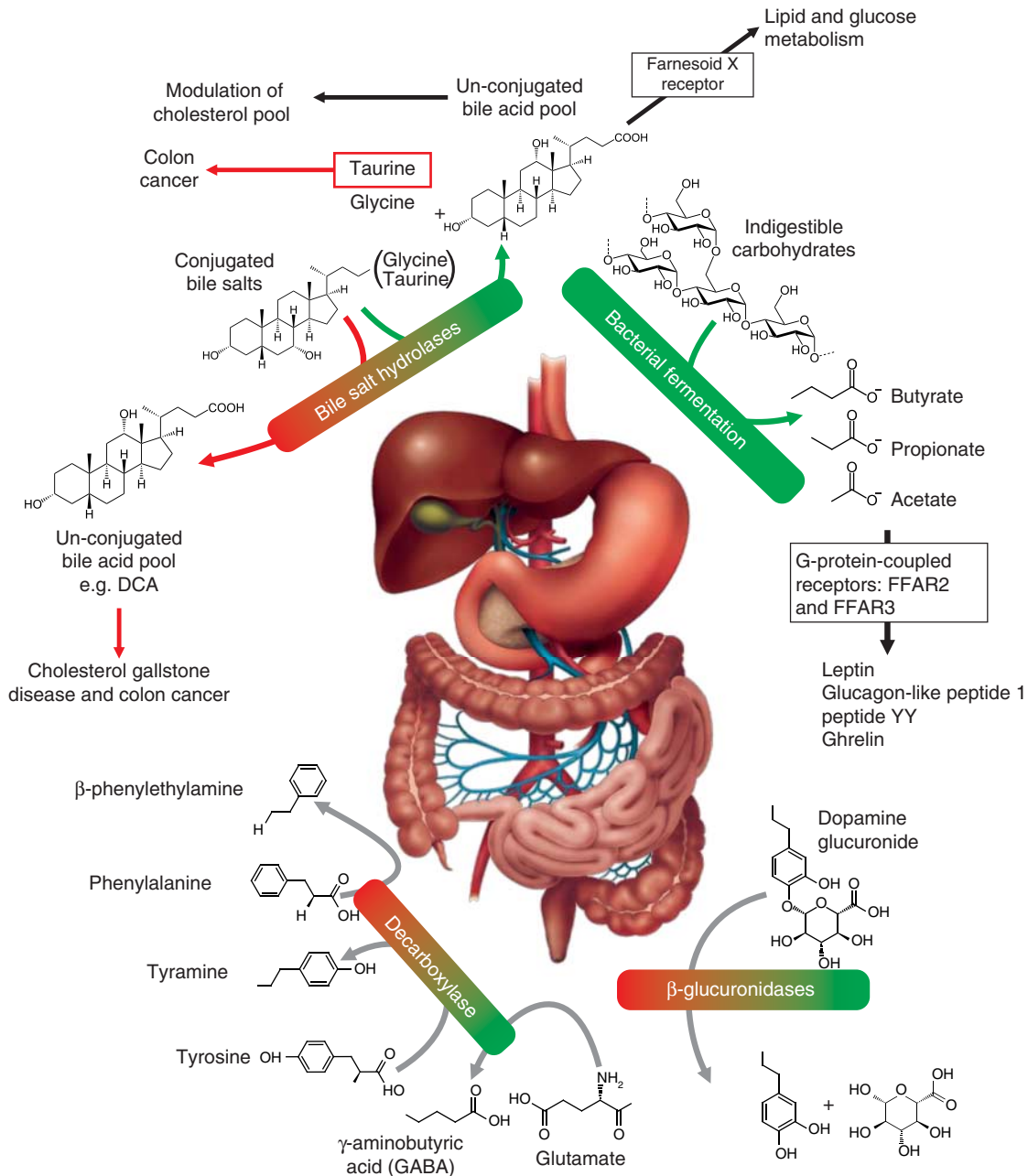
**Figure 1**

Schematic representation of areas of the rodent system that are significantly impacted by the absence of a normal microbiome; all the changes are those that are measured/observed in the sterile animal (adapted from Smith K,

McCoy KD & Macpherson AJ 2007 Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Seminars in Immunology* 19 59–69. (doi:10.1016/j.smim.2006.10.002).

(predominantly a C₂₄ structure (Russell 2003))-derived moiety that is wholly derived from cholesterol. This surfactant nature allows them to associate with fat molecules to form micelles, which are ultimately absorbed by the host, thus facilitating fat metabolism. Additionally, being a surfactant allows them to be antimicrobial also, as they can disrupt the plasma membrane of the bacteria, causing them to lyse and die; thus in a niche where food is plentiful, bile helps to prevent the bacteria in the small intestine from overgrowing and becoming a health issue. A secondary role for bile involves regulating the host's cholesterol levels, on a typical day, ~0.5 g of this steroid is used to synthesise bile acids in hepatocytes and accounts for 90% of the cholesterol usage (Russell 2003). Once the hormonal signal has been sent to the gall bladder, the bile acids are excreted into the small intestine, where they interact with the dietary lipids and fat-soluble vitamins. These complexes are eventually reabsorbed in the terminal ileum; this process is part of the enterohepatic circulation that ensures that 95% of the bile acids are recovered from the gut. The remaining 5% that escapes this pathway

enters the large intestine, where it becomes available for metabolism by bacteria. Interestingly, the gut bacteria have evolved several enzymes capable of modifying the primary bile acids such as the taurine- and glycine-conjugated cholic and chenodeoxycholic acids and removing the taurine and glycine parts of the molecules to produce secondary bile salts, such as cholic, lithocholic and deoxycholic acids. While some of these secondary bile acids are excreted in the faeces, a significant proportion are passively absorbed and returned to the liver. These secondary bile acids then enter the enterohepatic circulation and the general bile metabolite pool. The bacterial enzymes responsible for the deconjugation of either taurine or glycine are collectively known as bile salt hydrolases (BSHs), cholylglycine hydrolases or bile acid hydrolases (EC 3.5.1.24) and catalyse the hydrolytic removal of taurine or glycine from the corresponding primary bile acids. However, as with many gut functions, the diversity and abundance of BSHs are highly variable as are their substrate ranges (Jones *et al.* 2008). Additionally, studies on germ-free rodents (i.e. sterile or gnotobiotic)

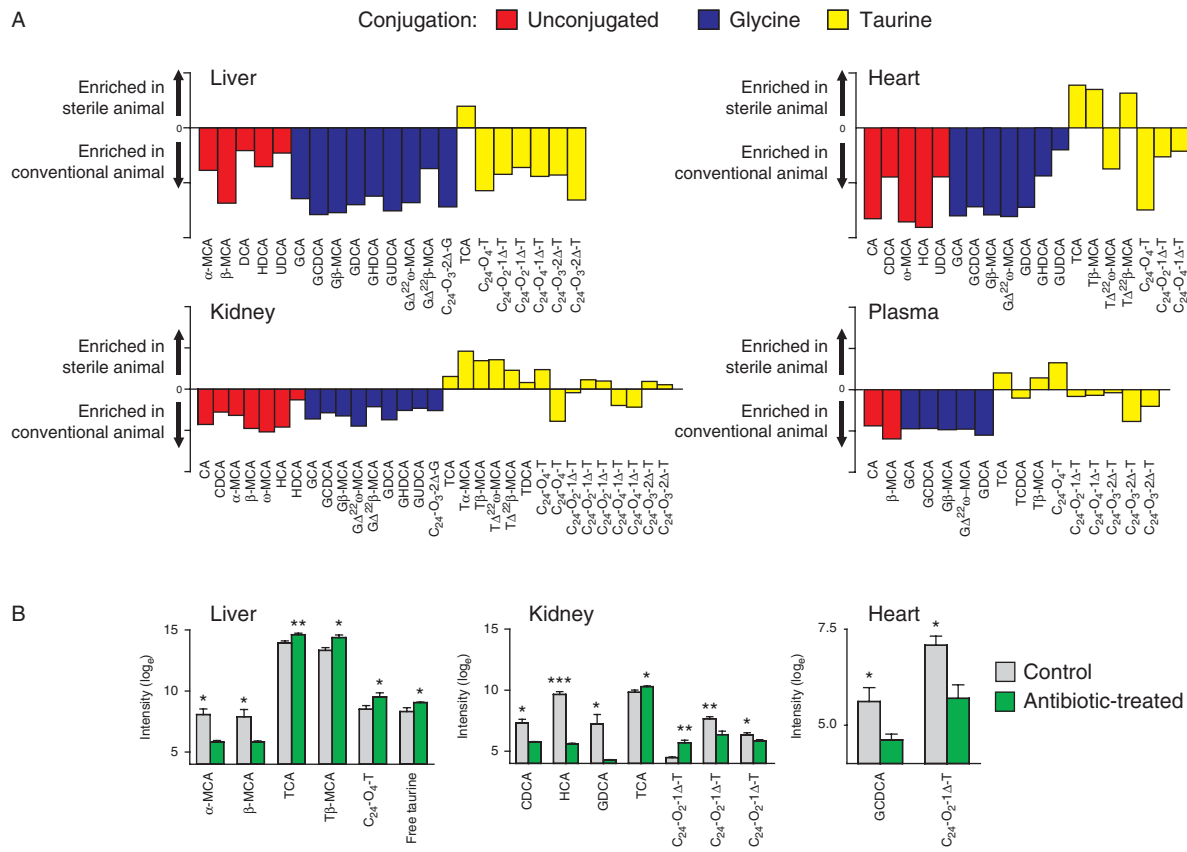
**Figure 2**

Schematic representation of some of the functions that the gut microbiota plays in the host. The four functions shown, those of bile salt hydrolases, bacterial fermentation, amino acid decarboxylases and glucuronidases, can

play a role in producing molecules that interact with the host's endocrine system. The red/green colour scheme implies that it is still unclear whether these functions are beneficial or detrimental to the host's physiology.

have shown that the bile pool is significantly and dramatically altered in other non-liver or non-gut compartments too, e.g. heart tissue (Fig. 3), which begs the question of the significance of having a specific BSH profile in the gut and the host's own physiology. Bile acids interact at an endocrinological level via three major signalling mechanisms, as ligands for the

G-protein-coupled receptor TGR₅, activators of the MAPK pathways and activators of the nuclear hormone receptors such as farnesoid X receptor α (FXR α ; NR1H4). While the primary bile acids are of significant interest as they have been shown to regulate lipid, energy and glucose metabolism, the secondary bile acids can also interact with these receptors. However, the availability of the

**Figure 3**

Differences in the bile profiles of sterile and normally colonised rats. (A) If the bar is above the origin (set at 0), the bile salt is enriched in the sterile animal when compared with the conventional animal and vice versa.

(B) Changes in bile metabolites when rats are treated with the antibiotics streptomycin and penicillin. (Values are means of the scaled data \pm S.E.M. Statistical significance was determined using Student's *t*-test. **P*<0.05;

P*<0.01; *P*<0.001 (modified from Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, Nicholson JK & Holmes E 2011 Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. *PNAS* 108(Suppl 1) 4523–4530. (doi:10.1073/pnas.1006734107)). Unconjugated bile acids are those that are produced by bacterial metabolism of the host conjugated acids.

secondary bile acids is not as tightly controlled since it is driven by the variable and dynamic diversity and expression of BSHs in the gut. Hence, the gut microbiota can be thought of as another environmental factor controlling an up-and-coming endocrine factor that is not stable and influenced by diet and medications.

SCFAs and the endocrine system

One of the most fundamental contributions that the gut microbiota makes to host function is the provision of SCFAs. These relatively simple molecules, predominantly butyrate, acetate and propionate, are all produced when certain classes of bacteria ferment a variety of carbon sources anaerobically. Although SCFAs are the end products of metabolism for some bacteria, they are a significant source of energy for the host. Both termites

(Warnecke *et al.* 2007) and ruminants (Brulc *et al.* 2009) have evolved a mutualistic relationship with the organisms that synthesise SCFAs, and it seems that humans too have co-evolved with these bacterial functions. The G-protein-coupled receptors free fatty acid receptor 2 (FFAR2, GPR43) and FFAR3 (GPR41) are the two endogenous receptors that have been identified to interact with SCFAs (Brown *et al.* 2003, Le Poul *et al.* 2003, Nilsson *et al.* 2003). FFAR2 and FFAR3 interact with the SCFAs (μ M–mM range) with a carbon chain length greater than six atoms and are those that are most likely to have evolved in response to the fermentation products of the gut bacteria. Both receptors are found in a variety of tissues including the gut and have been shown to be expressed in enteroendocrine cells that are producing peptide YY (PYY; Table 1). SCFAs have been shown to stimulate the release of PYY and 5-hydroxytryptamine (5-HT) from the

Table 1 Characteristics of free fatty acid receptor 2 and free fatty acid receptor 3

Nomenclature	FFAR2	FFAR3
Agonist (FFA)	Short-chain C3~C4~C2	Short-chain C3>C4>>C2
Gene	<i>GPR43</i>	<i>GPR41</i>
Localisation	19q13.1	19q13.1
Expression outside of the gut	Adipose tissue	Adipose tissue
Physiological role	PYY secretion 5-HT secretion	Leptin production PYY secretion

ileum and colon (Cherbut *et al.* 1998, Fukumoto *et al.* 2003). Mice that have had their FFAR3 knocked out (Samuel *et al.* 2008) show an associated reduction in the expression of PYY, increased intestinal transit rate and reduced harvest of energy (SCFAs) from the diet. These authors concluded that FFAR3 is involved in the regulation of host energy balance, which is ultimately driven by the gut microbiota and its metabolites. Additionally, the FFARs have been proposed to be involved in glucose intolerance and thus diabetes (Tolhurst *et al.* 2012). Tolhurst *et al.* have shown in their study that both FFAR2 and FFAR3 are more greatly expressed in glucagon-like peptide-1 (GLP1)-secreting cells. Additionally, when mice that were homozygous negative for both FFARs were exposed to SCFAs, there was a significant reduction in SCFA-triggered GLP1 secretion. The other significant role that these bacterially derived metabolites play is the stimulation of leptin production in adipocytes (Xiong *et al.* 2004); however, the exact mechanism is still being elucidated (Zaibi *et al.* 2010). Since leptin is involved in a wide range of physiological processes, such as feeding behaviour, reproduction and metabolic rate, any molecules that stimulate its production are of significant interest. All of the SCFAs have been shown to stimulate plasma leptin levels (Yonekura *et al.* 2003, Xiong *et al.* 2004), and when propionate is delivered orally, these levels are raised and the effect can be blocked by small-interfering RNAs targeted towards GPR41 in Ob-Luc cells (Xiong *et al.* 2004).

Although SCFAs have been shown to interact with their own cognate receptors, they have also been shown to modulate hepatic glucose production in humans (Thorburn *et al.* 1993). The intake of fibre, which acts as a prebiotic and is only metabolised by the gut bacteria, has been reported to be inversely associated with the risk of type 2 diabetes and heart disease (Pereira *et al.* 2002). The hypothesis that fibre is digested eventually to SCFAs is an important one in the area of the gut microbiota, because it

assumes that fibre ingested will be digested to SCFAs and that this leads to eventual benefits to the host. However, this treats the gut microbiota as a 'black box' and does not consider that individuals' capacity to metabolise the different components of the fibre and to ferment these degradation products to SCFAs is conditional on them having the necessary bacterial functions present in the first place. Bearing this in mind, it may be explained why the evidence is not strong as to the beneficial role of fibre in the reduction of the risk of developing diabetes and other diseases (Williams 2012, Caricilli & Saad 2013).

The brain-gut axis

The hypothalamic–pituitary–adrenal (HPA) axis has received much attention in recent years, in particular, its association with the gut and its resident microbiota. It is becoming increasingly apparent that this association can both positively and negatively affect host health, with implications for the gut microbiota itself and the endocrine system.

Studies using *in vitro* and *in vivo* models have hinted at the possible underlying mechanisms of the effect of stress on the gut through the HPA axis. It has been shown that this axis responds to stress (Herman *et al.* 2003) through the secretion of corticotropin-releasing hormone (CRH) from the hypothalamus, in turn triggering the release of ACTH from the pituitary gland and driving the release of catecholamines and cortisol from the cortex (Mawdsley & Rampton 2005). CRH has been shown to stimulate the release of pro-inflammatory cytokines such as TNF α , IL1 β and IL6 in *in vitro* and *in vivo* murine models (Agelaki *et al.* 2002), while the *in vivo* stimulation of β -adrenergic receptor, a receptor for the catecholamines nor-epinephrine and epinephrine, has been shown to increase the levels of circulating IL1 and IL6 (Johnson *et al.* 2005). The production of cytokines, in particular, TNF α , has been shown to affect the gut epithelial barrier function in both *in vitro* (Schmitz *et al.* 1999) and in human studies, where one study has shown the restoration of the gut epithelial barrier function in Crohn's disease patients through the administration of infliximab, a chimeric MAB against TNF α (Suenart *et al.* 2002). Bacterial cells can also disrupt the function of the gut epithelial membrane through lipopolysaccharide (LPS) and its interaction with myosin light chain kinase, a regulator of tight junctions (Shen *et al.* 2006). It has also been shown that the stress response of the HPA axis and 'leakiness' of the gut epithelial barrier in mice can be attenuated through feeding of the probiotic organism *Lactobacillus farciminis* (Ait-Belgnaoui *et al.*

2012). This highlights the two-way nature of the HPA axis and our resident gut microbiota.

A very common diagnosis in gastroenterological practice worldwide is irritable bowel syndrome (IBS), which is characterised by abnormal bowel function, bloating and abdominal pain or discomfort relieved by defaecation (Spiller *et al.* 2007). IBS significantly affects quality of life (Simren *et al.* 2006) and is a substantial economic burden to healthcare systems (Nyrop *et al.* 2007). IBS has been reported to be present in up to 10% of the population and responsible for 3.6% of GP consultations. So, understanding this syndrome is an important goal, and recently, in a landmark study, Dinan *et al.* (2006) have shown that in an IBS cohort the levels of cortisol and the pro-inflammatory cytokines IL6 and IL8 are elevated. Prior to this study, they had also used a bacterial intervention, in the form of two separate probiotics (*Lactobacillus salivarius* UCC4331 or *Bifidobacterium infantis* 35624), and shown that *B. infantis* is able to normalise the IL10:IL12 ratio. Hence, this again leads us to the conclusion that the variable levels of some organisms in the gut may be responsible for diseases/syndromes as they signal to a variety of host effectors.

Microbially derived endocrine molecules

Endocrine molecules are not solely produced by the human body; gut microbes can also produce these molecules. It has been shown previously that there is a significant level of dopamine production in the human gut (Eisenhofer *et al.* 1997). The importance of the gut microbiota in the generation of this compound has been overlooked, and in a subsequent piece of work, it has been shown that the gut microbiota plays a critical role in the production of norepinephrine and dopamine in the gut (Asano *et al.* 2012). This production is due to the expression of β -glucuronidases by commensal gut bacteria, generating dopamine and norepinephrine through the cleavage of their inactive conjugated forms. Gut microbes can also produce non-noradrenergic, non-cholinergic transmitters such as nitric oxide, which plays a pivotal role in the regulation of gastric emptying (Orihata & Sarna 1994), through the anaerobic reduction of nitrate to nitrogen (Sobko *et al.* 2005, Cutruzzolà 2012). The inhibitory transmitter γ -aminobutyric acid can be generated by *Lactobacillus brevis* and *Bifidobacterium dentium* (Barrett *et al.* 2012); both of these organisms can be isolated from humans (Rönkä *et al.* 2003, Ventura *et al.* 2009). Studies such as these are highlighting the previously unrealised importance of our own gut microbiota in generating compounds that interact with our own endocrine system.

Diabetes and gut bacteria

Diabetes is a chronic metabolic disorder that affects an estimated 347 million people throughout the world (WHO 2012). Type 1 diabetes, also known as juvenile diabetes, is a state of absolute deficiency of insulin, while type 2 diabetes is a state of relative insulin deficiency in the presence of obesity and insulin resistance; the prevalence of the latter is increasing among children due to the adoption of a sedentary lifestyle. There is increasing evidence that the gut microbiota plays a role in the development of this disorder. In the non-obese diabetic (NOD) murine model, which was also deficient for the adapter protein MYD88, researchers have shown that the gut microbiota plays a role in the development of type 1 diabetes (Wen *et al.* 2008). In this study, the islets of Langerhans of germ-free, specific pathogen-free and altered Schaedler flora (Dewhirst *et al.* 1999) *Myd88* knockout NOD mice were histologically compared; the germ-free mice exhibited a greater level of islet infiltration. The authors showed that the gut commensal bacteria can modify the development of this disease. With this in mind, research has been carried out to determine which bacterial species and groups in humans are most prevalent in the sufferers of type 1 diabetes. In a recent study (Giongo *et al.* 2011), the distal gut microbiota of four children with autoimmunity, who went on to develop type 1 diabetes, has been compared with that of four healthy children through pyrosequencing of faecal DNA extracted at three time points. The results highlighted differences between the two major phyla in the gut, *Bacteroidetes* and *Firmicutes*. There was an increase in the number of DNA sequences from the *Bacteroidetes* phylum as autoimmunity developed to type 1 diabetes and a reduction in the number of DNA sequences obtained from the *Firmicutes* phylum. In contrast, the four healthy children exhibited the opposite pattern, with the number of *Bacteroidetes* sequences decreasing and that of *Firmicutes* sequences increasing as each time point was analysed. It was also shown that the healthy control children had a higher diversity of bacterial species than the children with autoimmunity, suggesting a link between low bacterial species diversity and type 1 diabetes. This low diversity is indicative of a non-normal gut microbiota, which has been suggested to combine with intestinal leakiness and altered intestinal immune responsiveness to generate a 'perfect storm' for the development of type 1 diabetes (Vaarala *et al.* 2008).

The gut microbiota, through LPS, has been shown to exert a pro-inflammatory effect and when combined with

a high-fat diet leads to the onset of insulin resistance and, therefore, the development of type 2 diabetes in a mouse model (Cani *et al.* 2007, 2008). In accordance with what has been observed in type 1 diabetes, a metagenomic analysis of faecal samples of type 2 diabetes sufferers has shown a slight level of dysbiosis in their gut bacterial make-up; type 2 diabetes sufferers exhibited higher levels of opportunistic pathogens (Qin *et al.* 2012). Arguably more important is the fact that this research showed a reduction in the number of butyrate-producing bacteria, a SCFA that modulates the activity of NF- κ B (Inan *et al.* 2000), hinting that the loss of bacterial species may not be as important as the loss of functions that they play.

Microbial modulation of neurotransmitters: serotonin

One metabolic pathway of interest is the metabolism of tryptophan, a precursor for a number of metabolites, but particularly for the production of serotonin or 5-HT. Serotonin is a well-documented monoamine neurotransmitter that has been extensively studied due to its hypothesised role in the regulation of learning, mood, sleep, anxiety and other psychiatry-related afflictions. In recent years, serotonin has also gained increasing interest for its role in the area of gut pathophysiology (Gershon & Tack 2007) and its role as a signalling molecule linking the brain and the gut. Research has been fairly conclusive in demonstrating that bacteria can affect serotonin levels indirectly by stimulating secretion. However, recent evidence suggests a role for the gut microbiota in actually modulating the levels of the serotonin precursor tryptophan and hence having a control over serotonin levels in the host. Research by Desbonnet *et al.* (2008) has suggested a role for tryptophan producers such as *B. infantis* as probiotics specifically to aid in combating psychiatric disorders such as depression. Other groups have also shown a role for bacteria in modulating neurotransmitter precursors (Rhee *et al.* 2009, Wikoff *et al.* 2009, Heijtz *et al.* 2011).

Around 90% of serotonin is located in the enterochromaffin cells of the human gastrointestinal tract (Keszthelyi *et al.* 2009) and, therefore, must be intimately associated with the gastrointestinal mucosa and its microbiota. Enterochromaffin cells release serotonin in response to certain bacterial stimuli such as LPS, enzymes and other bacterial toxins to help increase gastric transit and rid the host of the toxin as quickly as possible (result is often diarrhoea). As a result of a bacterial stimulus, there is release of serotonin, which acts as a signalling molecule to activate primary sensory neurons to communicate with

the brain and ultimately alter secretory reflexes. Consequently, an ongoing brain–gut interaction is developed, exerting various effects on gut physiology. This relationship has resulted in serotonin potentially being one of the most significant molecules related to the pathophysiology of intestinal disorders such as inflammatory bowel disease and IBS and is also intrinsically linked to the anxiety and depression associated with such disorders.

If serotonin is in charge of mood and sleep and low levels are associated with the onset of depression, etc., then varying levels of its precursor, tryptophan, are likely to have an effect on available serotonin levels (Heijtz *et al.* 2011). Studies have shown that germ-free rats have depleted levels of tryptophan, but upon administration of certain bacteria, such as bifidobacterial species, tryptophan levels are increased (Desbonnet *et al.* 2008), thus suggesting that bacteria can alter the available serotonin pool and ultimately elicit communication between the gut and the brain. Some microbes, such as *Candida* spp., *Streptococcus* spp., *Escherichia* spp. and *Enterococcus* spp., have been shown to directly produce serotonin (Cryan & Dinan, 2012), although much more research is needed into the impact that this has on the host.

Conclusions

In the last decade, the paradigm that the human genome is the predominant driver of host health has shifted towards a more superorganism-based viewpoint, with the microbiome playing a significant role in influencing host physiology and function. This review has not sought to cover all the areas where there is evidence for a role of the host's microbiota and has unapologetically focused on the gut microbiota, since the gut is the best-studied and most densely populated niche. There are numerous other niches in the human body that are colonised and in which the adapted microbiome interacts with the host, and in due course, these will be explored and their interactions with the host described. We hope to have made it more obvious to the reader that the microbiome needs to be understood in more depth and integrated into the endocrine system as it is being integrated into the immune system. In the future, more 'omic' approaches will further uncover more associations between the human microbiome and the endocrine system, which may be drugable and thus modulated to the benefit of the host.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Author contribution statement

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References

- Agelaki S, Tsatsanis C, Gravanis A & Margioris AN 2002 Corticotropin-releasing hormone augments proinflammatory cytokine production from macrophages *in vitro* and in lipopolysaccharide-induced endotoxin shock in mice. *Infection and Immunity* **70** 6068–6074. (doi:10.1128/IAI.70.11.6068-6074.2002)
- Ait-Belgnaoui A, Durand H, Cartier C, Chaumaz G, Eutamene H, Ferrier L, Houdeau E, Fioramonti J, Bueno L & Theodorou V 2012 Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology* **37** 1885–1895. (doi:10.1016/j.psyneuen.2012.03.024)
- Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K, Koga Y & Sudo N 2012 Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **303** G1288–G1295. (doi:10.1152/ajpgi.00341.2012)
- Barrett E, Ross RP, O'Toole PW, Fitzgerald GF & Stanton C 2012 γ -Aminobutyric acid production by culturable bacteria from the human intestine. *Journal of Applied Microbiology* **113** 411–417. (doi:10.1111/j.1365-2672.2012.05344.x)
- Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ *et al.* 2003 The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *Journal of Biological Chemistry* **278** 11312–11319. (doi:10.1074/jbc.M211609200)
- Brulc JM, Antonopoulos DA, Miller ME, Wilson MK, Yannarell AC, Dinsdale EA, Edwards RE, Frank ED, Emerson JB, Wacklin P *et al.* 2009 Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. *PNAS* **106** 1948–1953. (doi:10.1073/pnas.0806191105)
- Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C *et al.* 2007 Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* **56** 1761–1772. (doi:10.2337/db06-1491)
- Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM & Burcelin R 2008 Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* **57** 1470–1481. (doi:10.2337/db07-1403)
- Caricilli AM & Saad MJA 2013 The role of gut microbiota on insulin resistance. *Nutrients* **5** 829–851. (doi:10.3390/nu5030829)
- Chen W, Liu F, Ling Z, Tong X & Xiang C 2012 Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PLoS ONE* **7** e39743. (doi:10.1371/journal.pone.0039743)
- Cherbut C, Ferrier L, Roze C, Anini Y, Blottiere H, Lecannu G & Galmiche JP 1998 Short-chain fatty acids modify colonic motility through nerves and polypeptide YY release in the rat. *American Journal of Physiology* **275** G1415–G1422.
- Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, Marchesi JR, Falush D, Dinan T, Fitzgerald G *et al.* 2011 Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *PNAS* **108** 4586–4591. (doi:10.1073/pnas.1000097107)
- Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HMB, Coakley M, Lakshminarayanan B, O'Sullivan O *et al.* 2012 Gut microbiota composition correlates with diet and health in the elderly. *Nature* **488** 178–184. (doi:10.1038/nature11319)
- Collins FS, Lander ES, Rogers J & Waterson RH 2004 Finishing the euchromatic sequence of the human genome. *Nature* **431** 931–945. (doi:10.1038/nature02945)
- Collins SM, Surette M & Bercik P 2012 The interplay between the intestinal microbiota and the brain. *Nature Reviews. Microbiology* **10** 735–742. (doi:10.1038/nrmicro2876)
- Cryan JF & Dinan TG 2012 Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews. Neuroscience* **13** 701–712. (doi:10.1038/nrn3346)
- Cutruzzolà F 2012 Nitric oxide synthesis by bacterial cytochrome cd1 nitrite reductases. *Nitric Oxide* **27**(Suppl) S10. (doi:10.1016/j.niox.2012.04.037)
- Desbonnet L, Garrett L, Clarke G, Bienenstock J & Dinan TG 2008 The probiotic *Bifidobacterium infantis*: an assessment of potential antidepressant properties in the rat. *Journal of Psychiatric Research* **43** 164–174. (doi:10.1016/j.jpsychires.2008.03.009)
- Dethlefsen L, Huse S, Sogin ML & Relman DA 2008 The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biology* **6** e280. (doi:10.1371/journal.pbio.0060280)
- Dewhirst FE, Chien CC, Paster BJ, Ericson RL, Orcutt RP, Schauer DB & Fox JG 1999 Phylogeny of the defined murine microbiota: altered Schaedler flora. *Applied and Environmental Microbiology* **65** 3287–3292.
- Dinan TG, Quigley EMM, Ahmed SMM, Scully P, O'Brien S, O'Mahony L, O'Mahony S, Shanahan F & Keeling PWN 2006 Hypothalamic–pituitary–gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker? *Gastroenterology* **130** 304–311. (doi:10.1053/j.gastro.2005.11.033)
- Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE & Relman DA 2005 Diversity of the human intestinal microbial flora. *Science* **308** 1635–1638. (doi:10.1126/science.1110591)
- Eisenhofer G, Aneman A, Friberg P, Hooper D, Fandriks L, Lonroth H, Hunyady B & Mezey E 1997 Substantial production of dopamine in the human gastrointestinal tract. *Journal of Clinical Endocrinology and Metabolism* **82** 3864–3871. (doi:10.1210/jc.82.11.3864)
- Fischbach MA & Sonnenburg JL 2011 Eating for two: how metabolism establishes interspecies interactions in the gut. *Cell Host & Microbe* **10** 336–347. (doi:10.1016/j.chom.2011.10.002)
- Fukumoto S, Tatewaki M, Yamada T, Fujimiya M, Mantyh C, Voss M, Eubanks S, Harris M, Pappas TN & Takahashi T 2003 Short-chain fatty acids stimulate colonic transit via intraluminal 5-HT release in rats. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **284** R1269–R1276.
- Gershon MD & Tack J 2007 The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology* **132** 397–414. (doi:10.1053/j.gastro.2006.11.002)
- Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, Drew JC, Ilonen J, Knip M, Hyöty H *et al.* 2011 Toward defining the autoimmune microbiome for type 1 diabetes. *ISME Journal* **5** 82–91. (doi:10.1038/ismej.2010.92)
- Harrell L, Wang Y, Antonopoulos D, Young V, Lichtenstein L, Huang Y, Hanauer S & Chang E 2012 Standard colonic lavage alters the natural state of mucosal-associated microbiota in the human colon. *PLoS ONE* **7** e32545. (doi:10.1371/journal.pone.0032545)
- Heijtz RD, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forsberg H & Pettersson S 2011 Normal gut microbiota modulates brain development and behavior. *PNAS* **108** 3047–3052. (doi:10.1073/pnas.1010529108)

- Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC & Cullinan WE 2003 Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo–pituitary–adrenocortical responsiveness. *Frontiers in Neuroendocrinology* **24** 151–180. (doi:10.1016/j.yfrne.2003.07.001)
- Human Microbiome Project Consortium (HMPC), Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, Creasy HH, Earl AM, FitzGerald MG *et al.* 2012 Structure function and diversity of the healthy human microbiome. *Nature* **486** 207–214. (doi:10.1038/nature11234)
- Inan MS, Rasoulpour RJ, Yin L, Hubbard AK, Rosenberg DW & Giardina C 2000 The luminal short-chain fatty acid butyrate modulates NF- κ B activity in a human colonic epithelial cell line. *Gastroenterology* **118** 724–734. (doi:10.1016/S0016-5085(00)70142-9)
- Jalanka-Tuovinen J, Salonen A, Nikkilä J, Immonen O, Kekkonen R, Lahti L, Palva A & de Vos WM 2011 Intestinal microbiota in healthy adults: temporal analysis reveals individual and common core and relation to intestinal symptoms. *PLoS ONE* **6** e23035. (doi:10.1371/journal.pone.0023035)
- Jernberg C, Löfmark S, Edlund C & Jansson JK 2010 Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* **156** 3216–3223. (doi:10.1099/mic.0.040618-0)
- Johnson JD, Campisi J, Sharkey CM, Kennedy SL, Nickerson M, Greenwood BN & Fleshner M 2005 Catecholamines mediate stress-induced increases in peripheral and central inflammatory cytokines. *Neuroscience* **135** 1295–1307. (doi:10.1016/j.neuroscience.2005.06.090)
- Jones BV, Begley M, Hill C, Gahan CGM & Marchesi JR 2008 Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *PNAS* **105** 13580–13585. (doi:10.1073/pnas.0804437105)
- Kachrimanidou M & Malisiovas N 2011 *Clostridium difficile* infection: a comprehensive review. *Critical Reviews in Microbiology* **37** 178–187. (doi:10.3109/1040841X.2011.556598)
- Keszthelyi D, Troost FJ & Masclee AAM 2009 Understanding the role of tryptophan and serotonin metabolism in gastrointestinal function. *Neurogastroenterology and Motility* **21** 1239–1249. (doi:10.1111/j.1365-2982.2009.01370.x)
- Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT & Ley RE 2011 Succession of microbial consortia in the developing infant gut microbiome. *PNAS* **108** 4578–4585. (doi:10.1073/pnas.1000081107)
- Kolmeder CA, de Been M, Nikkilä J, Ritamo I, Mättö J, Valmu L, Salojärvi J, Palva A, Salonen A & de Vos WM 2012 Comparative metaproteomics and diversity analysis of human intestinal microbiota testifies for its temporal stability and expression of core functions. *PLoS ONE* **7** e29913. (doi:10.1371/journal.pone.0029913)
- Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J *et al.* 2003 Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *Journal of Biological Chemistry* **278** 25481–25489. (doi:10.1074/jbc.M301403200)
- Li JV, Ashrafian H, Bueter M, Kinross J, Sands C, le Roux CW, Bloom SR, Darzi A, Athanasiou T, Marchesi JR *et al.* 2011 Metabolic surgery profoundly influences gut microbial–host metabolic cross-talk. *Gut* **60** 1214–1223. (doi:10.1136/gut.2010.234708)
- Louis P & Flint HJ 2009 Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiology Letters* **294** 1–8. (doi:10.1111/j.1574-6968.2009.01514.x)
- Macfarlane GT & Macfarlane S 2011 Fermentation in the human large intestine: Its physiologic consequences and the potential contribution of prebiotics. *Journal of Clinical Gastroenterology* **45** S120–S127. (doi:10.1097/MCG.0b013e31822fecfe)
- Marchesi JR 2010 Prokaryotic and eukaryotic diversity of the human gut. *Advances in Applied Microbiology* **72** 43–62.
- Mawdsley JE & Rampton DS 2005 Psychological stress in IBD: new insights into pathogenic and therapeutic implications. *Gut* **54** 1481–1491. (doi:10.1136/gut.2005.064261)
- Nilsson NE, Kotarsky K, Owman C & Olde B 2003 Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. *Biochemical and Biophysical Research Communications* **303** 1047–1052. (doi:10.1016/S0006-291X(03)00488-1)
- Nyrop KA, Palsson OS, Levy RL, Korff MV, Feld AD, Turner MJ & Whitehead WE 2007 Costs of health care for irritable bowel syndrome, chronic constipation, functional diarrhoea and functional abdominal pain. *Alimentary Pharmacology & Therapeutics* **26** 237–248. (doi:10.1111/j.1365-2036.2007.03370.x)
- O’Hara AM & Shanahan F 2006 The gut flora as a forgotten organ. *EMBO Reports* **7** 688–693. (doi:10.1038/sj.embor.7400731)
- Orihata M & Sarna SK 1994 Inhibition of nitric oxide synthase delays gastric emptying of solid meals. *Journal of Pharmacology and Experimental Therapeutics* **271** 660–670.
- Pennisi E 2003 A low number wins the GeneSweep pool. *Science* **300** 1484. (doi:10.1126/science.300.5625.1484b)
- Pereira MA, Jacobs DR, Pins JJ, Ratz SK, Gross MD, Slavin JL & Seaquist ER 2002 Effect of whole grains on insulin sensitivity in overweight hyperinsulinemic adults. *American Journal of Clinical Nutrition* **75** 848–855.
- Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T *et al.* 2010 A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464** 59–65. (doi:10.1038/nature08821)
- Qin JJ, Li YR, Cai ZM, Li SH, Zhu JF, Zhang F, Liang SS, Zhang WW, Guan YL, Shen DQ *et al.* 2012 A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* **490** 55–60. (doi:10.1038/nature11450)
- Ravussin Y, Koren O, Spor A, Leduc C, Gutman R, Stombaugh J, Knight R, Ley RE & Leibel RL 2012 Responses of gut microbiota to diet composition and weight loss in lean and obese mice. *Obesity* **20** 738–747. (doi:10.1038/oby.2011.111)
- Rhee SH, Pothoulakis C & Mayer EA 2009 Principles and clinical implications of the brain–gut–enteric microbiota axis. *Nature Reviews. Gastroenterology and Hepatology* **6** 306–314. (doi:10.1038/nrgastro.2009.35)
- Rönkä E, Malinen E, Saarela M, Rinta-Koski M, Aarnikunnas J & Palva A 2003 Probiotic and milk technological properties of *Lactobacillus brevis*. *International Journal of Food Microbiology* **83** 63–74. (doi:10.1016/S0168-1605(02)00315-X)
- Russell DW 2003 The enzymes, regulation and genetics of bile acid synthesis. *Annual Review of Biochemistry* **72** 137–174. (doi:10.1146/annurev.biochem.72.121801.161712)
- Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M *et al.* 2008 Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. *PNAS* **105** 16767–16772. (doi:10.1073/pnas.0808567105)
- Scanlan PD, Shanahan F, O’Mahony C & Marchesi JR 2006 Culture-independent analyses of the temporal variation of the dominant faecal microbiota and targeted bacterial sub-groups in Crohn’s disease. *Journal of Clinical Microbiology* **44** 3980–3988. (doi:10.1128/JCM.00312-06)
- Schmitz H, Fromm M, Bentzel CJ, Scholz P, Detjen K, Mankertz J, Bode H, Epple HJ, Riecken EO & Schulzke JD 1999 Tumor necrosis factor- α (TNF α) regulates the epithelial barrier in the human intestinal cell line HT-29/B6. *Journal of Cell Science* **112** 137–146.
- Shen L, Black ED, Witkowski ED, Lencer WI, Guerriero V, Schneeberger EE & Turner JR 2006 Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure. *Journal of Cell Science* **119** 2095–2106. (doi:10.1242/jcs.02915)
- Simren M, Svedlund J, Posserud I, Björnsson ES & Abrahamsson H 2006 Health-related quality of life in patients attending a gastroenterology

- outpatient clinic: functional disorders versus organic diseases. *Clinical Gastroenterology and Hepatology* **4** 187–195. (doi:10.1016/S1542-3565(05)00981-X)
- Smith K, McCoy KD & Macpherson AJ 2007 Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Seminars in Immunology* **19** 59–69. (doi:10.1016/j.smim.2006.10.002)
- Sobko T, Reinders CI, Jansson EÅ, Norin E, Midtvedt T & Lundberg JO 2005 Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite. *Nitric Oxide* **13** 272–278. (doi:10.1016/j.niox.2005.08.002)
- Spiller R, Aziz Q, Creed F, Emmanuel A, Houghton L, Hungin P, Jones R, Kumar D, Rubin G, Trudgill N *et al.* 2007 Guidelines on the irritable bowel syndrome: mechanisms and practical management. *Gut* **56** 1770–1798. (doi:10.1136/gut.2007.119446)
- Suenaert P, Bulteel V, Lemmens L, Noman M, Geypens B, Assche GV, Geboes K, Ceuppens JL & Rutgeerts P 2002 Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. *American Journal of Gastroenterology* **97** 2000–2004. (doi:10.1111/j.1572-0241.2002.05914.x)
- Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, Nicholson JK & Holmes E 2011 Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. *PNAS* **108**(Suppl 1) 4523–4530. (doi:10.1073/pnas.1006734107)
- Thorburn A, Muir J & Proietto J 1993 Carbohydrate fermentation decreases hepatic glucose output in healthy subjects. *Metabolism* **42** 780–785. (doi:10.1016/0026-0495(93)90249-N)
- Tims S, Derom C, Jonkers DM, Vlietinck R, Saris WH, Kleerebezem M, de Vos WM & Zoetendal EG 2013 Microbiota conservation and BMI signatures in adult monozygotic twins. *ISME Journal* **7** 707–717. (doi:10.1038/ismej.2012.146)
- Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, Cameron J, Grosse J, Reimann F & Gribble FM 2012 Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. *Diabetes* **61** 364–371. (doi:10.2337/db11-1019)
- Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP *et al.* 2009 A core gut microbiome in obese and lean twins. *Nature* **457** 480–484. (doi:10.1038/nature07540)
- Vaarala O, Atkinson MA & Neu J 2008 The 'perfect storm' for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. *Diabetes* **57** 2555–2562. (doi:10.2337/db08-0331)
- Ventura M, Turrone F, Zomer A, Foroni E, Giubellini V, Bottacini F, Canchaya C, Claesson MJ, He F, Mantzourani M *et al.* 2009 The *Bifidobacterium dentium* Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. *PLoS Genetics* **5** e1000785. (doi:10.1371/journal.pgen.1000785)
- Warnecke F, Luginbuhl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT, Cayouette M, McHardy AC, Djordjevic G, Aboushadi N *et al.* 2007 Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* **450** 560–565. (doi:10.1038/nature06269)
- Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, Hu C, Wong FS, Szot GL, Bluestone JA *et al.* 2008 Innate immunity and intestinal microbiota in the development of type 1 diabetes. *Nature* **455** 1109–1113. (doi:10.1038/nature07336)
- WHO 2012 Diabetes. World Health Organisation. Available at: <http://www.who.int/mediacentre/factsheets/fs312/en/index.html> [Accessed: 29/11/2012].
- Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC & Siuzdak G 2009 Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *PNAS* **106** 3698–3703. (doi:10.1073/pnas.0812874106)
- Williams PG 2012 Evaluation of the evidence between consumption of refined grains and health outcomes. *Nutrition Reviews* **70** 80–99. (doi:10.1111/j.1753-4887.2011.00452.x)
- Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM & Yanagisawa M 2004 Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. *PNAS* **101** 1045–1050. (doi:10.1073/pnas.2637002100)
- Yap IK, Li JV, Saric J, Martin FP, Davies H, Wang Y, Wilson ID, Nicholson JK, Utzinger J, Marchesi JR *et al.* 2008 Metabonomic and microbiological analysis of the dynamic effect of vancomycin-induced gut microbiota modification in the mouse. *Journal of Proteome Research* **7** 3718–3728. (doi:10.1021/pr700864x)
- Yonekura S, Senoo T, Kobayashi Y, Yonezawa T, Katoh K & Obara Y 2003 Effects of acetate and butyrate on the expression of leptin and short-form leptin receptor in bovine and rat anterior pituitary cells. *General and Comparative Endocrinology* **133** 165–172. (doi:10.1016/S0016-6480(03)00162-X)
- Zaibi MS, Stocker CJ, O'Dowd J, Davies A, Bellahcene M, Cawthorne MA, Brown AJH, Smith DM & Arch JRS 2010 Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. *FEBS Letters* **584** 2381–2386. (doi:10.1016/j.febslet.2010.04.027)
- Zhang H, Dibaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, Parameswaran P, Crowell MD, Wing R, Rittmann BE *et al.* 2009 Human gut microbiota in obesity and after gastric bypass. *PNAS* **106** 2365–2370. (doi:10.1073/pnas.0812600106)

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