Effects of *Brassica* on the human gut microbiota

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Abstract

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Brassica vegetables, such as broccoli, are characterised by the presence of sulphur-containing compounds, termed glucosinolates, which are associated with potential health benefits for humans. Glucosinolates are metabolised in the gut by members of the gut microbiota, producing biologically active breakdown products, such as isothiocyanates. The effects of consuming *Brassica* on the composition of the gut microbiota, and the bacterial mechanisms employed for glucosinolate metabolism, are unclear, and forms the basis of the research presented in this thesis.

Culturing human faecal microbiotas in an *in vitro* batch fermentation model identified the bacterial-mediated reduction of glucoraphanin and glucoiberin to glucoerucin and glucoiberverin, respectively. An *Escherichia coli* strain was found to exhibit reductase activity on glucoraphanin and the broccoli-derived compound *S*-methylcysteine sulphoxide, through the reduction of the sulphoxide moiety. Within this fermentation model, the relative proportions of members of the genus *Lactobacillus* were found to significantly increase when the microbiota was repeatedly exposed to a broccoli leachate, and 16S rDNA sequencing identified these as *L. fermentum*. Metabolite analysis detected relatively high concentrations of lactate and short-chain fatty acids when faecal microbiotas were cultured in the presence of broccoli leachates, compared to a glucose control media.

A human dietary study investigating the effects of *Brassica* on the microbiota composition revealed a significant association between dietary *Brassica* and changes to the relative proportions of a number of bacteria, many of which belong to the *Clostridiales*. Further studies are required to reveal the nature of this association, and whether the presence of glucosinolates may have been a factor.

The work presented in this thesis highlights the strong connection between diet, the gut microbiota, and the potential health benefits to the host that may be derived from the bacterial metabolism of dietary compounds.

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Abbreviations

16S rDNA	16S ribosomal deoxyribonucleic acid
^{1}H NMR	Proton nuclear magnetic resonance
4-MIND	4-methoxyindolylmethyl
ACN	Acetonitrile
AHVLA	Animal Health and Veterinary Laboratories agency
amiRNA	Artificial microRNA
ANOVA	Analysis of variance
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine triphosphate
BAT	Bile acid transporter
BCAT	Branched-chain aminotransferase
BF	Burkino Faso
BHI	Brain heart infusion
BITC	Benzyl isothiocyanate
BL media	Broccoli leachate-containing media
BMI	Body Mass Index
$C_6H_{17}N_3O_7$	Ammonium citrate dibasic
$CaCl_2.6H_2O$	Calcium chloride hexahydrate
Cat.	Catalogue number
$CH_3COONa.3H_2O$	Sodium acetate trihydrate
CNM	Chemostat nutrient media
CO_2	Carbon dioxide
COG	Clusters of Orthologous Groups of proteins
C-S	Carbon-sulphur
CYP	Cytochrome P450 enzyme
D_2O	Deuterium oxide
DES	DNA elution solution
DHAP	Dihydroxyacetone phosphate
Diff	Difference
DmsABC	Dimethyl sulphoxide reductase
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EBL	Effects of *Brassica* on human gut lactobacilli
E-NAC	Erucin *N*-acetylcysteine
ENGAGE	The conversion of encapsulated glucoraphanin, gut microbiota phylogeny and genotype
ESM	Epithiospecifier modifier
ESP	Epithiospecifier protein
Fe^{2+}	Ferrous ion
Ffh	Fifty-four-homolog
FimA	Type-1 fimbrial protein

FMO	Flavin monooxygenase
FMT	Faecal microbiota transplantation
γ-Glu	γ-Glutamyl
GC-MS	Gas chromatography-mass spectrometry
G-ERN	Glucoerucin
GGP	γ-glutamyl peptidase
GIP	Gastric inhibitory peptide
GI	Glucoiberin
Glc	Glucose
Gly	Glycine
GL media	Glucose media
Gln	Glutamine
GLP-1	Glucagon-like peptide 1
Glu	Glutamate
GP	General practitioner
GPR	G protein-coupled receptors
GR	Glucoraphanin
GSH	Glutathione
GST	Glutathione S-transferase
H^+	Hydron
H_2	Hydrogen gas
H_2O	Water
H_2S	Hydrogen sulphide
HAG	High aliphatic glucosinolate
HCl	Hydrochloric acid
HIG	High indolic glucosinolate
His	Histidine
HPLC	High-performance liquid chromatography
HREC	Hertfordshire Research Ethics Council
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
Ile	Isoleucine
ITC	Isothiocyanate
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Monopotassium phosphate
LAB	Lactic acid bacteria
LB	Luria broth
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Leu	Leucine
Lys	Lysine
Max.	Maximum
MeOH	Methanol
Mg^{2+}	Magnesium ion
$MgSO_4$	Magnesium sulphate
MID	Multiplex identifier

MRP	Multidrug resistance associated-protein
MS	Mass spectrometer
$MgSO_4.6H2O$	Hexahydrite
MRM	Multiple reaction monitoring
MRS	Man Rogosa Sharpe
Msr	Methionine sulphoxide reductase
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Monosodium phosphate
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium azide
NAT	*N*-acetyltransferases
NCT	National Clinical Trial
Nic	Nicotinamide
Nico	Nicotinate
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
NSP	Nitrile specifier protein
Nucleo	Nucleosides/tides
OD_{600}	Optical density at 600 nm
OTUs	Operational taxonomic units
PapC	Pyelonephritis-associated pili C
PBS	Phosphate buffered saline
PC	Principal component
PCA	Principal component analysis
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PD	Phylogenetic distance
PDMS/DVB	Polydimethylsiloxane/divinylbenzene
Pgp-1	P-glycoprotein-1
Phe	Phenylalanine
PPS	Protein precipitation solution
Pro	Proline
Pyro	Pyroglutamate
PYY	Peptide YY
QIIME	Quantitative Insights Into Microbial Ecology
QTL	Quantitative trait locus
rDNA	Ribosomal deoxyribonucleic acid
REC	Research Ethics Council
RDP	Ribosomal database project
RNA-Seq	RNA sequencing
RT	Retention time
SCFA	Short-chain fatty acid
SD	Standard deviation
SEWS-M	Salt/ethanol wash solution
SF	Sulforaphane

SF-NAC	Sulforaphane *N*-acetylcysteine
SIN	Sinigrin
SMC	S-methylcysteine
SMCSO	S-methylcysteine sulphoxide
SOT	Sulphotransferase
Sp.	Species
SPME	Solid phase microextraction
SUR	S-alkyl-thiohydroximate lyase
TBE	Tris/borate/ethylenediaminetetraacetic acid
TFP	Thiocyanate forming protein
tRFLP	Terminal restriction fragment length polymorphism
Trp	Tryptophan
TSP	Sodium 3-(Trimethylsilyl)-propionate- d_4
Tyr	Tyrosine
UGT	Uridine diphosphate glycosyltransferase
UK	United Kingdom
Ura	Uracil
USA	United States of America
UV	Ultraviolet
Val	Valine
Var.	Variety
vs	Versus

Symbols

±	plus/minus
%	percentage
˚C	degrees Celsius
bp	base pair
cm	centimetre
g	gram
GF/A	Glass fibre grade A
GF/B	Glass fibre grade B
GF/D	Glass fibre grade D
hr	hour
Hz	hertz
id	internal diameter
Kb	Kilobase
Kg/m^2	Kilograms per square metre
L	Litre
µg/ml	micrograms per millilitre
µl	microliter
µmol/g	micromoles per gram
µm	micrometre
µM	micromoles per litre
M	Moles per litre
mbar	millibar
mg	milligram
mg/ml	milligrams per millilitre
MHz	Megahertz
min	minute
ml	millilitre
ml/min	millilitre per minute
mm	millimetre
mM	millimoles per litre
ms	milliseconds
ng/ml	nanograms per millilitre
nm	nanometre
pH	potential of Hydrogen
ppm	parts per million
rpm	revolutions per minute
s	seconds
T0	pre-inoculation
T12	12 hours post-inoculation
T24	24 hours post-inoculation
x g	times gravity
w/v	mass per volume

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CHAPTER ONE

1. General introduction

1.1 Dietary Brassica *vegetables*

The family *Brassicaceae* consists of approximately 3,500 species within 350 genera, including the genus *Brassica* [1]. Several important food crops are cultivars within the species *Brassica oleracea*, including broccoli (var. *italica*), cauliflower (var. *botrytis*), and cabbage (var. *capitata*). In addition, members of *B. rapa* (e.g. pak choi), *B. napus* (e.g. swede), and *B. juncea* (e.g. mustard greens) are also regularly consumed worldwide [2]. *Brassica* vegetables are a rich source of fibres, vitamins, minerals, carotenoids, and phenolic compounds, and contain relatively high levels of sulphur-containing compounds, such as *S*-methylcysteine sulphoxide (SMCSO) and glucosinolates, which add to the characteristic flavour and aroma of these vegetables [3, 4]. Traka *et al* performed total sulphur analysis on several broccoli cultivars, including the Beneforté® high-glucoraphanin hybrids, and found that much of the sulphur was incorporated into sulphate (29.4%), methionine-derived glucosinolates (21.4%), and SMCSO (13.6%) [5].

1.1.1 Glucosinolate structure

Glucosinolates are typical of the order Capparales, but are also found in the genus *Drypetes*, of the family *Putranjivaceae*. There are between 120 and 200 known glucosinolates, which all share a basic chemical structure (**Figure 1.1**) [6, 7]. Glucosinolates are β-thioglucoside *N*-hydroxysulphates, with a sulphur-linked β-D-glucopyranose moiety and a variable side-chain (*R*), which is derived from one of eight amino acids: alanine, phenylalanine, leucine, isoleucine, tyrosine, tryptophan, valine, methionine, and chain-elongated homologs of methionine and phenylalanine [7-10].

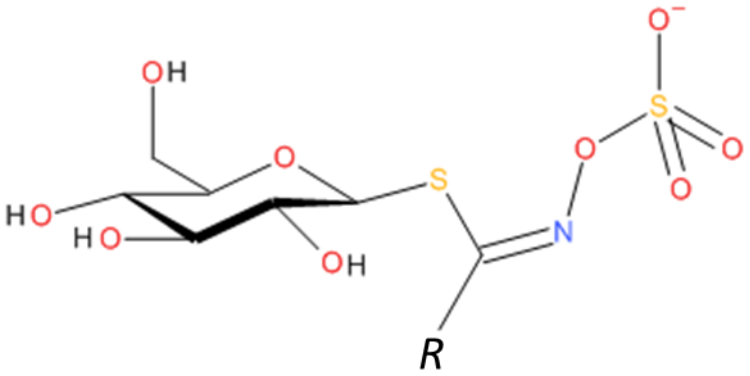


Figure 1.1: The basic structure of the glucosinolate molecule, where *R* denotes the variable side-chain.

Each glucosinolate contains a central carbon atom that is bound to a thioglucose group via a sulphur atom, to a sulphate group via a nitrogen atom, and to a side group [11]. The variation in side groups amongst glucosinolates is responsible for the varying biological activities of these compounds. Aliphatic glucosinolates are derived from alanine, leucine, isoleucine, methionine or valine, whereas those that are derived from phenylalanine or tyrosine are aromatic glucosinolates, and indole glucosinolates are derived from tryptophan [12]. The structures of some of the more abundant glucosinolates observed in nature can be found in **Table 1.1** and **Table 1.2**.

Table 9.1: The most abundant aliphatic glucosinolates found in nature.

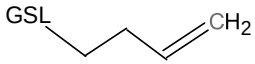
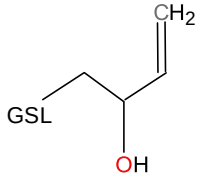
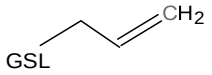
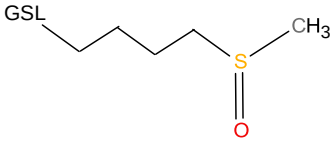
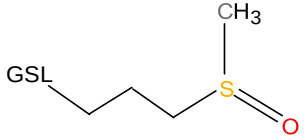
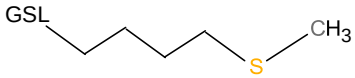
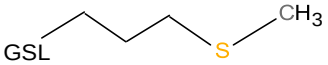
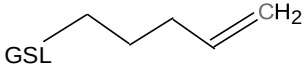
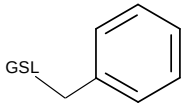
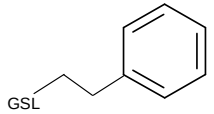
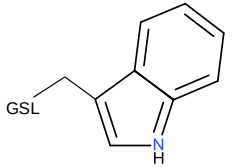
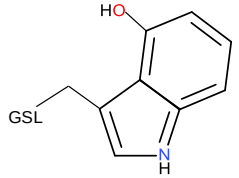
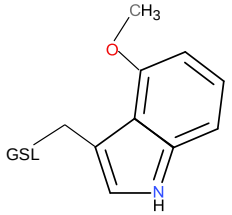
Class	Semi-systematic name	Trivial name	*R* group
Aliphatic	3-Butenyl	Gluconapin	
	(R)-2-Hydroxy-3-butenyl	Progoitrin	
	2-Propenyl	Sinigrin	
	4-Methylsulphinylbutyl	Glucoraphanin	
	3-Methylsulphinylpropyl	Glucoiberin	
	4-Methylthiobutyl	Glucoerucin	
	3-Methylthiopropyl	Glucoiberverin	
	4-Pentenyl	Glucobrassicanapin	

Table 1.2: The most abundant aromatic and indolic glucosinolates found in nature.

Class	Semi-systematic name	Trivial name	*R* group
Aromatic	Benzyl	Glucotropaeolin	
	2-Phenylethyl	Gluconasturtiin	
Indolic	3-Indolylmethyl	Glucobrassicin	
	4-Hydroxy-3-indolylmethyl	4-hydroxyglucobrassicin	
	4-Methoxy-3-indolylmethyl	4-Methoxyglucobrassicin	

1.1.2 Biosynthesis of glucosinolates

Biosynthesis of glucosinolates can be divided into three stages: chain elongation of pre-cursor amino acids (methionine & phenylalanine only), formation of the core glucosinolate structure, and secondary modifications of the amino acid side-chain.

1.1.2.1 Chain elongation

The largest group of chain-elongated glucosinolates are the methionine-derived glucosinolates [13]. Using the plant model, *Arabidopsis thaliana*, the first deamination reaction was identified as being performed by branched-chain amino acid aminotransferase 4 (BCAT4) [14, 15], whilst the transamination that yields homomethionine was accomplished by BCAT3 (**Figure 1.2**).

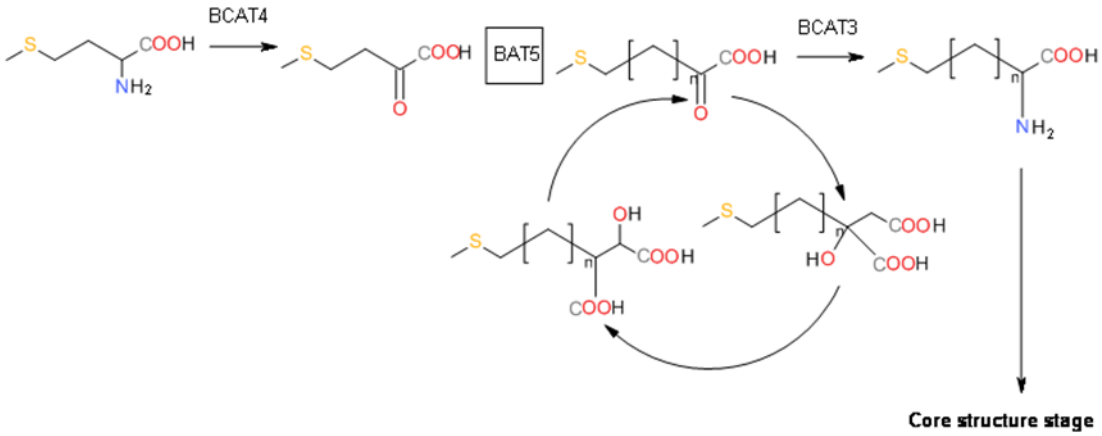


Figure 1.2: The chain elongation stage in the biosynthetic pathway for aliphatic and indolic glucosinolates in *Arabidopsis* (adapted from [15]). Abbreviations: BCAT = branched-chain amino acid aminotransferase; BAT = bile acid transporter.

BCAT4 is localised in the cytosol, whilst the remaining enzymes involved in chain elongation are localised in the chloroplast. The biosynthesis of the core glucosinolate structure occurs in the cytosol, indicating that 2-oxo acids are imported into the

chloroplast and the resulting chain-elongated amino acids are exported back out to the cytosol [12, 15, 16]. Based on co-expression analysis, and its transactivation by three aliphatic glucosinolate regulators (MYB28, MYB29 and MYB76), the chloroplast-localised bile acid transporter BAT5 was identified as a candidate transporter [15, 17, 18]. Work involving *bat5* knockdown mutants, *BAT5* amiRNA knockdown plants, and *in planta* feeding studies suggest that BAT5 functions as an importer of 2-oxo acids into the chloroplast, but the role of BAT5 has yet to be conclusively established [17-19].

1.1.2.2 Core structure

The construction of the glucosinolate core begins with cytochromes P450, of the CYP79 family, converting the precursor amino acids to aldoximes (**Figure 1.3**) [15, 20-23]. The aldoximes are oxidised to either nitrile oxides or *aci*-nitro compounds by cytochromes P450, of the CYP83 family. CYP83A1 converts the aliphatic aldoximes, and CYP83B1 metabolises the phenylalanine-derived and tryptophan-derived acetaldoximes [15, 24-26]. Following the conjugation of the activated aldoximes to a sulphur donor, these products are converted to thiohydroximates by the carbon-sulphur (C-S) lyase, SUR1 [27]. The thiohydroximates are then *S*-glycosylated by glucosyltransferases of the UGT74 family to form desulphoglucosinolates. Finally, the desulphoglucosinolates are sulphated by the sulphotransferases SOT16, SOT17 and SOT18 to form glucosinolates. SOT16 metabolises tryptophan- and phenylalanine-derived desulphoglucosinolates, whilst SOT17 and SOT18 favour long-chained aliphatic substrates [28].

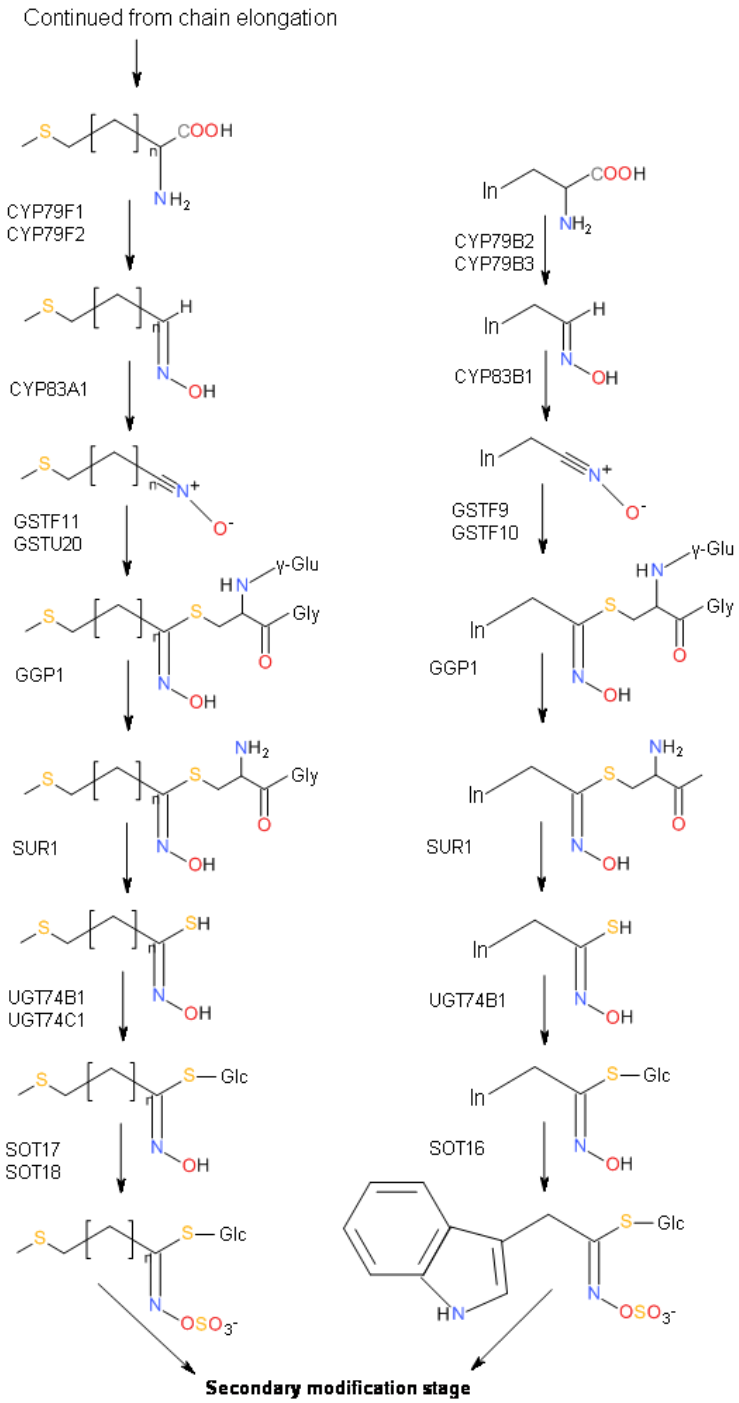


Figure 1.3: Biosynthesis of the core glucosinolate structure for aliphatic (left) and aromatic/indolic glucosinolates (right) in *Arabidopsis* (adapted from [15]). Abbreviations: Glc = glucose; Gly = glycine; In = 3'-indolyl; γ-Glu = γ-glutamyl

1.1.2.3 Side-chain modification

The biological activity of glucosinolates is largely determined by the structure of the side-chain. Secondary modifications for aliphatic glucosinolates include alkenylations, hydroxylations, oxygenations and benzoylations, whilst those of aromatic and indolic glucosinolates consist of hydroxylations and methoxylations [15]. Quantitative trait locus (QTL) analyses have identified four gene loci responsible for side-chain variability of aliphatic glucosinolates in *Arabidopsis*, named *GS-ELONG* (related to methionine side-chain elongation)*, GS-OX, GS-AOP* and *GS-OH* [29]. Studies on the *Brassicaceae*-specific subgroup of flavin monooxygenase (FMO) genes ($FMO_{GS\text{-}OX1\text{-}5}$) have shown that $FMO_{GS\text{-}OX1\text{-}5}$ *S*-oxygenate short- and long-chained aliphatic glucosinolates, albeit with different chain-length specificity [15, 30, 31].

1.1.2.4 Exploiting the regulatory gene **MYB28**

A complex network of transcription factors regulates the biosynthesis of glucosinolates in response to both abiotic and biotic stress. Of these factors, the R2R3 MYB family are the best characterised and can be divided into two groups: high aliphatic glucosinolate (HAG) 1-3, and high indolic glucosinolate (HIG) 1-3. HAG 1-3 is comprised of MYB28, MYB76 and MYB29, which are specifically involved in the control of aliphatic glucosinolate synthesis [32-36]. High-glucoraphanin broccoli hybrids were developed by crossing standard broccoli cultivars with the wild broccoli species, *Brassica villosa* [5]. These hybrids were commercialised under the name Beneforté® broccoli, and contain 2.5 – 3 times the glucoraphanin content of standard broccoli cultivars. The increased glucoraphanin content is considered to be due to the hybrids being heterozygous for *Myb28 B. villosa* allele and a *Myb28* broccoli allele [5]. The Myb28 transcription factor has been demonstrated to play an important role in the biosynthesis of methionine-derived glucosinolates, and sulphate assimilation [33, 35, 37].

1.1.3 Plant myrosinase enzymes

Glucosinolates are hydrolysed by glycosylated thioglucosidases called myrosinases (E.C 3.2.1.147), which produce an unstable thiohydroximate-*O*-sulphonate (**Figure 1.4**).

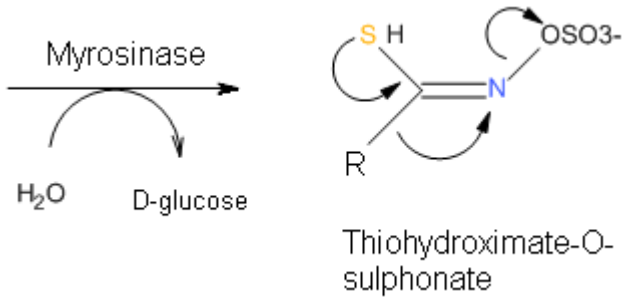


Figure 1.4: The production of the thiohydroximate-*O*-sulphonate though the action of plant myrosinase.

Glucosinolates are spatially separated from myrosinases within the plant, but tissue damage, such as can be caused by infection, pest attack, or mechanical damage, results in cellular breakdown and exposes the glucosinolates to the myrosinase enzyme. *O*-glycosidases are ubiquitous in plants, but myrosinase is the only known *S*-glycosidase, and it is strongly activated by ascorbic acid [38]. Myrosinase is a member of the glycoside hydrolase family 1, but unlike other members of the family, it lacks the acid/base residue in its active site [39-41]. Although its active site resembles that of the *O*-glycosidases, they differ with respect to one of the catalytic residues: whereas *O*-glycosidases have a carboxylate residue, which functions as the general base, myrosinase has a glutamine residue [39]. The hydrolysis of glucosinolates by myrosinases releases glucose and sulphate (via a Lossen-like rearrangement), and produces an isothiocyanate (ITC), thiocyanate, nitrile, epithionitrile, oxazolidine-2-thione, or other less common products (**Figure 1.5**) [42, 43].

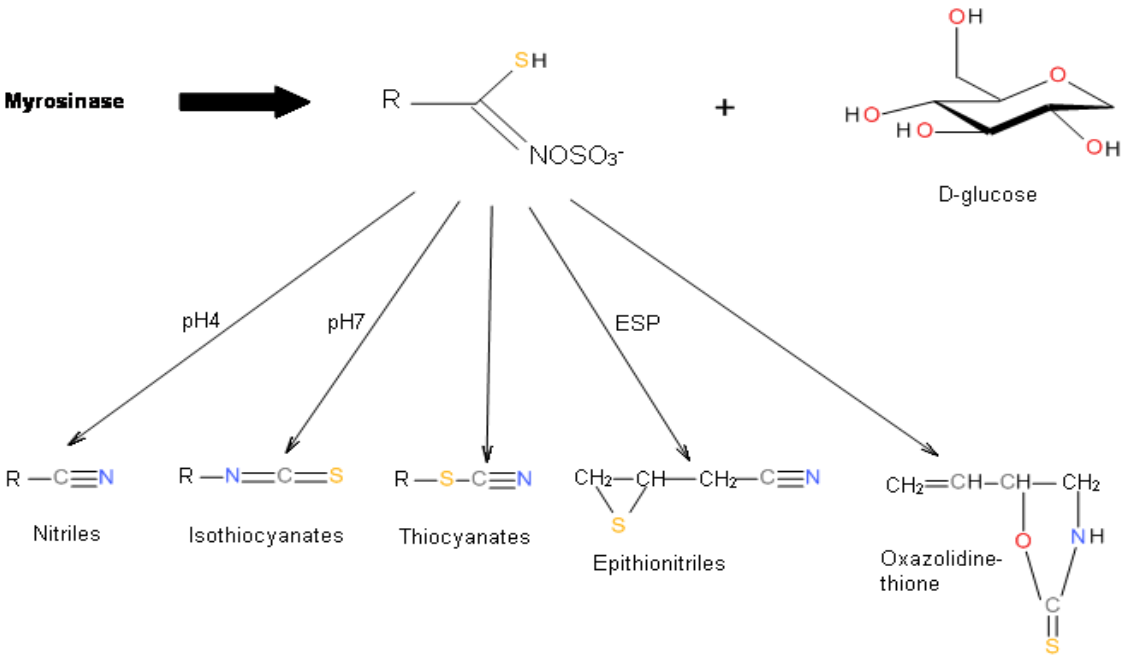


Figure 1.5: The generation of glucosinolate hydrolysis products by myrosinase, via the formation of thiohydroximate-*O*-sulphonate. The conditions that are believed to favour particular hydrolysis products are indicated (adapted from [43]). Abbreviations: ESP = Epithiospecifier protein.

1.1.4 Glucosinolate hydrolysis products

Several factors influence the formation of different hydrolysis products, such as the glucosinolate structure, pH, and the presence of metal ions (e.g. Fe^{2+}) or specifier proteins. For example, ITCs are generally produced at a neutral pH (~pH 7), whereas nitriles are thought to be favoured at a lower pH (~pH 4) [44]. A recent study observed nitrile production within a pH range of 3.7 – 7.6 during bacterial fermentations with glucosinolates, however it is possible that this may be reflective of differences between the plant and bacterial enzyme activities [45]. It has also been shown that nitriles are produced when glucosinolates undergo non-enzymatic hydrolysis catalysed by Fe^{2+} [46].

The most prevalent products are ITCs, which result from a spontaneous Lossen-like rearrangement in the absence of the epithiospecifier protein (ESP) [42]. ITCs play an important part in plant defence and are toxic to a diverse range of organisms including insects, bacteria, fungi and nematodes. It has been shown that ITCs can cleave disulphide bonds and react with amino acids *in vitro* [47]. The volatility and polarity is determined by the molecular structure of the ITC and this enables access to different targets within an organism. An increase in molecular weight increases the toxicity, and aromatic ITCs have been found to be more toxic than aliphatic ITCs to various organisms [48-50]. Nitriles, epithionitriles, and organic thiocyanates are formed instead of ITCs when myrosinase and specifier proteins are both present.

1.1.4.1 Epithiospecifier protein

If ESPs are present during the myrosinase-catalysed hydrolysis of glucosinolates, these specifier proteins can redirect hydrolysis to generate nitriles or epithionitriles. It is believed that ferrous ions allow the formation of an intermediate between the thiohydroximate and ESP [42, 51-53]. Although the proteins do not form a stable complex, physical contact between ESP and myrosinase is necessary for epithionitrile formation to occur, and it has been predicted that ESPs contain a series of β-sheets called Kelch-motifs, which may play an important role in the interaction between ESP and myrosinase [54-56]. Another factor in the formation of nitriles or epithionitriles is the QTL named *epithiospecifier modifier 1* (*ESM1*), which acts in concert with ESP to epistatically affect nitrile formation by repressing the production of nitriles in favour of the formation of ITCs from methionine-derived glucosinolates [57].

1.1.4.2 Thiocyanate forming protein

The formation of organic thiocyanates, via a thiocyanate forming protein (TFP), is not as prevalent as that of nitriles and epithionitriles. TFP activity has only been identified in a limited number of plant species including *Thlaspi arvense*, *Eruca sativa*, *Alliaria petiolata*, and *Lepidium sativum* [58]. The TFPs currently identified have differing substrate/product specificities, with some TFPs limited to benzylthiocyanate formation, and others limited to allylthiocyanate formation, from benzyl- and allylglucosinolate hydrolysis, respectively [58]. To date, organic thiocyanates have only been found to be generated from three glucosinolates: allylglucosinolate, benzylglucosinolate, and 4-methylthiobutylglucosinolate [56]. As with ESP, TFP also contain Kelch repeats [59]. The structure of the glucosinolate is an important factor that contributes to whether ESP or TFP will generate epithionitriles or organic thiocyanates, respectively. Glucosinolates must have a terminal double bond in their side-chain for epithionitriles to be formed, and organic thiocyanate formation has only been found in glucosinolates that have side-chains that can form a stable carbocation [56]. If these structures are absent, simple nitriles will be generated upon myrosinase-catalysed hydrolysis in the presence of specifier proteins [60-64].

1.1.4.3 Nitrile specifier protein

The nitrile specifier protein (NSP) was first identified in the larvae of *Pieris rapae* (cabbage white butterfly). It does not contain Kelch motifs, but it is made up of sequence repeats of approximately 200 amino acids each, and contains a signal peptide for excretion into the gut lumen. In the presence of NSP, myrosinase-catalysed hydrolysis of glucosinolates results in the generation of simple nitriles, instead of ITCs, which are not harmful to the larvae and are excreted in the faeces [56, 65, 66]. However, this protein did not display sequence similarity to plant ESP and, at the time, a comparable plant NSP had not been found. In 2009, six *A. thaliana* genes with a

relatively high degree of sequence similarity to *A. thaliana* ESP were identified and, of these, five were found to exhibit NSP activity (*NSP1*, *NSP2*, *NSP3*, *NSP4*, and *NSP5*). The sixth gene was deficient for specifier activity and was hypothesised to represent the ancestor of the gene family [52, 67]. A recent study performed phylogenetic analyses on putative specifier proteins in *Brassicaceae*, and concluded that ESPs likely evolved from NSPs at least 36 million years ago, with TFP activity evolving from ESPs [68]. Whilst ESP and TFP also have nitrile-specifier activity, NSP contributes exclusively to the formation of simple nitriles.

1.2 Potential health benefits of glucosinolates

Numerous studies have investigated the potential health benefits of *Brassica* vegetables, and glucosinolates specifically. Research indicates that the consumption of high levels of *Brassica* vegetables is associated with a decreased risk in developing various cancers, including lung, breast, prostate, pancreatic, and gastrointestinal cancers [69-73]. Other beneficial effects linked to a diet rich in *Brassica* include the maintenance of a healthy heart [74-77], a reduction in circulating levels of pro-inflammatory cytokines [78], a reduced risk of type 2 diabetes [79-81], and the positive modulation of mitochondrial function [82]. The majority of the research on potential health benefits of glucosinolates has focused on the biological activity of sulforaphane, the ITC formed through the hydrolysis of glucoraphanin.

1.3 Bioavailability of glucosinolates

Evidence indicates that the breakdown products of glucosinolates may have an impact on the risk of developing chronic diseases, but these compounds can only have an effect *in vivo*, if they are present at an appropriate site and at a sufficient concentration [83]. To ascertain their mechanisms of action and how they may influence health maintenance, it is necessary to consider which factors may limit the release of glucosinolates from the food matrix, the extent of absorption, and the fate of these compounds in an *in vivo* setting [83]. In general, the bioavailability of a dietary compound is dependent on several processes: Liberation, the release and dissolution of a compound from its food matrix; Absorption, the transfer of the compound from the site of administration to the blood circulation; Distribution, the diffusion of the compound or its transference to the extravascular space from the intravascular space; Metabolism, the biochemical conversion or transformation of the compound into forms that allow for efficient disposal; and Excretion, the disposal of metabolites/conjugates or unchanged compounds from the body through renal, biliary or pulmonary processes [83].

1.3.1 Liberation of glucosinolates

For efficient intestinal absorption to occur, compounds that reach the intestinal mucosal surface must be in a form that enables access to the enterocyte, or the ability to pass through the tight junctions of the epithelial layer [83]. As glucosinolates are encased within the plant tissue, it is necessary for this matrix to undergo disintegration to allow the dissolution of the glucosinolates into the intestinal milieu [83]. The amounts of glucosinolates present, methods of food processing, and the food matrix affects both liberation and absorption [84]. Chopping, cooking, and bacterial fermentation result in cell damage, degradation of glucosinolates, and the formation of different hydrolysis

products. Boiling has been found to cause a 30 - 60% reduction in glucosinolate content, whilst blanching only results in a minor reduction in glucosinolate levels, suggesting that the predominant reason for glucosinolate loss during cooking is the leaching of glucosinolates into the cooking liquor, rather than thermal degradation [83, 85, 86]. The complexity of the food matrix can have a large effect on glucosinolate availability; glucosinolates present in a soup or juice will be more accessible than those embedded within plant tissue. In conjunction with ionisability and lipophilicity, aqueous solubility determines the dissolution of a compound and, due to the sulphate group and thioglucose moiety, intact glucosinolates are water-soluble [83].

The initial release of intact glucosinolates is likely to occur during the mastication of the vegetable. Due to the lack of myrosinase activity in human saliva, the formation of hydrolysis products from glucosinolates is dependent on the activity of the plant myrosinase during mastication. Whether glucosinolate hydrolysis occurs during mastication of cooked *Brassica* vegetables can depend on the choice of cooking method, as it has been shown that microwaving vegetables completely abolishes myrosinase activity [87, 88]. Processing techniques determines the degree of cell disruption, and ESP activity, as well as the activity of myrosinase [83]. A high level of thermal cell disruption results in a large release of glucosinolates, and whilst myrosinase is relatively heat stable, ESP is heat sensitive. After processing, if active myrosinase is present, the release of the glucosinolates via cell disruption allows for enzymatic glucosinolate hydrolysis, and relatively high amounts of the corresponding hydrolysis products.

1.3.2 Digestion of glucosinolates

In a study evaluating the concentration of glucosinolates in the ileal digesta of pigs, it was proposed that approximately 60% of glucosinolates are able to reach the colon intact [89]. Similar results were found in two rat studies, with 55% of the initial dose of glucosinolates recovered from the gastrointestinal tract in one study, and the second study reporting 50% of the initial dose in the rat large intestine [90, 91]. It is thought that the losses of total glucosinolates during peptic and small intestinal digestion is due to three main processes: acidic hydrolysis, non-specific binding, and hydrolysis by plant myrosinase [83].

The majority of glucosinolates were found to be relatively stable when exposed to acidic conditions (pH 2) that mimic those found in the empty stomach [92]. A study examining the effects of incubating rapeseed meal with pepsin-HCL and the contents of porcine small intestine found a ~15% reduction in total glucosinolate contents after *in vitro* gastric digestion, and a 25 - 37% reduction when this was followed by an *in vitro* simulation of a 4-hour digestion in the small intestine [93]. The pepsin-HCL and porcine small intestinal treatments had a larger effect on glucosinolate content than was observed when glucosinolates were tested under acidic conditions [92, 93]. It was suggested that processes, such as non-specific binding and further degradation of the plant material, would have likely had a substantial effect on the observed glucosinolate losses [83].

A study examining the bioavailability of glucosinolates in rats observed a loss of the initial dose of glucosinolates in the intestinal contents (24%) and intestinal tissue homogenate (42%), which was suggested to be a result of non-specific binding of glucosinolates to the homogenised material [94]. A combination of the acidic conditions in the stomach and the activity of digestive enzymes, results in the lysis of vegetative

cells, and the subsequent release of glucosinolates and myrosinase [83]. Thus the hydrolysis of glucosinolates by active plant myrosinase, can occur during the passage of glucosinolates to the colon, leading to the production of the bioactive compounds in both the upper and lower gastrointestinal tract

1.3.3 Absorption & distribution of glucosinolates

It has been proposed that intact glucosinolates are absorbed through the gut epithelium [95-97]. This was later supported by a study that examined the transport of allyl glucosinolates in everted gut sacs from the small intestine and colon of rodents, in which the transport rate of intact glucosinolates was observed to be both structure and side-chain dependent [94]. This study suggested that intact glucosinolates could only be absorbed by passive diffusion or facilitated transport [94]. Recent research has reported the presence of intact glucosinolates in plasma, albeit at relatively low concentrations [98-102]. The permeation of tissue membranes must occur to enable the entry of ITCs, and this is thought to happen in the same manner as described for intestinal absorption [103]. ITCs must be present as unbound compounds or L-cysteine derivatives in order to passively diffuse into the cells, with passive diffusion being driven by GSH conjugation within the cell [103-106]. Studies on the cellular effects of ITCs in the presence or absence of excessive GSH or L-cysteine indicated that the equilibrium between bound and free ITC is an important factor in enabling the passage of cellular membranes, and that organ specific effects of ITCs *in vivo* may be linked to organ-specific differences in GSH concentration [83, 107]. After absorption into epithelial cells of the gut, ITCs spontaneously conjugate with GSH (typically present at concentrations of 1 – 10 mM), an action that may be promoted by glutathione transferases [108]. This is followed by the export of the GSH conjugate to the systemic circulation via the multidrug resistance associated protein-1 (MRP1), MRP2 and P-glycoprotein-1 (Pgp-1) [108-110].

1.3.4 Metabolism & excretion of glucosinolates and their products

Even if there is a high degree of absorption for a compound, bioavailability may be restricted depending on the speed and degree of metabolism. It has been observed that the total urinary excretion of dithiocarbamates is higher following consumption of ITCs, compared to consumption of the corresponding intact glucosinolates [111-114]. Shapiro *et al* reported a 6-fold increase in the cumulative excretion of dithiocarbamates after ITC consumption, and a significantly faster peak excretion (4 hours vs 8 hours), when compared to glucosinolate consumption [114]. Several studies have reported variation between individuals regarding the excretion of glucosinolate hydrolysis products from a known dose of glucosinolates, with recovery rates ranging between ~1% and 45% [91, 111-114].

Following intestinal absorption of ITCs, the liver is the second metabolic barrier [83]. Due to the high levels of GSH, and GST activity, the liver is a site of increased conjugation of GSH with ITCs, enabling the accumulation of ITCs [83, 104, 115]. A result of the accumulation of ITCs is a concomitant decrease of GSH, and elevation of the binding of ITCs to cellular macromolecules [106]. The ITC structure influences the extent of this binding and the rates of enzymatic and non-enzymatic conjugation with GSH [83, 115]. Several studies have indicated that the reduction of glucosinolates and ITCs may occur in the liver, with evidence that glucosinolates, ITCs, and ITC conjugates can undergo enterohepatic circulation [98, 116].

The formation of the corresponding mercapturic acids from the GSH conjugates predominantly takes place in the kidney. The isopeptide bond, between the γ-carbonyl of the glutamate residue and the amino group of cysteine, is cleaved through the activity of γ-glutamyltranspeptidase, with the resulting *S*-cysteinylglycine conjugate undergoing further breakdown by aminopeptidase M or cysteinylglycinase (**Figure 1.6**)

[83]. The resultant *S*-cysteine conjugate is *N*-acetylated into the equivalent *N*-acetyl-*S*-cysteine conjugate through the activity of *N*-acetyltransferases (NAT), and excreted in the urine [83, 104, 117]. The formation of the mercapturic acid derivatives can occur prior to excretion in the kidney, due to the high concentrations of GSH in the liver, and high NAT activities in both the liver and kidney [83, 91, 116].

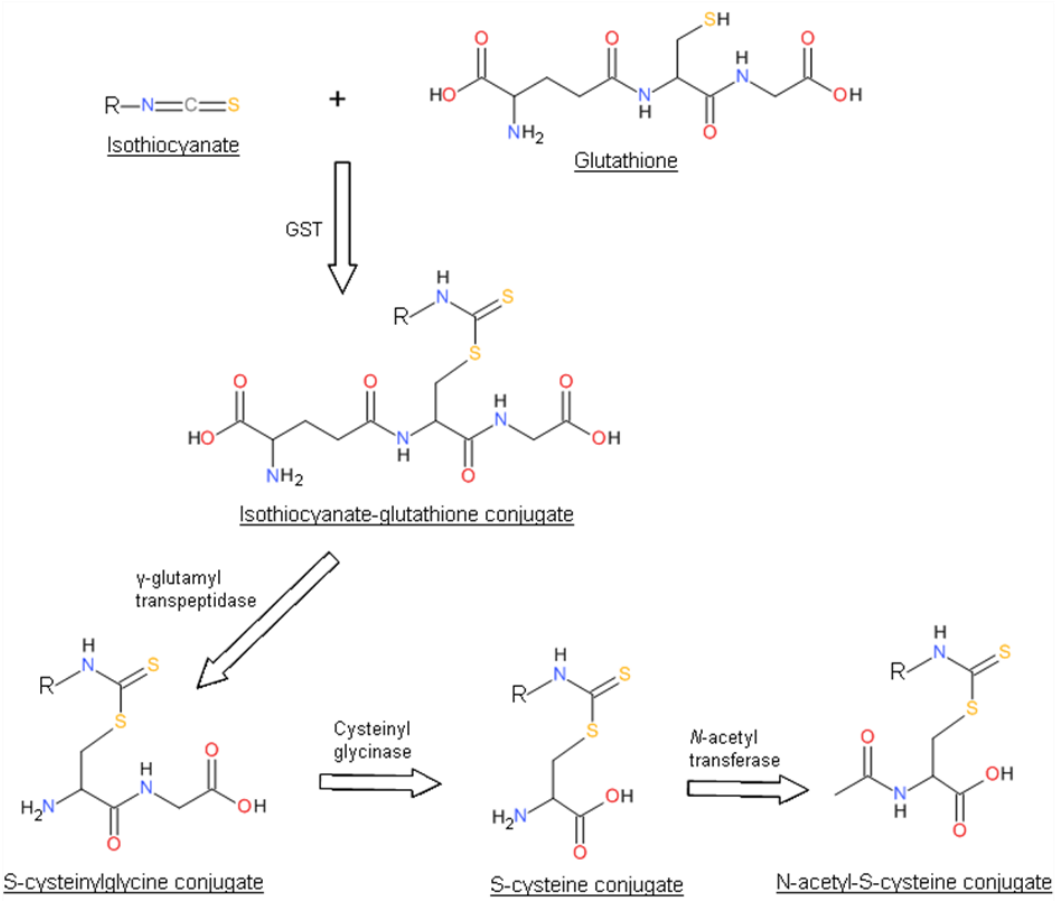


Figure 1.6: The mercapturic acid pathway. Following the hydrolysis of glucosinolates, isothiocyanates conjugate with glutathione, and sequential cleavage reactions produce the mercapturic acids, which are excreted in the urine.

The metabolism of specific ITCs, and their potential chemopreventive effects, may be influenced by polymorphisms within the genes encoding GST enzymes. It has been demonstrated that individuals with *GSTM1* and/or *GSTT1* null genotypes benefit from an increased protective effect of high cruciferous vegetable intake on the risk of lung cancer [118]. However, other studies suggest that *GSTM1* positive individuals receive

an increased protection from cancer following the ingestion of cruciferous vegetables than *GSTM1* null individuals, and that the *GSTM1* null genotype is associated with a greater rate of excretion of sulforaphane metabolites [119-121]. It is likely that the effects of GST polymorphisms on cancer protection from cruciferous vegetables are more complex than first thought, and may differ dependent on which glucosinolates/ITCs are consumed.

1.4 Bacterial metabolism of glucosinolates

Through the use of *in vitro* experiments, in which pure or mixed cultures of bacteria were incubated with glucosinolates, it has been confirmed that various bacterial species that populate the human gut, such as *Bacteroides thetaiotaomicron, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Lactobacillus agilis*, and particular *Bifidobacterium* sp. and *Peptostreptococcus* sp., are able to metabolise glucosinolates in culture [90, 122-126]. One of the earliest studies into this was performed in 1972 using papaya (*Carica papaya L.*) seeds, which are known to contain a high concentration of benzyl glucosinolates [127]. Homogenates of mature papaya seeds were found to produce an odour of hydrogen sulphide (H_2S), and tested positive for sulphide after an overnight incubation at room temperature. Gram-negative, rod-shaped bacteria were identified from the seed homogenates using microscopy techniques, and it was postulated that the production of H_2S might have resulted from the microbial degradation of benzyl ITC (BITC) [127]. The addition of chloramphenicol, or heat-treating the homogenate, inhibited the formation of H_2S [127]. Of the bacteria isolated from the BITC-enriched papaya pulp homogenates, 14 were classified as members of *Enterobacter cloacae,* with the remaining two classified as the genera *Citrobacter* and *Hafnia* from the family *Enterobacteriaceae*. The *E. cloacae* isolates were found to produce H_2S from BITC, whilst the *Citrobacter* and *Hafnia* isolates, as

well as the *E. cloacae* type strain, were found to generate either minimal or no sulphide when tested. Further study with one of the *E. cloacae* isolates (strain P6A) identified that the kinetics of benzylamine formation from BITC corresponded to the production of H_2S [127].

In 1995 a research group investigated 42 *Lactobacillus* strains for the ability to degrade sinigrin. Three of these strains (R16, KG25, and KG31) were linked to a decrease in sinigrin content, with *Lactobacillus agilis* R16 found to exhibit the greatest sinigrin-degrading ability [125]. Experimental work with resting cells demonstrated that the sinigrin-degrading activity of *L. agilis* R16 could be induced by culturing the bacteria in the presence of sinigrin. Following incubation of the induced cells with a mixture of sinigrin and glucose, the glucose disappeared after 1 hour, whilst the sinigrin levels remained constant. After 4 hours, 80% of the sinigrin had been degraded, and after 24 hours this too had been fully depleted [125]. The sinigrin-degrading activity was found to be associated with the presence of intact cells; when cell-free extracts were used, sinigrin degradation was not observed. This is supported by other work, such as that of Ohtsuru and Hata, in which it was found that the myrosinase-like enzyme obtained from the fungus *Aspergillus niger* was unstable, even in the presence of stabilising agents [128, 129]. This suggested that functioning bacterial myrosinase activity may either be cell-associated or intracellular [125].

Bifidobacterium species are commonly found in human faecal microflora and they are known to have beneficial effects on the host [123, 130, 131]. The ability to hydrolyse glucosinolates was investigated in three strains of *Bifidobacterium* species: *B. adolescentis* JCM 7045*, B. longum* JCM 7050, and *B. pseudocatenulatum* JCM 7040. This was performed *in vitro*, using sinigrin and glucotropaeolin, and the digestive products were analysed using gas chromatography-mass spectrometry (GC-MS) [123].

Degradation of sinigrin and glucotropaeolin was observed with all three strains, and was associated with a decrease in the pH of the medium (7.1 to 5.2). The major products identified were the nitrile derivatives of the two glucosinolates, which may have been a result of the increasing acidity of the medium. Sinigrin degradation was found to be similar in all three strains, 20 - 48% and 72 - 83% of the initial amount of 0.5 mM was degraded in the 24 and 48 hour cultivation, respectively, and the degradation of glucotropaeolin by *B. adolescentis* followed a similar pattern [123]. No sinigrin-digestive activity was seen when the supernatant of the 12 hour-cultured *B. adolescentis* was tested, indicating that any glucosinolate-digesting enzymes were unlikely to have been secreted into the medium. However, an apparent activity to degrade sinigrin was perceived in a cell-free extract prepared from sonically crushed cells of *B. adolescentis* [123]. This activity increased in the presence of 0.5 mM L-ascorbic acid, which indicates that ascorbic acid may be an activator of bacterial myrosinase, as has been observed for plant myrosinase [123].

In 2001, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was employed to study the biotransformation of sinigrin and glucotropaeolin by the human colonic microbiota *in vitro* [132]. Additionally, it was investigated whether glucosinolate metabolism by the gut microbiota would be hindered by the availability of glucose, by incubating stool samples with a mixture of sinigrin and glucotropaeolin in the presence, or absence, of glucose. They showed that sinigrin and glucotropaeolin were transformed by the human faecal flora into allylamine and benzylamine, respectively, and that the presence of glucose had no effect on the nature of the metabolites, or the rate of glucosinolate transformation [132]. These results contrasted with those of previous studies that showed that the human microbiota generated ITCs from glucosinolates [90, 91, 133]. The presence of allylamine and benzylamine, in conjunction with the low amounts of ITCs recovered (10 - 20%), may indicate that the

glucosinolates were degraded to form ITCs, and the ITCs were then hydrolysed to form allylamine and benzylamine. This is supported by work performed by Tang *et* al. that showed that BITC could be converted to benzylamine when incubated with a suspension of *E. cloacae* isolated from papaya pulp [127, 132].

Between 2009 and 2011, two human dietary studies were performed in an attempt to identify the relationship between the gut microbiota and the metabolism of glucosinolates, using terminal restriction fragment length polymorphism (tRFLP) [124, 134]. In one study, urinary ITC levels of subjects were examined after a standardised broccoli meal, enabling the selection of high- and low-ITC excreters. Correlations between the levels of urinary ITCs excreted and differences in the composition of the faecal microbiotas were then investigated [124]. Following a 48 hour anaerobic incubation with glucoraphanin, it was shown that the faecal bacteria from the high-ITC excreters were able to degrade more glucoraphanin than those from the low-ITC excreters. However, the overall bacterial community structure did not differ significantly between the two groups, either in faecal samples or in *ex vivo* faecal bacterial cultivation samples [124]. This may indicate functional redundancy in glucosinolate metabolism for gut bacteria; although not closely related phylogenetically, bacterial species may have the same metabolic function. This is supported by previous work, as bacteria that are able to hydrolyse glucosinolates have been found in several different phylogenetic families, including Actinobacteria, Firmicutes and Bacteroidetes [90, 122, 123, 125, 126, 135]. It has been hypothesised that a relatively high level of lateral gene transfer may occur between bacterial species within the gut environment [136-138]. Lateral gene transfer could explain the diversity between gut-associated bacteria that exhibit glucosinolate hydrolysis activity, and suggest that metabolomic or metatranscriptomic approaches may be the best path towards understanding microbial glucosinolate hydrolysis.

The same group performed a human dietary study concerned with the effects of a *Brassica* diet on the composition of the gut microbiota. Participants were asked to consume a diet rich in *Brassica*, but restricted of other vegetables, fruits, high-fibre and whole grain foods [134]. tRFLP analysis of the composition of the faecal microbiotas after the *Brassica* diet, was compared to the microbiota composition after following the dietary restrictions without the supplementation of *Brassica* vegetables. Modifications to the microbiota composition associated with the *Brassica* diet was observed, with changes to the relative proportions of bacterial species putatively identified as *Eubacterium hallii*, *Phascolarctobacterium faecium*, *Alistipes putredinis*, and *Eggerthella* sp. [134]. However, it was not reported whether the proportions of these bacteria were positively or negatively associated with the consumption of the *Brassica* diet. The bacterial sequences obtained from the dietary study were compared to a reference set of 96 16S rDNA sequences, which were obtained from a faecal sample of a single individual. This reference set is unlikely to represent the less dominant members of the bacterial community, and does not take into consideration the variability of the gut microbiota between individuals. Therefore, limitations in the analysis suggests that further research would be required to elucidate the effects of *Brassica* diets on the community composition of the human gut microbiota.

1.4.1 Bacterial reduction of glucosinolates

Research into the bacterial metabolism of glucosinolates has indicated that gut bacteria are able to convert glucosinolates to their reduced form via a reduction reaction. A study performed by Kassahun *et al* showed interconversion between sulforaphane and erucin in a rat model [116]. When rats were dosed with erucin via intraperitoneal injection, urine analysis detected erucin *N*-acetylcysteine accounting for 28.5% of the initial dose, and sulforaphane *N*-acetylcysteine which represented 66.7% of the initial

ITC dose. However, dosing with sulforaphane led to the urinary excretion of 60.2% of the dose as sulforaphane *N*-acetylcysteine, and 11.6% in the form of erucin *N*-acetylcysteine [139]. This suggested that although the reduction of sulforaphane occurred, the oxidation of erucin to sulforaphane was more prevalent. Another study investigated the metabolism of glucoraphanin in rats and detected the reduced analogue, glucoerucin, in the bile [98]. Thus indicating that glucoraphanin could be reduced *in vivo*, and that enterohepatic circulation may play a role in glucosinolate metabolism. A study performed in mice in 2014 reported that the reduction of sulforaphane to erucin was observed, and that erucin was the preferred form in the liver, kidney, and bladder [140]. Evidence for the interconversion of sulforaphane and erucin has also been reported in humans [141-143].

Direct evidence that the reduction of glucosinolates is performed by the human gut microbiota was first reported in 2012 [142]. A human faecal microbiota was cultured in a media containing glucoraphanin in an anaerobic batch fermentation model. Glucoerucin was observed in the media after 8 hours, with the amount increasing after 24 hours, and erucin nitrile was also detected. The conversion of glucoraphanin did not occur with heat-sterilised faecal samples, indicating that the reduction of glucoraphanin was a bacterial-mediated reaction. Recently, two studies from independent research groups identified human-derived bacterial isolates that had the ability to reduce glucosinolates and ITCs under *in vitro* conditions [45, 144]. *E. coli* Nissle 1917, *E. coli* VL8, and *E. cloacae* reduced glucoraphanin and glucoiberin to glucoerucin and glucoiberverin, respectively. The reduction reaction targeted the sulphoxide group present on glucoraphanin and glucoiberin, and was not influenced by oxygen levels, or pH [144]. *E. coli* VL8 was shown to also convert sulforaphane to erucin, suggesting that the sulphoxide of ITCs are also a target of the reductase enzyme (**Figure 1.7**) [45]. Luang-In *et al* suggested that the glucosinolate core skeleton induced the reductase

reaction, and that the bacterial reduction of glucosinolates may reflect different mechanisms of glucosinolate metabolism by human gut bacteria [45]. To date, all bacteria exhibiting glucosinolate reductase activity are members of the *Enterobacteriaceae*, which may reflect a phylogenetic-specific mechanism of glucosinolate metabolism.

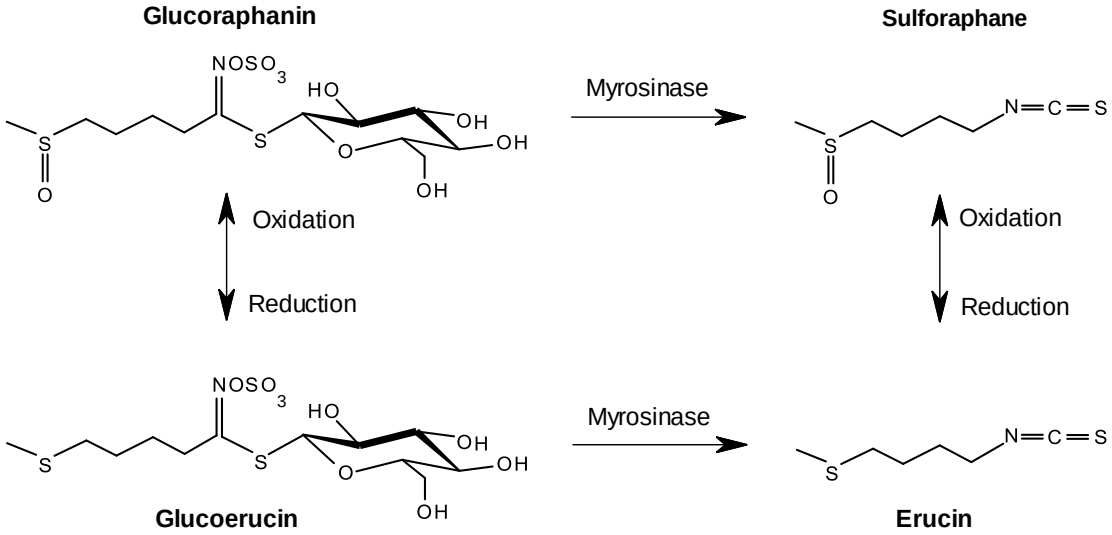


Figure 1.7: Potential methods of glucosinolate hydrolysis, and glucosinolate/ITC interconversion, by human gut bacteria.

1.4.2 *S*-methylcysteine sulphoxide

Both SMCSO and glucosinolates are thought to play a role in plant defence, largely through the biological activity of their hydrolysis products [145-148]. Although glucosinolates are often the focus of *Brassica* research, SMCSO is present at higher concentrations (1 - 2% dry weight) than glucosinolates (0.1 – 0.6% dry weight) in *Brassica* vegetables [149, 150]. However, as SMCSO is more prone to thermal degradation than glucosinolates, the difference in content may not be reflected in the gastrointestinal tract following the consumption of cooked vegetables [151].

In 1973, SMCSO was linked to cases of severe haemolytic anaemia that was reported to develop in ruminants that were fed a diet consisting mainly of *Brassica* crops [150]. The effects of SMCSO consumption was found to be dependent on the amount consumed (~15 to 20 g SMCSO per 100 kg liveweight per day could produce a haemolytic response), and the species of the animal [150]. Cattle and goats were the most susceptible, with sheep, fowl, and rats also affected, whilst rabbits, mice, hamsters, and guinea pigs did not exhibit symptoms of anaemia following consumption of SMCSO-containing crops [3, 150, 152-157]. Further research indicated that the differential response between species was likely due to differences in bacterial metabolic activity in the digestive system [157-159]. It was proposed that the ruminal bacteria were able to degrade SMCSO to the causative agent, dimethyl disulphide, that led to the depletion of glutathione in erythrocytes, which were subsequently damaged by reactive oxygen species [150, 160, 161]. These studies culminated in the development of cultivars of *Brassica* species with significantly reduced levels of SMCSO that are now used as cattle fodder, and have led to a steep reduction in *Brassica*-associated anaemia [150, 162, 163].

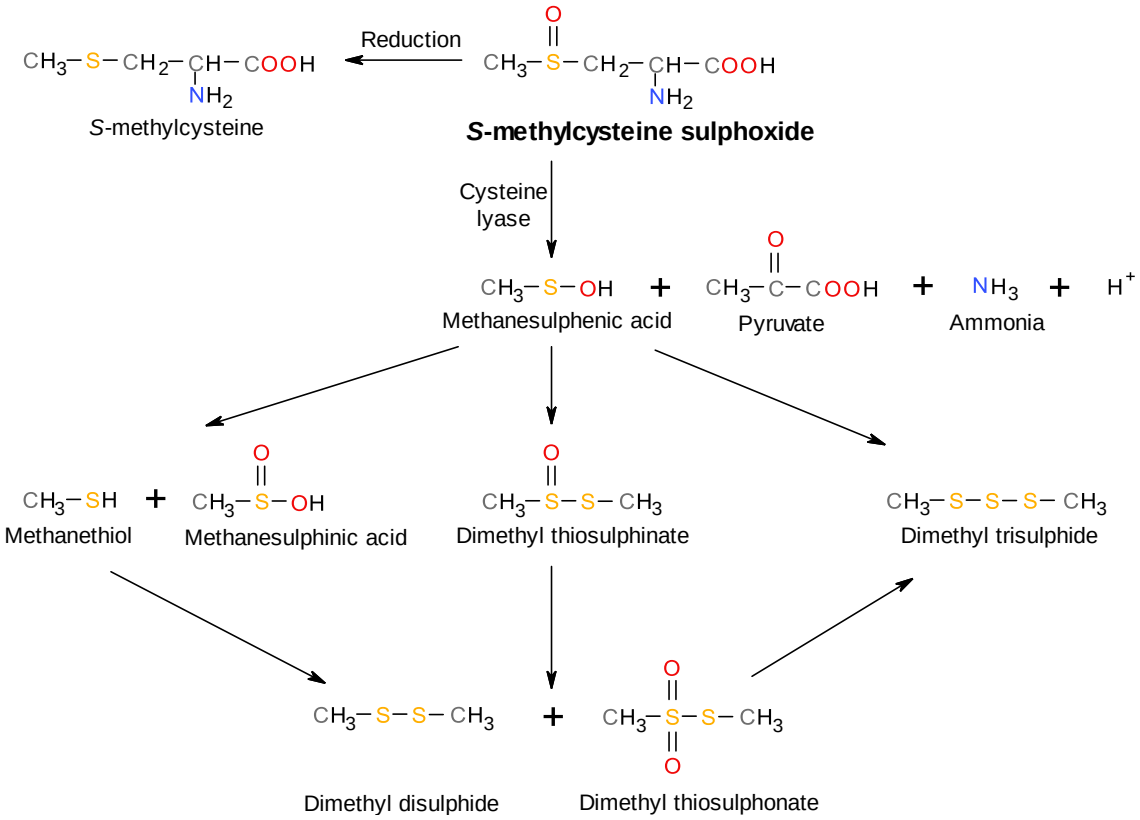


Figure 1.8: Metabolic schematic for the reduction or degradation of *S*-methylcysteine sulphoxide (SMCSO). Schematic displaying the metabolic products formed through the degradation of SMCSO by the action of cysteine lyase, and the reduced analogue of SMCSO.

Members of the human gut microbiota have been reported to exhibit the cysteine sulphoxide lyase activity necessary to degrade SMCSO (**Figure 1.8**) [164-166]. However, the amount of SMCSO present in 150 g of *Brassica*, such as broccoli, cauliflower, or Brussel's sprouts would represent a dose of ~0.5 g/100 kg body weight in a 70 kg individual [150]. Therefore, the levels of SMCSO that could be expected to be found in the human gut are far lower than the amounts that were observed to cause negative effects on cattle health. Conversely, there are indications that the bacterial degradation of low levels of SMCSO may produce beneficial effects on human health. SMCSO, and its breakdown product dimethyl thiosulphinate, were found to produce a 33% reduction in micronucleus formation in mice treated with the carcinogen

benzo[α]pyrene [167]. Dimethyl thiosulphinate has also been associated with an increased life expectancy in mice inoculated with a fibrosarcoma cell line, and an inhibition of the *in vitro* proliferation of human colon and prostate cancer cell lines [150, 168-170]. The SMCSO metabolite dimethyl thiosulphonate has been observed to exert anti-mutagenic effects against mitomycin C in *Drosophila melanogaster* and mice [171], and aflatoxin B_1 in treated rat bone marrow cells [172]. Dimethyl thiosulphonate was also found to reduce the occurrence of aberrant crypt foci, and induce a regression of the associated lesions, in azoxymethane-treated rats [173, 174]. As well as anti-mutagenic properties, SMCSO has also been reported to have cholesterol- and triglyceride-lowering effects, and anti-hyperlipidemic and anti-diabetic properties in rats [175-179].

1.5 Human gut microbiota

It has been suggested that there are tenfold more microbes in the human body than there are human cells, and that they encode 100-fold more unique genes than the human genome [180, 181]. The majority of these microbes reside in the gut and, as well as affecting human physiology, they contribute to energy harvest from food [182, 183]. It has been approximated that the colon contains ~1.5 kg of bacteria, with an approximate density of 10^{12} cells per gram of intestinal content [184, 185]. In the early years of human development, the gastrointestinal tract is colonised by a myriad of microbes, dominated by bacteria, which are collectively known as the gut microbiota. Many of the bacteria are derived from the mother during birth, and the mode of delivery (vaginal or caesarean section) influences the early microbiota of the infant. Vaginal birth is associated with a microbiota containing *Lactobacillus* and *Prevotella* species, whilst the microbiota of babies delivered by caesarean section harbour increased numbers of *Staphylococcus* and *Propionibacterium* species [186-188].

1.5.1 Effects of the gut microbiota on human health

The gut bacteria interact intimately with our body and contribute to human health, playing a critical role in the development of the immune system [189-192]. An altered gut microbiota (dysbiosis) has been associated with inflammatory bowel disorders (IBD) [193], type 2 diabetes [194-196], irritable bowel syndrome (IBS) [197, 198], coeliac disease [199, 200], obesity [201, 202], and colorectal cancer [203]. Faecal microbiota transplantation (FMT) is a therapeutic strategy that aims to restore the balance of the microbiota, and reduce the risk of developing health issues associated with dysbiosis [204, 205]. At present this therapy has had most success against *Clostridium difficile* infections [206, 207], but has also shown promise in treating IBD and IBS [208, 209]. Although some health complaints may be linked to specific members of the microbiota [210, 211], others are thought to be associated with a reduction in species diversity [181, 196, 212-214]. A recent surge in research has focused on the gut-brain axis, with studies indicating that the gut microbiota may be associated with some brain disorders, and can influence mood and sociality, largely through bacterial metabolites and their effects on gut hormones [215-221].

'Colonisation resistance' is the name given to the microbial barrier, formed by the gut microbiota, which inhibits the colonisation of the gut by pathogenic organisms [222]. Members of the microbiota can provoke a physiological response from the host e.g. stimulation of peristalsis, chemically modify bile salts, or induce immunological processes [223]. Alternately, the bacteria can directly interact with the potential pathogens by i) occupying mucosal receptors and the mucin layer to inhibit colonisation [224, 225], ii) creating a physiologically restrictive environment through the production of short-chain fatty acids, lowering the pH, or altering the oxidation-reduction potential,

iii) successfully competing for growth factors and nutrients, or iv) producing antimicrobial substances [223].

1.5.2 Community composition of the gut microbiota

Microbes in the gut are faced with a selective pressure from both the host and microbial competitors, which will generally lead to a homeostasis of the ecosystem in which some species will occur in high abundance, whilst many others will be present in a low abundance [226]. Members of two bacterial phyla, Firmicutes and Bacteroidetes, dominate the distal gut microbiota, constituting more than 90% of the bacteria present in the total microbiota, followed by species of Proteobacteria, Actinobacteria and Verrucomicrobia [181, 202, 227, 228]. The clostridial clusters IV and XIVa are the most abundant members of the Firmicutes, with a lower representation of the cluster IX group, and it is thought that bacteria belonging to these groups may comprise approximately 60% of the colonic microbiota [229]. In addition to bacteria, the gut ecosystem is also known to contain members of the methanogenic archaea, which have been suggested to be temporally stable members of the gut microbiota, viruses, and bacteriophages [181, 228]. It is known that certain life changes such as medication, illness, and travel can affect the microbiota homeostasis in a temporary manner. The temporal instability of the microbiota caused by overseas travelling may be due to a combination of factors, such as stress, exposure to new environmental microbes, or disturbances of the circadian rhythms [228].

Many studies have focused on identifying the composition of the human gut microbiota, based on the 16S ribosomal-RNA-encoding gene, or analysis of phylogenetic microarrays [181, 202, 226, 230-236]. These studies have found variation in community composition and relative abundances within and between individuals, but it appears that the gene pool is largely conserved, indicating functional redundancy within the ecosystem [181, 202, 226, 227, 237]. In 2010, Qin *et al* used metagenomic sequencing

to analyse the faecal samples of 124 European individuals [181]. In total the cohort contained 1,000 – 1,150 bacterial species, with each individual having a minimum of 160 species [181]. They identified 75 species common to more than 50% of individuals, and 57 species common to more than 90% of individuals [181]. Another group performed metagenomic analysis of human faecal samples, and it was posited that the gut microbiota may be stratified into three groups, termed enterotypes, characterised by a dominance of either *Bacteroides*, *Prevotella*, or *Ruminococcus* species [226]. There is some evidence that these enterotypes may be the result of the effects of long-term dietary intake, as individuals with high levels of *Bacteroides* are often associated with a diet high in protein and fat, whilst those rich for *Prevotella* tend to consume high levels of fibre [238, 239].

Although much can be learned from studying the abundant species or genera within the gut microbiota, this alone cannot reveal the entire functional complexity of the gut ecosystem. It has been reported that low-abundance *E. coli* within one individual contributed more than 90% of two abundant proteins, PapC (COG3188) and FimA (COG3539), which are associated with bacterial pilus assembly [226]. It is likely that some low-abundance species, such as methanogens, may perform specialised functions that are beneficial to the host [240, 241].

1.5.3 Intestinal lactic acid bacteria

An example of important low-abundance species can be found in the lactic acid bacteria (LAB), which include *Lactobacillus* species. Along with the genus *Bifidobacterium*, lactobacilli are important early colonisers of the gut. A culture-based study performed in 1999 investigated differences in the gut microbiotas of infants delivered vaginally or by caesarean section [242]. It was found that the intestinal colonisation of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* species was delayed in

infants delivered by caesarean section, compared to the infants born vaginally. Maternally-derived *Lactobacillus* and *Bifidobacterium* have been shown to be present in breast milk, and the oligosaccharides present in the milk sustain the growth of the infant microbiota [243-245]. The genus *Lactobacillus* contains bacteria that are considered to be beneficial to human health, and yet they have been reported to constitute less than 1% of the total adult bacterial community [246].

Human gut lactobacilli have been demonstrated to play a role in maintaining microbiota homeostasis through immunomodulatory properties, the elimination of intestinal pathogens, the release of acidic compounds that subsequently lowers the pH, and the production of anti-microbial/anti-inflammatory compounds [247-251]. The LAB includes *Streptococcus*, *Weisella*, *Lactococcus*, *Enterococcus*, and *Leuconastoc*, as well as *Lactobacillus*, and are classified as either homofermenters or heterofermenters based on the end-products of fermentation (**Figure 1.9**) [252]. The LAB are characterised by the production of lactate, which can be used by other intestinal bacteria to generate short-chain fatty acids (SCFA) [253]. These bacterial waste products are considered to have a beneficial impact on human health, and will be discussed in more detail in section 1.5.6.

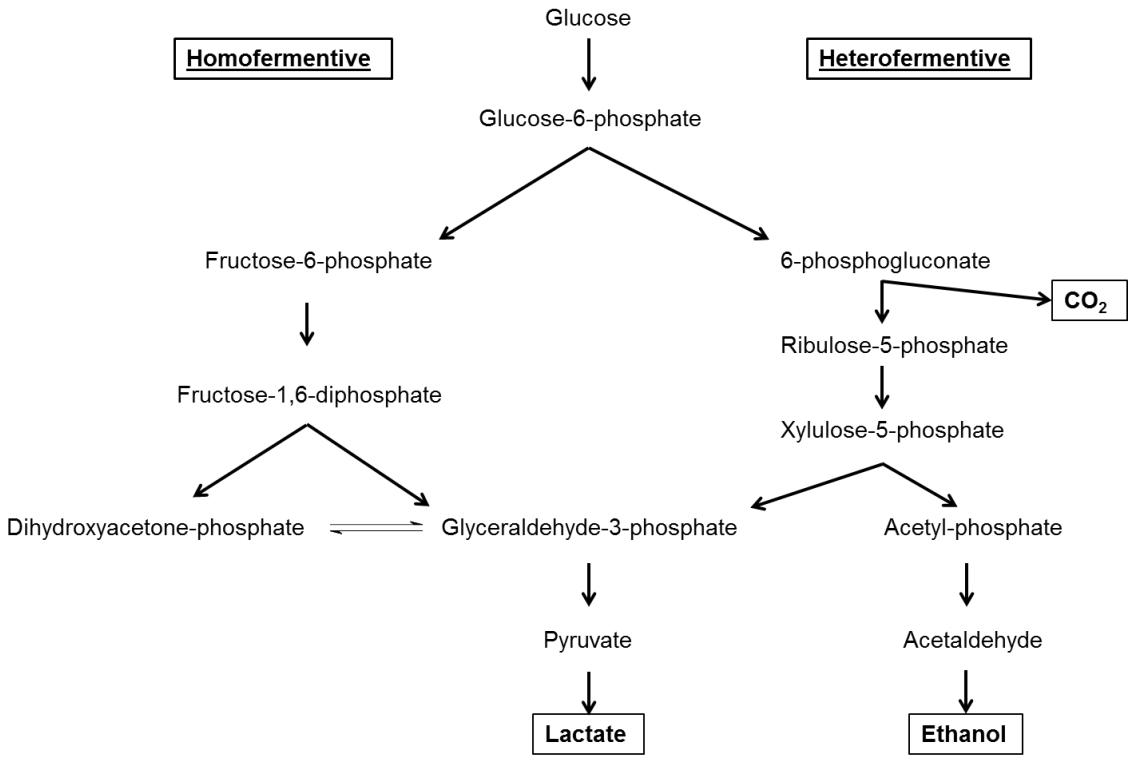


Figure 1.9: Schematic displaying glucose fermentation strategies by the lactic acid bacteria.

1.5.4 Dietary effects on the gut microbiota

Diet has a substantial effect on the composition of the gut microbiota, and subsequently human health. Human dietary interventions have reported compositional changes to the gut microbiota after short periods [254-258], and long-term dietary habits have been implicated in shaping the stable microbiota structure [238, 239, 259]. A study performed in 2010 investigated differences in the composition of the gut microbiota in children living in the urban city of Florence, compared to children in a village in Burkino Faso (BF) [238]. Members of the Bacteroidetes and Actinobacteria were more abundant in the microbiotas of children from BF, whilst relative proportions of Proteobacteria and Firmicutes were higher in those of the Florentine children. *Xylanibacter*, *Prevotella*, *Treponema*, and *Butyrivibrio* species were only observed in the children of BF, and this was considered to be a consequence of the high plant-

based diet consumed in BF, compared to the high-fat diets common in the Western world [238]. A higher total amount of SCFAs was found in the faeces of the BF children, compared to the Florentine children, and this would seem to correlate with the high-fibre plant-based diet consumed by the BF children.

When the microbiotas of the Hadza hunter-gatherer tribes in Tanzania were sequenced, gender-based differences were found, and this was hypothesised to be a consequence of the tasks assigned to men and women within the tribes [259]. Whilst the male members of the tribe may travel far from the camp in search of honey and meat, the female members stay close to the camp foraging for plants and tubers. All the food that is gathered is shared between the other members of the tribe, but snacking throughout the day leads to an increased consumption of the foods obtained by each group [259]. This snacking pattern may be responsible for the higher levels of *Treponema* that were detected in the microbiota of the Hadza women, compared to the men. The genus *Treponema* contains species that are efficient degraders of cellulose and xylan, which would be present at relatively high levels in the female colon due to the increased consumption of plant material [259]. The diets of the BF children and Hadza tribal members are alike in terms of being high in fibre and low in fat, and this was reflected in enrichment for *Prevotella*, *Treponema*, and *Butyrivibrio,* and a depletion of *Bacteroides* in both cohorts, compared to the Florentine children tested [238, 259].

The effect of a short-term diet on the long-term human gut microbiota composition was recently investigated in two independent studies [239, 254]. In 2011, Wu *et al* examined how two 10-day diets, characterised as high-fibre/low-fat and low-fibre/high-fat, would affect the composition of the gut microbiota in ten individuals [239]. The microbiotas of the subjects were characterised based on the proposed enterotype

hypothesis, and the observation that a dominance of *Prevotella* is associated with a high-fibre diet, whilst a large relative abundance of *Bacteroides* may be linked to a high fat/protein diet [240]. Within 24 hours of consuming either of the diets, notable changes in the microbiota were observed, but the changes were not significant enough to cause a switch between the enterotype clusters associated with high-fibre or high-protein/fat consumption [239]. The other human dietary study was performed by David *et al* in 2014 to examine how a plant-based diet may affect the gut microbiota, compared to an animal-based diet [254]. As with the Wu *et al* study, notable changes in the microbial communities were observed after a single day; the animal-based diet caused a significant increase in β-diversity compared to the pre-intervention baseline samples. Of the two diets, it was observed that the animal-based diet had the greatest effect on the gut microbiota, including an increase in the relative proportions of *Bilophila wadsworthia* [254]. It was posited that this was a reflection of an increase in bile acid secretion, which is known to reflect a high fat intake [260]. Increased levels of bile acids have been linked to the inhibition of some members of the Bacteroidetes and Firmicutes, and experiments using mice indicate a possible connection to the development of IBD and cancer [261-263]. These studies highlight the importance of the diet regarding the gut microbiota and human health, which may enable the use of long-term dietary changes as a means to combat the development of disease.

Several studies have investigated the differences in the gut microbiota between omnivorous, vegetarian, and vegan humans [264-268]. Vegan subjects have been found to exhibit lower microbial counts of *Bifidobacterium*, *Bacteroides*, *E. coli,* and other *Enterobacteriaceae* species, compared to omnivorous subjects [264, 268]. It has been suggested that the reduced numbers of both *E. coli* and *Enterobacteriaceae* may be linked to low levels of dietary protein and a lower gut pH, whilst an increase in *Bacteroides* species have been linked to a protein-rich diet [238, 268]. A vegan diet is

associated with a higher consumption of carbohydrates and a higher fibre intake compared to omnivores, and this is known to lower the stool pH [269].

1.5.5 Effect of pH changes on gut bacteria

The bacterial fermentation of dietary fibres results in the production of acidic compounds, such as lactate and SCFAs, which lowers the pH of the intestinal milieu. Intestinal bacteria that are able to ferment fibres are generally more tolerant of acidic environments, than non-fermenting bacteria. Studies focusing on the effects of pH on bacterial communities have observed marked differences in community composition within a pH range of 5.5 – 6.7 [270, 271]. Experiments culturing bacterial isolates and faecal bacterial communities at varying pH within a range of 5.5 – 6.7, have observed differential growth, community shifts, and changes to bacterial metabolite production [270, 271]. At a pH of 5.5 increased proportions of 19 of 23 Gram-positive anaerobes tested, and specifically members of the *Roseburia* group, were observed, whilst the proportions of *Bacteroides* species and *E. coli* decreased [270, 271]. Conversely, the proportions of *E. coli* and members of the *Bacteroides* increased when the pH was altered to 6.7 [270, 271]. *Eubacterium rectale*, which had accounted for 50% of all 16S rDNA sequences at pH 5.5, was not detected at pH 6.7 [270]. pH-associated changes to the microbiota composition have been fund to correlate to changes in the production of SCFAs. Butyrate concentrations were higher at pH 5.5 compared to a pH of 6.5, and this was directly related to the decreased proportions of the butyrate-producing members of the *Roseburia* group [271]. Propionate levels peaked when the medium had a pH of 6.5, and this coincided with an increase in the levels of the genus *Bacteroides,* which have been reported to contain propionate-producing species [271-273]. Relatively small changes in the colonic pH can significantly affect the composition of the gut microbiota, and this can occur through the fermentation of dietary fibres, and subsequent production of SCFAs.

1.5.6 Short-chain fatty acid production

The most prevalent SCFAs found in the human colon and stools are acetate, butyrate, and propionate, which have been reported to be present at a molar ratio of 60:20:20 [274-276]. Total colonic SCFA concentrations range from ~70 – 140 mM in the proximal colon, and ~20 – 70 mM in the distal colon, and this is dependent on the diet [277]. The diet and the gut microbiota are inextricably linked through the production of metabolites, such as SCFAs. The consumption of fibre leads to bacterial fermentation and the production of SCFAs, which lowers the pH of the intestinal milieu and aids in the prevention of overgrowth of pH-sensitive pathogenic bacteria, such as *Clostridium* and *Enterobacteriaceae* species [270, 278, 279]. There are multiple pathways that various bacteria can employ to generate SCFAs from hexoses, pentoses, fucose, and rhamnose sugars (**Figure 1.10**).

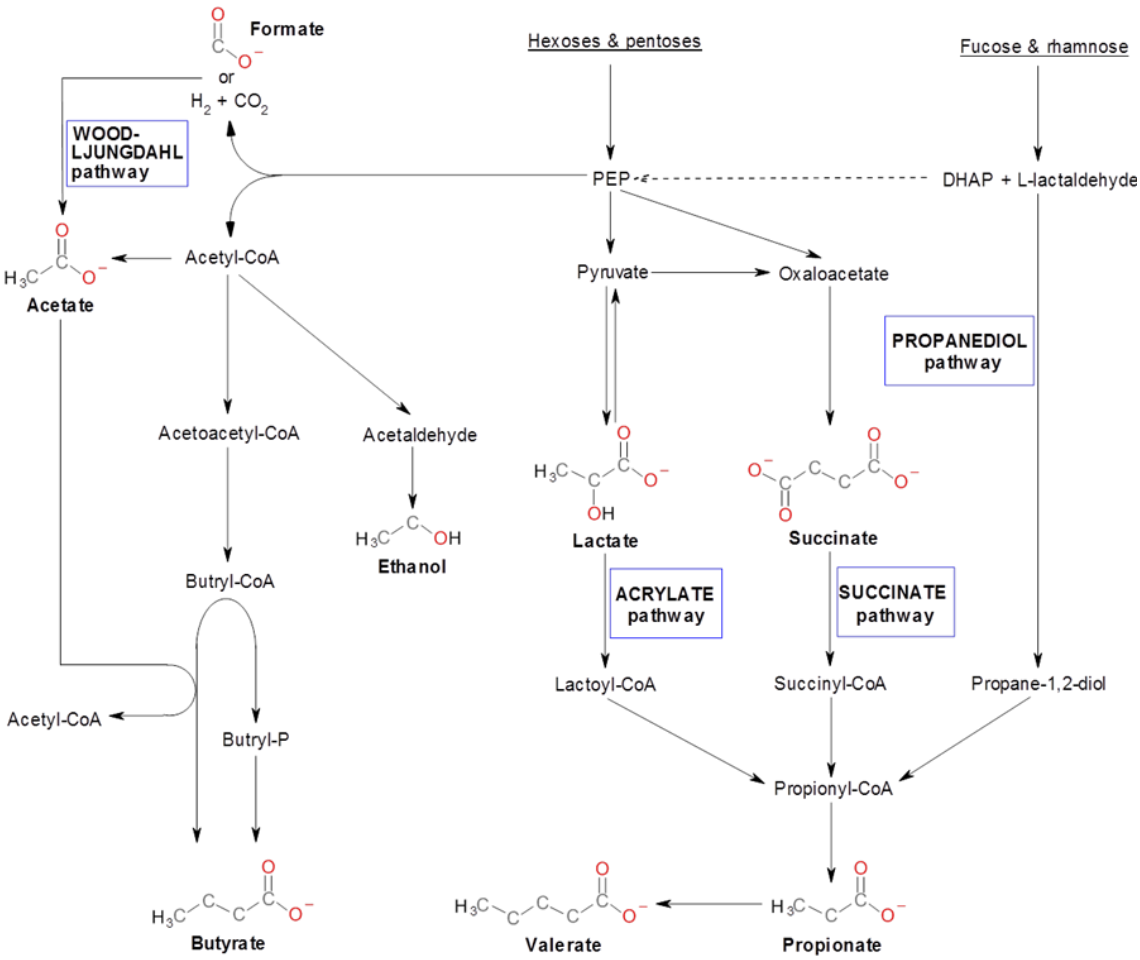


Figure 1.10: Schematic of microbial short-chain fatty acid (SCFA) production. Metabolism of sugars by the human gut microbiota may lead to the production of the SCFAs, acetate, butyrate, propionate, or valerate, by various metabolic pathways (the acrylate, propanediol, succinate, or Wood-Ljungdahl pathways) (adapted from Louis *et al* [280]). Abbreviations: DHAP = dihydroxyacetone phosphate ; PEP = phosphoenolpyruvate

Although SCFAs are bacterial waste products, produced to balance redox equivalent production, these compounds can be absorbed from the gut lumen and elicit beneficial properties on human health [281]. The nature of these beneficial properties differs dependent on the SCFA. Acetate is the only SCFA to reach relatively high concentrations in the peripheral blood (0.10 – 0.15 mM) and serves as an energy source for peripheral tissues [282, 283]. Acetate has also been shown to cross the blood-brain barrier and act to suppress appetite by influencing central hypothalamic mechanisms [284]. Propionate has been implicated in the enhancement of satiety and

is used by the liver as an energy source, as well as exhibiting anti-inflammatory, anti-carcinogenic, anti-lipogenic, and cholesterol-lowering activity [282, 285-287]. Butyrate is perhaps the most widely studied SCFA, and is utilised as an energy source by the colonic epithelia [282]. Butyrate has been shown to affect genes and pathways involved in epithelial integrity, fatty acid oxidation, and apoptosis, by inducing histone hypermethylation through the inhibition of histone deacetylases [288-290].

SCFAs have been shown to exert activities by binding to the G protein-coupled receptors (GPRs) GPR41 and GPR43 [291, 292]. GPR41 is activated by propionate and butyrate, which leads to the expression of $G_{i/o}$ protein in the peripheral nervous system, adipocytes, and the gut, promoting activation and growth of the sympathetic nervous system [291-293]. Activated GPR43 has been linked to the decrease of inflammatory cytokines in the intestine, and is more responsive to propionate and acetate, rather than butyrate, activating both $G_{i/o}$ and G_q proteins in the gut and adipose tissue [291-293]. The production of SCFAs has also been shown to affect energy homeostasis and levels of gut peptides. Butyrate has been associated with a decrease in the rate of digestion and intestinal transit, leading to an increase in plasma insulin and satiety promotion, through an increase in the plasma levels of gastric inhibitory peptide (GIP), glucagon-like peptide 1 (GLP-1), peptide YY (PYY), insulin, and amylin [291-293]. Propionate has also been found to increase the plasma levels of GIP, insulin, and amylin, although to a lesser degree than observed for butyrate [291-293]. Whilst acetate has not been found to affect these hormones, it has been reported to increase the release of leptin from fat cells [291, 292].

1.6 Bacterial reductases

Bacterial reductases are multi-purpose enzymes that are key to various processes that are crucial to the survival of bacteria. Respiration is a fundamental process which involves the transfer of electrons from low-redox-potential donors, and concludes in the reduction of a high-redox-potential electron acceptor [294]. The greatest amount of respiratory flexibility is found in bacteria and archaea, due to the wide range of electron acceptors that they are able to utilise. It has been reported that these organisms are able to use halogenated organics [295-297], radionuclides [298, 299], transition metals [300], metalloid oxy-anions [301, 302], and a range of nitrogen-based [303-306] and sulphur-based compounds [307-309]. Simala-Grant and Weiner researched the substrate specificity of the dimethyl sulphoxide reductase, DmsABC, of *E. coli* and found that it has a very broad substrate specificity [310]. It was identified that the anaerobic growth of *E. coli* on sulphoxide compounds was solely due to the activity of this reductase [310].

Some bacterial reductase enzymes are able to protect proteins from oxidative damage. Methionine residues are primary targets for oxidation, and this can lead to the loss of biological activity [311]. Methionine sulphoxide reductases (Msr) A and B are a class of highly conserved enzymes that are able to reverse methionine oxidation, and restore the biological activity of oxidised proteins [312]. Msr was first discovered in the cytosol of *E. coli,* and recently it was identified that these protein repair mechanisms are also present in the bacterial cell envelope [313]. Methionine residues within the protein compartment of the bacterial signalling recognition particle, Ffh, have been shown to be prone to oxidation resulting in a perturbation of the targeting of membrane proteins [313, 314]. Experiments using *E. coli msr* knockout mutants identified that msr activity was essential for the normal functioning of Ffh during aerobic growth [314].

Bacterial reductases are also involved in maintaining homeostasis through the modification of proteins [315], the synthesis of DNA [316, 317], xenobiotic metabolism [318], and detoxification processes [319, 320]. The bacterial reduction of dietary compounds, such as sulphoxide-containing glucosinolates and cholesterol have also been reported [45, 142, 144, 321]. The reduction of dietary compounds may increase the range of biologically active metabolites, potentially with different biological activities, derived from the parent compound, and lead to a variety of potential health benefits for the human host from a single compound.

1.7 Thesis aims

1. To use an *in vitro* batch fermentation model to culture human faecal microbiotas in a broccoli leachate-containing media, in order to investigate:

   i) The metabolic fate of broccoli-derived glucosinolates when exposed to the human faecal microbiota.

   ii) The effects on the human faecal microbiota following a repeated exposure to a broccoli leachate.

   iii) Changes to bacterial metabolism caused by a repeated exposure to a broccoli leachate.

   iv) The identity of human gut bacteria able to metabolise glucosinolates and SMCSO.

2. To examine the effects of two *Brassica* diets on the human gut microbiota through the use of a human intervention study.

CHAPTER TWO

2 Materials and methods

2.1 General reagents

A 0.02 M sodium acetate solution was generated by adding 1.2 ml acetic acid glacial (Cat. # 01074131, Biosolve) to 990 ml 0.22 µm filtered water, adjusted to a pH of 5.0 using sodium hydroxide, and adding 0.22 µm filtered water to a total volume of 1 L.

DEAE Sephadex A25 (Cat. # 17-0170-01) and SP Sephadex C25 (Cat. # 17-0230-01) were obtained from GE Healthcare Life Sciences, and prepared in the same manner. Sephadex (26 g) was added to 200 ml 0.02 M sodium acetate, and filtered with Whatman GF/D 15 cm filter paper (Cat. # WHA1823150, Sigma Aldrich). The solid fraction was resuspended in 200 ml 0.02 M sodium acetate, and filtered a second time before it was resuspended in 2x volume 0.02 M sodium acetate.

Sinigrin hydrate was supplied from Sigma Aldrich (Cat. # 85440), and glucotropaeolin was obtained from Dr Shikha Saha at the Institute of Food Research. A 16 mM stock solution was prepared of each for use as internal standards by adding 63.6 mg of sinigrin hydrate, or 72 mg of glucotropaeolin, to 10 ml of 0.22 µm filtered water.

Dulbecco's phosphate-buffered saline, no calcium, no magnesium (Cat. # 14200-067) was purchased from Life Technologies, and aliquots were diluted 10-fold in 0.22 µm filtered water prior to use.

Sulforaphane was obtained from LKT Laboratories (Cat. # S8045). The synthesis of the sulforaphane conjugates of nitrile, *N*-acetyl-cysteine, cysteine, cysteine-glycine and glutathione, and erucin *N*-acetyl-cysteine were all produced by Dr Paul Needs at the Institute of Food Research. A 1 mg/ml stock solution of each was prepared in 0.22 µm filtered water.

Acetonitrile (Cat. # 10407440) and methanol (Cat. # 10674922) were purchased from Fisher Scientific, and were of high-performance liquid chromatography (HPLC) grade with a purity of 95% or greater.

Dansyl chloride reagent was prepared by dissolving 0.2691 g dansyl chloride in 100 ml HPLC grade acetonitrile (ACN). A 0.1 M boric acid solution was generated by dissolving 0.6183 g boric acid in 100 ml of 0.22 µm filtered water.

A 20 mM borate buffer was produced by dissolving 3.8 g sodium tetraborate decahydrate in 500 ml of 0.22 µm filtered water, and the pH was adjusted to 9.2 using 0.1 M boric acid solution.

A 50 mM ammonium acetate buffer was prepared by dissolving 3.854 g ammonium acetate in 1 L of 0.22 µm filtered water, and adjusted to pH 5 using 0.1 M acetic acid.

A 0.1% ammonium acetate buffer was generated by mixing two components; A1 and A2. A1 was produced by weighing 0.25 g of ammonium acetate (Cat. # 1161030250, Merck Millipore) and adding 0.22 µm filtered water to a final volume of 250 ml. A2 was produced by adding 250 µl glacial acetic acid to a volumetric flask, and adding 0.22 µm filtered water to a final volume of 250 ml. A1 was added to A2 until a pH of 4 was reached, before the buffer solution was filtered through 0.60 µm filter paper (Cat. # DAWP04700, Merck Millipore).

ACN with 0.1% acetic acid was generated by adding 250 µl glacial acetic acid to a volumetric flask, and adding HPLC grade ACN to a final volume of 250 ml.

Sulfatase (Type H-1 from *Helix pomatia*) (Cat. # S9626, Sigma Aldrich) was purified before use. Sulfatase (300 mg) was dissolved in ice-cold 0.22 µm filtered water (12 ml),

ice-cold ethanol (12 ml) was added, and the solution was mixed thoroughly. Following centrifugation (3,000 x g, 4˚C, 6 minutes), the supernatant was mixed with ice-cold ethanol (1.5x volume), and centrifuged under the same conditions as previously stated, the pellet was dissolved in 0.22 µm filtered water (8 ml). Aliquots (2 ml) were passed consecutively through columns of DEAE Sephadex A25 and SP Sephadex C25 and stored at -20˚C.

2.2 Generating a broccoli leachate

Beneforté® broccoli (purchased from Marks & Spencer) was steamed in 180 g batches on the second tier of a pre-heated domestic steamer for 3 minutes. Immediately after steaming, the broccoli was cooled on dry ice, and stored at -20˚C. The frozen broccoli was freeze-dried using a benchtop modulyo freeze drier (Edwards Vacuum), and milled using a domestic coffee-bean grinder. Two grams of powdered broccoli were added to tubes containing 40 ml of 0.22 µm filtered water (room temperature). The tubes were vortexed and incubated at room temperature for 60 minutes, prior to centrifugation at 5,292 x g, 21˚C, for 10 minutes. The supernatant was passed through a Whatman GF/D filter under vacuum, and the water content was reduced approximately 10-fold using a BUCHI Rotavapor R-210 rotary evaporator set to 60˚C, 72 mbar. The concentrated broccoli leachate was filtered under vacuum through a succession of decreasing pore size filters in the following order: Whatman GF/A (Cat. # 1820125, Sigma Aldrich), Whatman GF/B (Cat. # 1821090, Sigma Aldrich), nitrocellulose membrane 60 µm (Cat. # DAWP04700, Merck Millipore), nitrocellulose membrane 40 µm (Cat. # HAWP04700, Merck Millipore), and nitrocellulose membrane 0.22 µm (Cat. # GSWP04700, Merck Millipore). A 600 ul aliquot was analysed as described below (section 2.5). The remaining leachate was filtered through a Sartolab BT500 0.2 µm bottle top filter unit (Cat. # 18085E, Sartorius) into a sterile bottle and stored at 4˚C.

2.3 Bacterial media

Chemostat nutrient media (CNM) is a medium designed to provide the nutrients necessary for the cultivation of bacteria found in the human gastrointestinal tract. Luria Broth (LB) is a widely used bacterial culture medium that is rich in nutrients, and often used to culture members of the *Enterobacteriaceae*. Man Rogosa Sharpe (MRS) is a medium that was designed for the cultivation of lactobacilli. Brain-heart infusion agar (BHI) is a general purpose solid growth medium that is rich in nutrients. The composition of each medium is shown in **Table 2.1**.

Table 2.1: Compositions of bacterial culture media

Media	Compositions in 1 L H_2O	pH
BHI agar	12.5 g brain infusion solids, 5 g beef heart infusion solids, 10 g proteose peptone, 5 g sodium chloride, 2 g glucose, 2.5 g disodium phosphate, 10 g agar	7.4 ± 0.2
CNM	2 g peptone water, 2 g yeast extract, 0.01 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4.6H_2O$, 0.01 g $CaCl_2.6H_2O$, 2 g $NaHCO_3$, 0.5 g cysteine.HCL.H_2O, 0.5 g bile salts, 2 ml Tween-80, 0.005 g Hemin, 10 µl vitamin K1	7.0 ± 0.2
LB	10g tryptone, 5g yeast extract, 10g NaCl	7.0 ± 0.2
MRS agar	8 g peptone, 5 g yeast extract, 5 g $CH_3COONa.3H_2O$, 2 g K_2HPO_4, 2 g $C_6H_{17}N_3O_7$, 5 ml salt solution (0.2 g $CaCl_2$ anhydrous, 0.2 g $MgSO_4$, 1 g K_2HPO_4, 1 g KH_2PO_4, 10 g $NaHCO_3$, 2 g NaCl in 1 L), 1 ml Tween 80, 20 g glucose, 3 g agar	6.5 ± 0.2

CNM was the basal bacterial medium used in the production of the broccoli leachate-containing (BL) media and the glucose (GL) media (chapter 3). These media were used for the *in vitro* batch fermentation culturing experiments using human faecal microbiotas (chapters 4, 5, and 6), and the faecal bacteria screening assays (chapter 7).

In chapter 7, MRS was used to grow bacterial isolates for identification by 16S rDNA sequencing, and LB was used in the bacterial screening assays investigating glucoraphanin metabolism by human faecal bacteria. BHI agar was used as described in section 2.8.5.

2.4 Liquid chromatography mass spectrometry analysis

2.4.1 Glucosinolate hydrolysis product analysis

Glucosinolate hydrolysis products and their conjugates were analysed by LC-MS/MS using a Phenomenex Luna C18(2) (100 x 2 mm id, 3 µm particle size) column connected to a model 1290 infinity 6490 Triple Quad LC-MS/MS system (Agilent Technologies) comprised of a degasser, binary pump, cooled autosampler, column oven, diode array detector and 6490 mass spectrometer. The samples were eluted at 0.25 ml/min with a gradient of increasing acidified ACN: solvent A (0.1% ammonium acetate buffer), solvent B (ACN with 0.1% acetic acid). The gradient increased from 5% acidified ACN to 100% over 9.1 minutes, prior to re-equilibration to 5% acidified ACN for 2.9 minutes. The LC eluent flow was sprayed into the mass spectrometer interface without splitting. ITCs and their conjugates were monitored by tandem MS using multiple reaction monitoring (MRM) with electrospray ionisation source in the positive ion mode. Identification was achieved on the basis of retention time and product ions, and quantification was performed through the use of calibration standards.

2.4.2 *S*-methylcysteine sulphoxide analysis

Cultured samples (100 µl) from the experiments using bacteria isolated from human faecal bacteria (chapter 7) were mixed with 250 µl dansyl chloride reagent (10 mM) and 650 µl borate buffer (20 mM; pH 9.2).The samples were incubated at room temperature for 30 min, prior to centrifugation at 17,000 x g for 10 min at room temperature. The supernatants (~ 800 µl) were transferred to autosampler vials for LC-MS analysis. *S*-methylcysteine sulphoxide (SMCSO) was analysed by LC-MS with a Waters Spherisorb ODS2 (4.6 x 250 mm id, 5 µm particle size) column connected to an 1100 series Single Quad LC-MS system (Agilent Technologies) comprised of a 1200 series degasser, binary pump, cooled autosampler, column oven, diode array detector and G1956B mass spectrometer. The samples were eluted at 0.9 ml/min with a gradient of increasing acidified methanol (MeOH): solvent A (50 mM ammonium acetate adjusted to pH 5 with 0.1 M acetic acid), solvent B (MeOH (1 L) containing 10 mM hydrochloric acid (826 µl 37% conc.)). The gradient increased from 30% acidified MeOH to 40% over 35 minutes, then increased from 40% to 75% over 25 minutes, and after 10 minutes this re-equilibrated to 30% acidified MeOH for 5 minutes. The LC eluent flow was sprayed into the mass spectrometer interface without splitting. SMCSO isomers were monitored by MS using full scan acquisition with electrospray ionisation source in the positive ion mode. Identification was achieved on the basis of retention time, and quantification was performed through the use of calibration standards.

2.5 Glucosinolate analysis

2.5.1 Desulphoglucosinolate extraction

Glucosinolates were measured using an adapted method that converts glucosinolates to their corresponding desulphoglucosinolates [322]. Briefly, 200 µl of the samples were added to 4.8 ml of 70% MeOH (70˚C), and 50 µl of 16 mM sinigrin was added to act as an internal standard. Samples were mixed, and 3 ml aliquots were added to ion exchange columns. Once the samples had passed through, the columns were washed with 0.22 µm filtered water (2x 0.5 ml) and 0.02 M sodium acetate (2x 0.5 ml), prior to the addition of purified sulphatase (75 µl). Following an overnight incubation at room temperature, the desulphoglucosinolates were eluted by the consecutive application of 0.22 µm filtered water (2x 0.5 ml and 0.25 ml), and the eluent was collected and analysed using HPLC.

2.5.2 Analysis of desulphoglucosinolates by high-performance liquid chromatography

The desulphoglucosinolates were analysed using a model 1100 HPLC system (Agilent Technologies) equipped with a degasser, binary pump, cooled autosampler, column oven and diode array detector, with a Waters Spherisorb ODS2 (4.6 x 250 mm id, 5 µm particle size) column attached. Filtered (0.22 µm) water and ACN were used as solvents with a flow rate of 1 ml/min, and a gradient of increasing ACN from 5% to 90% over 32 minutes, prior to re-equilibration to 5% for 14 minutes. Desulphoglucosinolate quantification was achieved, using absorbance at 229 nm, by comparison with the peak area ratio of the internal standard (sinigrin), and the relevant desulphoglucosinolate ultraviolet (UV) light relative response factor (**Table 2.2**).

Table 2.2: Desulphoglucosinolate UV relative response factors for HPLC analysis

Desulphoglucosinolate	Correction factor	Ref.
Desulphoglucobrassicin	0.29	[323]
Desulphoglucoerucin	1.00	[324]
Desulphoglucoiberin	1.13	[323]
Desulphoglucoiberverin	0.80	[325]
Desulphoglucoraphanin	1.13	[323]
Desulphoglucotropaeolin	0.95	[326]
Desulphogluconasturtiin	0.95	[326]
Desulphoprogoitrin	1.09	[326]
Desulphosinigrin	1.00	[326]
Desulpho4-hydoxyglucobrassicin	0.28	[326]
Desulpho4-methoxyglucobrassicin	0.20	[326]
Desulpho4-methoxyindolylmethyl	0.25	[327]

2.6 Metabolite analysis

2.6.1 Sample preparation

Cultured bacterial samples (2 ml) from the *in vitro* batch fermentation experiments were centrifuged at 9,600 x g at room temperature for 5 minutes. The supernatants were passed through a 0.2 µm syringe filter (Cat. # 16532K, Sartorius) into sterile tubes and stored at -20˚C.

Prior to analysis, the samples were thawed at room temperature, 600 µl was added to 70 µl of 0.4 mM phosphate buffer (K_2HPO_4 (2.82 g) and NaH_2PO_4 (0.54 g) [pH 7.4]) made up in 100% D_2O (total volume 200 ml), containing 0.1% NaN_3 (104 mg), and 2.5 mM sodium 3-(Trimethylsilyl)-propionate-d_4, (TSP) (34 mg) as a chemical shift reference. The samples were mixed, and 600 µl was transferred into a 5 mm NMR tube for spectral acquisition.

2.6.2 Proton nuclear magnetic resonance spectrometry analysis

The ^{1}H NMR spectra were recorded at 600 MHz on a Bruker Avance spectrometer (Bruker BioSpin GmbH) running Topspin 2.0 software and fitted with a cryoprobe and a 60-slot autosampler. Each ^{1}H NMR spectrum was acquired with 64 scans, a spectral width of 12,500 Hz, an acquisition time of 2.62 seconds, and a relaxation delay of 3 seconds. The “noesypr1d” pre-saturation sequence was used to suppress the residual water signal with a low-power selective irradiation at the water frequency during the recycle delay and a mixing time of 10 ms. Spectra were transformed using the Chenomx NMR Suite software with a 0.3 Hz line broadening, and were manually phased, baseline corrected, and referenced by setting the TSP methyl signal to 0 ppm.

2.7 Glucoraphanin stability assay

A broccoli leachate was prepared as described in section 2.2, and the glucoraphanin content was analysed in three technical replicates using HPLC (section 2.5). An aliquot of the broccoli leachate and CNM were added to six sterile 25 ml glass bottles to a volume of 19 ml, to produce a glucoraphanin concentration of 100 μM in a final inoculated volume of 20 ml. The tubes were transferred to an anaerobic cabinet (Don Whitley Scientific, UK) set at 37˚C to deoxygenate (85% nitrogen, 10% hydrogen, 5% carbon dioxide) for 12 hours.

A 5 g portion of the faecal sample was placed into a stomacher bag (Cat. # BA6141/STR, Seward Ltd, UK) using a sterilised spatula. Deoxygenated sterile 1x PBS was added to the stomacher bag to obtain a mass of 50 g. The stomacher bag was placed into the Stomacher 400 circulator (Seward Ltd, UK) and the faecal matter was homogenised at 230 rpm, for 45 seconds. A 10 ml aliquot of the homogenised faecal suspension was transferred into a 20 ml sterile glass bottle and placed into an anaerobic cabinet set at 37˚C. The faecal suspension was mixed, prior to 1 ml being added to three of the six bottles of deoxygenated media. The inoculated media were gently mixed and incubated in the anaerobic cabinet (37˚C) for 24 hours. Control samples were generated through the addition of 1 ml 0.22μm filtered water, in place of 1 ml faecal suspension, to the remaining three bottles of deoxygenated media. 2 ml aliquots were collected from each replicate, centrifuged at 9,600 x g for 5 minutes, and the supernatant was passed through a filter (0.2 μm) into a sterile tube. The filtered supernatants were stored at -20˚C prior to glucoraphanin analysis using HPLC (section 2.5).

2.8 Culturing human faecal microbiotas

2.8.1 Recruitment of faecal donors

Faecal material was obtained from participants recruited onto a human study (ENGAGE; ClinicalTrials.gov: NCT01927666) who gave written informed consent for their stools to be used in these experiments. Details of the faecal donors are displayed in **Table 2.3**. The ENGAGE study protocol was approved by the Human Research Governance Committee at the Institute of Food Research and Hertfordshire Research Ethics Committee (HREC 12/EE/0483). All study participants were assessed for eligibility on the basis of a health questionnaire and the results of clinical laboratory tests. All participants produced a urine sample for urinalysis which was screened for protein, blood, leukocytes, nitrites, glucose, ketones, bilirubin and urobilinogen via a dipstick urine test (Multistix® SG; Siemens).

The following exclusions applied:

- suffering from or had previously suffered from any gastrointestinal disease, gastrointestinal disorders and/or surgery including regular diarrhoea or constipation (excluding asymptomatic hiatus hernia), or the study intervention/procedure was indicated to be inadvisable.
- diagnosed with a long-term medical condition that may affect the study outcome e.g. cardiovascular disease, haemophilia, anaemia, diabetes, or glaucoma.
- was or had been pregnant in the last 12 months, or breast-feeding.
- on medication that may affect the study outcome.
- used antibiotics within the previous month or on long-term antibiotic therapy.
- regularly taking laxatives.
- intermittently used pre- or probiotics unless willing to abstain for a set period (continued regular use, defined as >3 times per week, was acceptable).

- taking certain dietary supplements or herbal remedies unless willing to abstain for a set period.
- regular/recent use of colonic irrigation or other bowel cleansing techniques.
- involved in another research project that includes dietary intervention and/or collection of blood.
- given blood sample to another research project within a set period (dependent on total volume).
- donated or intended to donate blood within a set period.
- Body Mass Index (BMI) less than 20 kg/m^2.
- unwilling to provide details of General Practitioner (GP).
- unable to provide written informed consent.
- related to or living with a member of the study team.
- unable to swallow capsules.
- no access to a freezer.
- clinical results at screening judged by the medical advisor to be indicative of a health problem or affect study outcome.

Table 2.3: Age, gender, Body Mass Index (BMI), and smoking status of the faecal donors.

Faecal donors	***Age (years)***	***Gender***	***BMI (kg/m^2)***	***Smoker***
Subject 1	44	Male	24.1	N
Subject 2	24	Female	23.3	N
Subject 3	52	Female	20.9	N
Subject 4	50	Female	38.4	N
Subject 5	61	Female	20.5	N

2.8.2 Preparation of broccoli leachate-containing media and glucose media

Three technical replicates of the broccoli leachate stock solution (section 2.2) were analysed, as described in section 2.5, to identify the concentration of glucoraphanin. A sterile inoculating loop was used to streak the broccoli leachate on a BHI agar plate, which was incubated anaerobically at 37˚C, to confirm that the leachate was not contaminated. The volumes of CNM and broccoli leachate necessary to make a final solution of 30 µmoles glucoraphanin BL media were added to five 50 ml sterilised glass bottles in a laminar flow hood. The bottles containing the BL media were transferred to an anaerobic cabinet set at 37˚C to deoxygenate for 12 hours. The glucose medium was generated by adding 113.79 mg of glucose (Cat. # G-7528, Sigma Aldrich) to a 1 L bottle to produce a medium containing 30 µmoles of glucose, prior to being autoclaved. CNM was added to the bottle up to 1 L, mixed, and 47.5 ml was dispensed into each of the five 50 ml sterilised bottles in a laminar flow hood. The bottles containing the 30 µmole GL medium were transferred to an anaerobic cabinet, set to 37˚C, to deoxygenate for 12 hours.

2.8.3 Culturing human faecal microbiotas in a broccoli leachate-containing media and a glucose media

A 17 g portion of faecal material was placed into a Seward stomacher bag using a sterilised spatula. Deoxygenated 1x PBS was added to the stomacher bag to obtain a mass of 170 g. The stomacher bag was placed into the Stomacher 400 circulator and the faecal matter was homogenised at 230 rpm, for 45 seconds. A 100 ml aliquot of the homogenised faecal suspension was transferred into a 150 ml sterile glass bottle and placed into an anaerobic cabinet set at 37˚C. The faecal suspension was inverted 4 times and 8 ml aliquots were transferred to sterile tubes for 16S rDNA phylogenetic

analysis. A 2 ml aliquot was taken from each of the four replicates of the BL and GL media for analysis of the pre-intervention samples. A 2.5 ml aliquot of the faecal suspension was added to the four replicates of each media, and these were gently mixed. The remaining bottle of the BL media and the GL media were not inoculated, and were incubated alongside the cultured media to act as indicators of possible contamination. Following 12 hours incubation, the cultured media were inverted 4 times, and aliquots were removed from each replicate and placed in sterile tubes for analysis using multiple techniques. Fresh media was prepared (as described in section 2.8.2) and seeded with a 2.5 ml aliquot from each replicate, to start the next cycle of culturing (**Figure 2.1**).

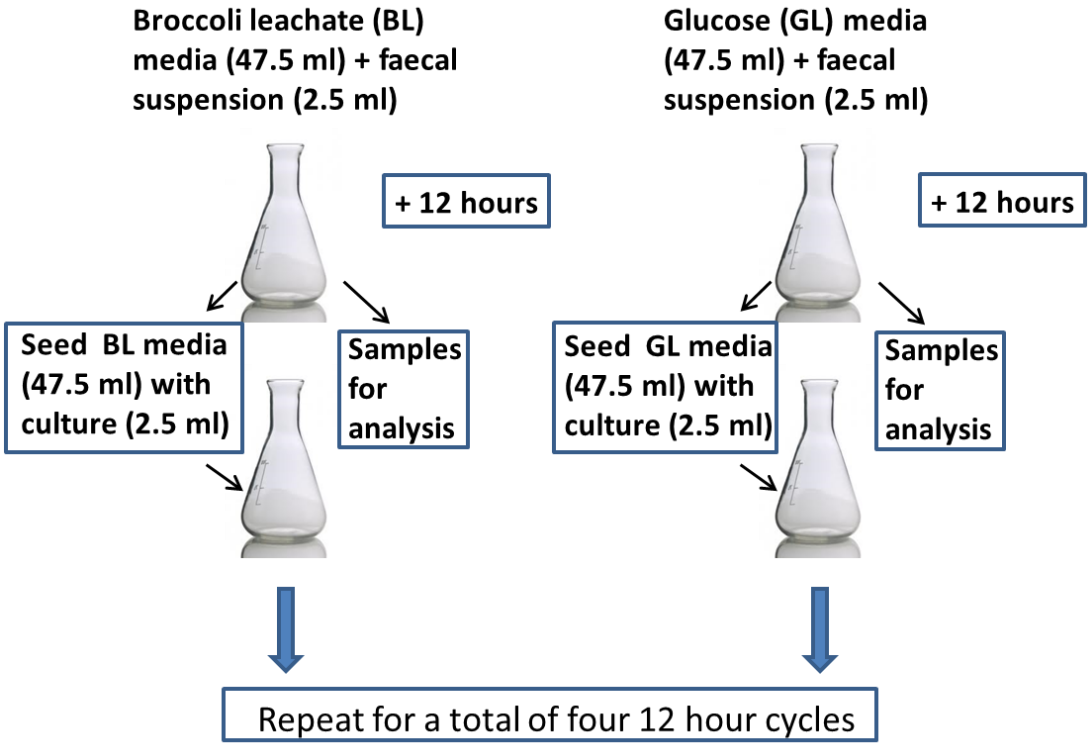


Figure 2.1: Experimental design for culturing human faecal microbiotas. Broccoli leachate (BL) media (47.5 ml) and glucose (GL) media (47.5 ml) were seeded with a faecal suspension (2.5 ml) and cultured for 12 hr at 37˚C, under anaerobic conditions. After a 12 hr incubation period, samples were taken for HPLC, LC-MS/MS, ^{1}H NMR spectroscopy, and 16S rDNA sequencing. A 2.5 ml aliquot was used to seed the respective fresh media, and this was repeated for four cycles of bacterial enrichment. This was performed identically for five human faecal microbiotas.

2.8.4 Sample storage

Three 2 ml aliquots, collected from each replicate after each 12 hour cycle, were centrifuged at 9,600 x g for 5 minutes; and the supernatant was passed through a filter (0.2 µm) into a sterile tube. The filtered supernatants were stored at -80˚C for HPLC (section 2.5), LC-MS/MS (section 2.4.1), and ^{1}H NMR spectroscopy (section 2.6) analysis. A further 8 ml aliquot, collected from each replicate after each 12 hour cycle, was centrifuged at 23,500 x g, at 4˚C, for 15 minutes. The supernatant was discarded and the pellets were stored at -80˚C for 16S rDNA phylogenetic analysis. This was repeated for each of the five cultured human faecal samples.

2.8.5 Bacterial isolation from cultured microbiotas

Following the fourth 12 hour cycle, each of the four replicates from both the BL and GL cultured media were streaked onto separate BHI agar plates. The plates were incubated in an anaerobic cabinet at 37˚C for 2 days. Twelve bacterial colonies were selected from each of the eight plates. Each bacterial colony isolated from the BL media was transferred to a single well of a 96-well microtitre plate containing 300 µl of 30 µmole BL media. Bacterial colonies isolated from the GL media were transferred to wells containing 300 µl of 30 µmole GL media. The microtitre plate was incubated overnight in an anaerobic cabinet at 37˚C. The entire contents of each well were transferred to sterile tubes containing 500 µl of 50% glycerol, and these glycerol stocks were stored at -80˚C.

2.9 Phylogenetic analysis of cultured human faecal microbiotas

2.9.1 Extracting bacterial DNA

The bacterial pellets were thawed at room temperature, and the bacterial DNA was extracted using a FastDNA SPIN Kit for Soil (Cat. # 116560200, MP Biomedicals, UK) following a method adapted from Maukonen *et al* [328]. Briefly, 200 mg of sample was transferred to a sterile tube containing 978 µl sodium phosphate buffer and 122 µl MT buffer, vortexed, and incubated at 4˚C for 45 minutes. Samples were vortexed, transferred to Lysing Matrix E tubes, and mechanically disrupted three times with a FastPrep instrument (MP Biomedicals, UK) at 6.5 ms^{-1} for 60 seconds. The supernatant was transferred to a sterile tube containing 250 µl protein precipitation solution (PPS), mixed well, and centrifuged at 16,800 x g for 5 minutes. The supernatant was transferred to a sterile tube containing 1 ml Binding Matrix suspension, mixed for 2 minutes, then incubated at room temperature for 3 minutes. 600 µl of the mixture was added to a SPIN filter tube, centrifuged at 14,500 x g for 1 minute, then underwent three 500 µl DNase-free salt/ethanol wash solution (SEWS-M) wash steps, centrifuged at 14,500 x g for 1 minute between each step. DNA was eluted with 50 µl of DNase/Pyrogen free DNA elution solution (DES) and stored at -20˚C.

2.9.1.1 Assessing bacterial DNA extraction

A 1% (w/v) agarose solution was generated by adding 0.5 g agarose powder (Cat. # A9539, Sigma Aldrich) to 500 ml 0.5 mM Tris/borate/ethylenediaminetetraacetic acid (TBE) buffer, and microwaved (800 watts) for 2 – 3 min at full power until clear. The 1% (w/v) agarose solution was added to an electrophoresis gel tray in a horizontal gel electrophoresis system (Cat. # 1068BD, Life Technologies, Inc.), with a toothed comb

fitted (to a depth of ~6 mm) and left to set at room temperature (~30 min). TBE (5 mM) was added to a depth that covered the 1% (w/v) agarose gel. 2 µl Bioline Hyperladder I (Cat. # BIO-33053VTFP) was added to the outer well to act as a molecular weight marker, and 2 µl DNA and 0.5 µl 10x loading dye were added to the remaining wells. The gel was run at 100 volts (PPV 300/200.4, Northumbria Biologicals Ltd) until the samples had migrated towards the end of the gel (~45 min), as indicated by the loading dye. The gels were submerged in an ethidium bromide solution (5 µg-10 µg/ml ethidium bromide in water) for ~30 min and rinsed with water. DNA fragments were visualised and photographed using the AlphaImager HP system (Alpha Innotech) under UV trans-illumination.

Total DNA was quantified using the NanoDrop ND-1000 UV/vis spectrophotometer (NanoDrop Technologies, Inc., USA) and the ND-1000 version 3.8.1 computer software. The nucleic acid, DNA-50 settings were selected, DNase/Pyrogen free water (1.2 µl) was used as a blank, and 1.2 µl of each sample was analysed.

2.9.2 Amplification and high throughput 454 pyrosequencing of 16S rDNA gene regions

PCR amplification of the 16S rDNA gene and subsequent DNA sequencing was performed at the Animal Health and Veterinary Laboratories Agency (AHVLA) using 454 pyrosequencing, as described by Ellis *et al* [329]. Briefly, universal primers for the V4 and V5 regions of the 16S rDNA gene were used to amplify the extracted DNA. The primers U515F (5'-GTGYCAGCMGCCGCGGTA) and U927R (5'-CCCGYCAATTCMTTTRAGT) include at their 5' end one of two adaptor sequences (adaptor A and B) used in the 454-sequencing library preparation. The adaptor

sequences are linked to a unique 10 base multiplex identifier (MID) tag barcode, with a minimum 2 base difference between each pair, to enable sample identification. FastStart HiFi polymerase (Roche Diagnostics Ltd) was used to amplify the ribosomal gene regions using the following conditions: 94˚C for 3 minutes; 30 cycles of 94˚C for 30 seconds, 55˚C for 45 seconds, 72˚C for 1 minute; followed by one cycle of 72˚C for 8 minutes. Amplicon purification was performed using Ampure XP magnetic beads (Beckman Coulter), and a Picogreen assay (Invitrogen) was used to measure the sample concentrations prior to normalisation. Samples were pooled into batches of 8 and subjected to unidirectional sequencing from the forward primer on the GS FLX Titanium platform following the manufacturer's instructions (Roche Diagnostics).

2.9.3 Bioinformatic analysis of 16S rDNA

Sequencing reads were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline and Ribosomal Database Project (RDP) classifier [330, 331]. All sequences were filtered to meet the following criteria: read length within 200 and 1,000 bp; a maximum of 6 ambiguous bases; a minimum average quality score of 25 within a 50 bp window; and exact match to primer sequences. ChimeraSlayer was used to filter trimmed reads for chimeric sequences, and RDP classifier (version 2.10) was used for bacterial taxonomy assignment with a confidence value threshold of 50%, with trimmed reads clustered into OTUs at 97% identity level. Observed species (number of unique OTUs), PD whole tree (measure of phylogenetic diversity), chao1 (species richness), and the Shannon Index (species richness and evenness) were used to compute alpha diversity and rarefaction plots. Weighted and unweighted UniFrac distances were used to generate beta-diversity PCoA plots, which were visualised using the Emperor tool.

2.10 Identifying bacterial isolates

2.10.1 Reviving glycerol stocks of bacterial isolates

MRS agar plates were transferred to an anaerobic cabinet set at 37˚C, and placed on a grid template. Fifty glycerol stocks of bacterial isolates obtained from experiments culturing human faecal microbiotas (section 2.8.5), were transferred to the anaerobic cabinet. Two microliters were taken from each of the glycerol stocks and added to the two MRS agar plates, at a recorded grid position. One plate was incubated in the anaerobic cabinet at 37˚C, and the other was transferred to a static incubator set at 37˚C. Both plates were incubated for 12 hours.

2.10.2 Colony polymerase chain reaction

Colony polymerase chain reaction (PCR) was performed on 19 colonies of the revived glycerol stocks that exhibited strong growth. Each colony was transferred to a 50 µl PCR tube containing 10 µl of 0.22 µm filtered water, and resuspended. The resuspended colonies were boiled in a pre-heated Hybaid PCR Sprint thermal cycler (Thermo Scientific) set at 95˚C, for 5 min. This was used as the template DNA for the following PCR reactions. The PCR reaction mixture was generated by adding the following together: 10 µl 5x colourless GoTaq reaction buffer (Cat. # M792A, Promega, UK), 0.4 µl (25 mM) deoxynucleotide triphosphate (dNTP) Mix (Cat. # 10180740, Thermo Fisher Scientific, Inc.), 1 µl (20 µM) universal forward primer (AmpF 5'-GAGAGTTTGATYCTGGCTCAG -3') [332], 1 µl (20 µM) universal reverse primer (AmpR 5'- AAGGAGGTGATCCARCCGCA -3') [332], 36.35 µl 0.22 µm filtered water, 1 µl template DNA, and 0.25 µl GoTaq DNA polymerase (Cat. # M3175, Promega, UK), to make a final volume of 50 µl per reaction mixture. A negative control was generated as above with the template DNA replaced with filtered water.

The thermal cycler was programmed to perform the amplifications as previously described by Nueno-Palop & Narbad [333] with minor modifications: one cycle of 95˚C for 2 min followed by 25 cycles of 95˚C for 30 s, 55˚C for 30 s, and 72˚C for 60 s, and one cycle of 72˚C for 5 min. The PCR products were resolved by electrophoresis in a 1% (w/v) agarose gel and visualised by ethidium bromide staining (section 2.9.1.1). PCR amplified products were cleaned using QIAquick PCR Purification Kit (Cat. # 28104, Qiagen) according to the manufacturer's instructions. Two 15 µl aliquots of each cleaned PCR amplicon was added to a Eurofins sequencing tube, with 2 µl of the AmpF primer added to one aliquot, and 2 µl of the AmpR primer added to the other aliquot. The tubes were sent to Eurofins for 16S rDNA sequencing.

2.10.3 Bacterial isolate identification

The FinchTV software (Geospiza, Inc.) was used for an initial assessment of the quality of the 16S rDNA sequencing data returned from Eurofins. The paired samples, sequenced with the AmpF and AmpR primers, were assembled to form a single contig using SeqMan (DNASTAR, Inc.). The contig of each sample was checked for errors or mismatches, the validity of which was evaluated through the use of FinchTV. The quality-checked aligned sequences were uploaded to the online RDP SeqMatch tool (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp (RDP Data - release11_1; Seqmatch – version 3)). The search options enabled were type and non-type strains, environmental sequences and isolates, near full length sequences (≥ 1,200), and good quality sequences. The identification of the bacterial isolates was determined on the basis of the highest S_ab score [334].

2.11 Bacterial screening assays

2.11.1 Screening bacterial isolates for glucoraphanin reduction activity

A 0.15 M glucoraphanin stock solution was generated by dissolving a purified glucoraphanin extract (Intertek Group plc) in either CNM or LB. This was further diluted in the same medium to produce a purified glucoraphanin extract media (~6 mM). Glycerol stocks containing bacterial isolates (section 2.8.5), and an *Escherichia coli* DH5α stock (kindly donated by Ms Fatma Cebeci), were mixed, prior to 5 µl of each being added to tubes containing the purified glucoraphanin extract media. Control samples were generated through the addition of 5 µl 0.22 µm filtered water in place of 5 µl bacterial glycerol stock. Samples were incubated in either an aerobic or anaerobic cabinet set at 37˚C, for 24 hr or 72 hr, under static conditions. Optical density readings were taken at 600 nm wavelength using a 6715 UV/vis. spectrophotometer (Cat. # 671 5B0, Jenway) with control samples used as blanks. Samples were centrifuged at 21,100 x g for 5 minutes; and the supernatant was passed through a filter (0.2 µm) into a sterile tube. The filtered supernatants were stored at -20˚C for desulphoglucosinolate analysis by HPLC (section 2.5).

2.11.2 Screening bacterial isolates for glucoerucin metabolism

Glucoerucin (Carl Roth®) was dissolved in 0.22 µm filtered water to generate a 1 mg/ml stock solution (2.37 mM). CNM had the glucoerucin stock solution added to generate a 0.3 mM glucoerucin media. The *E. coli* 1B04 glycerol stock (section 2.8.5) was mixed before 10 µl was added to the glucoerucin media. Filtered water was used in control samples in place of the *E. coli* 1B04 glycerol stock. The samples were placed in an anaerobic cabinet set at 37˚C for 24 hr.

The cultures were centrifuged at 21,100 x g for 5 minutes; and the supernatant was passed through a filter (0.2 µm) into a sterile tube. The filtered supernatants were stored at -20˚C prior to desulphoglucoerucin analysis using HPLC (section 2.5).

2.11.3 Screening bacterial isolates for activity against *S*-methylcysteine sulphoxide

Powdered SMCSO (Cat. # M6626, Sigma Aldrich) was dissolved in filtered water to generate a 1 mg/ml stock solution (7.4 mM). The SMCSO stock solution was added to CNM to a final concentration of 0.3 mM. The *E. coli* 1B04 glycerol stock was mixed, and 5 µl was added to 995 µl of the SMCSO media. Control samples were generated through the addition of 0.22 µm filtered water in place of the *E. coli* 1B04 glycerol stock. The samples were placed in an anaerobic cabinet set at 37˚C for 24 hr. The samples were centrifuged at 21,100 x g for 5 minutes, and the supernatant was passed through a filter (0.2 µm) into a sterile tube. The filtered supernatants were stored at -20˚C prior to SMCSO derivative analysis using LC/MS (section 2.4.2), and ^{1}H NMR spectroscopy analysis (section 2.6).

2.12 Gas chromatography mass spectrometry analysis

CNM containing either glucoraphanin (~6 mM) or SMCSO (0.3 mM) (section 2.11.1 and 2.11.3, respectively) was inoculated with 2.5 µl of either *E. coli* 1B04 or *E. coli* DH5α, to a final volume of 0.5 ml. Filtered water was used in control samples in place of i) the tested substrate (glucoraphanin or SMCSO), ii) the bacterial strain (*E. coli* 1B04 or *E. coli* DH5α), and iii) both the substrate and the bacterial strain. Samples were prepared in triplicate. Gas chromatography mass spectroscopy analysis was

performed by Mr Paul Brett. A 65 µm PDMS/DVB solid-phase microextraction (SPME) fibre was exposed in the headspace of samples at room temperature for 20 minutes. Gas chromatography mass spectrometry (GC-MS) was performed with an Agilent 6890 series system, with the Agilent 5973 inert mass selective detector (Agilent Technologies, UK). A DB-WAX 30 m x 250 µm x 0.25 µm capillary column was used with helium as the carrier gas (splitless) at a constant flow of 1 ml/min. The SPME fibre was inserted into the GC-MS with the inlet temperature set to 250˚C. The oven temperature was kept at 40˚C for 2 minutes, so extracted compounds could be thermally desorbed, before increasing at a rate of 20˚C/min to a final temperature of 200˚C, which was held for 5 minutes. Mass spectra were obtained by electron ionisation over a range of 30 – 500 atomic mass units with six scans performed every second. Ion source temperature was 230˚C, with the quadrupole temperature set to 150˚C. Peaks were identified by comparing retention times, mass spectra and fragment ion fingerprints with the National Institute of Standards and Technology (NIST) library.

2.13 Effects of Brassica on human gut lactobacilli human dietary intervention study

Full details can be found in the Appendix in the form of annexes but, briefly, the study method was as follows.

2.13.1 Study recruitment

Ten participants were recruited onto the ‘Effects of *Brassica* on human gut lactobacilli’ (EBL) human study (ClinicalTrials.gov: NCT02291328), and gave written informed consent (Appendix: annex 6) for their biological samples to be used as described in the (Appendix: study protocol). The EBL study protocol was approved by the Human

Research Governance Committee at the Institute of Food Research and Norfolk Research Ethics Committee (14/EE/1078). All study participants were assessed for eligibility on the basis of a health questionnaire (Appendix: annex 7) and the results of clinical laboratory tests. All participants produced a urine sample for urinalysis which was screened for protein, blood, leukocytes, nitrites, glucose, ketones, bilirubin and urobilinogen via a dipstick urine test (Multistix® SG; Siemens).

The following exclusions applied:

- Those not within the range of 18 – 50 years old.
- Those with a BMI outside the range of 19.5 and 30 kg/m^2.
- Women who are or have been pregnant within the last 12 months, or are lactating and/or breast feeding.
- Those currently suffering from, or have ever suffered from, any diagnosed gastrointestinal disease, gastrointestinal disorders including regular diarrhoea and constipation (excluding hiatus hernia unless symptomatic) and/or have undergone gastrointestinal surgery, or the study intervention/procedure is contraindicated.
- Have been diagnosed with any long-term medical condition that may affect the study outcome (e.g. diabetes, haemophilia, cardiovascular disease, glaucoma, anaemia). These will be assessed on an individual basis.
- Those diagnosed with a long-term medical condition requiring medication that may affect the study outcome.
- Those regularly taking self-prescribed over the counter medications for digestive/gastrointestinal conditions.
- Those on long-term antibiotic therapy. Those who have been on a course of antibiotics are able to participate in/continue on the study once 4 weeks has elapsed from the end of the course of antibiotics, and this will be assessed on an individual basis.

- Those regularly taking laxatives (once a month or more).
- Those intermittently using pre and/or probiotics unless willing to abstain for 1 month prior to and during study period. If used regularly (3+ times a week, and for more than one month) and will continue throughout study period then do not exclude.
- Those on a diet programme, or those who plan to start a diet programme during the study, that may affect the study outcome (e.g. the 5:2 fasting diet) unless willing to abstain for 1 month prior to and during study period. These will be assessed on an individual basis.
- Those taking dietary supplements or herbal remedies (including those derived from *Brassica* plants) which may affect the study outcome – unless the participant is willing to discontinue taking them for 1 month prior to and during study period. Please note that some supplements may not affect the study and this will be assessed on an individual basis.
- Regular/recent (within 3 months) use of colonic irrigation or other bowel cleansing techniques.
- Recently returned to the UK following a period abroad, and who have suffered gastric symptoms during the period abroad or on return to the UK. These will be assessed on an individual basis.
- Parallel participation in another research project which involves dietary intervention and/or sampling of biological fluids/materials. Sampling of certain biological samples, such as saliva, may not affect the study and this will be assessed on an individual basis.
- Those who record blood in their stools or have two or more episodes of type 1, 2, or 7 stools during the study.
- Any person related to or living with any member of the study team.
- Those who are unwilling to provide GPs contact details.

- Those who are unable to provide written informed consent.
- Those who are not suitable to take part in this study because of their eligibility screening results.
- Those who do not have access to a freezer.
- Those who regularly consume more than 15 units of alcohol (women) or 22 units of alcohol (men) a week.
- Those who are allergic to any of the foods/ingredients within the diets supplied.

Details of the participants that were enrolled onto the study are displayed in **Table 2.4**.

Table 2.4: Age, gender, Body Mass Index (BMI), and smoking status of the EBL study participants.

Participant code	***Age (years)***	***Gender***	***BMI (kg/m^2)***	***Smoker***
EBL16	35	Male	28.5	N
EBL28	25	Female	23.5	N
EBL51	25	Female	24.8	N
EBL56	32	Female	21.1	N
EBL74	39	Female	20.3	N
EBL84	40	Male	24.4	N
EBL88	36	Male	26.0	N
EBL89	41	Female	25.3	Y
EBL92	28	Male	27.5	N
EBL97	34	Female	23.8	N

2.13.2 Effects of Brassica on human gut lactobacilli study design

A randomised, two-phase crossover dietary intervention study (*n* = 10), with one phase representing a low-*Brassica* diet, and the other phase a high-*Brassica* diet (**Figure 2.2**). The low-*Brassica* diet consisted of one portion of 84 g frozen broccoli and one portion of 84 g frozen cauliflower, purchased from a Sainsbury's superstore (J Sainsbury PLC, UK). Portions were to be steamed following the instructions provided (Appendix: annex 18), and a single portion of the participant's choice was to be consumed each week for two weeks. The high-*Brassica* diet consisted of six 84 g portions of frozen broccoli, six 84 g portions of frozen cauliflower, and six 300 g portions of a broccoli and sweet potato soup containing 84 g broccoli (Bakkavor Group Ltd, UK). Three portions of frozen broccoli, three portions of frozen cauliflower, and three broccoli and sweet potato soups were consumed each week, for a period of two weeks, prepared as instructed (Appendix: annex 18).

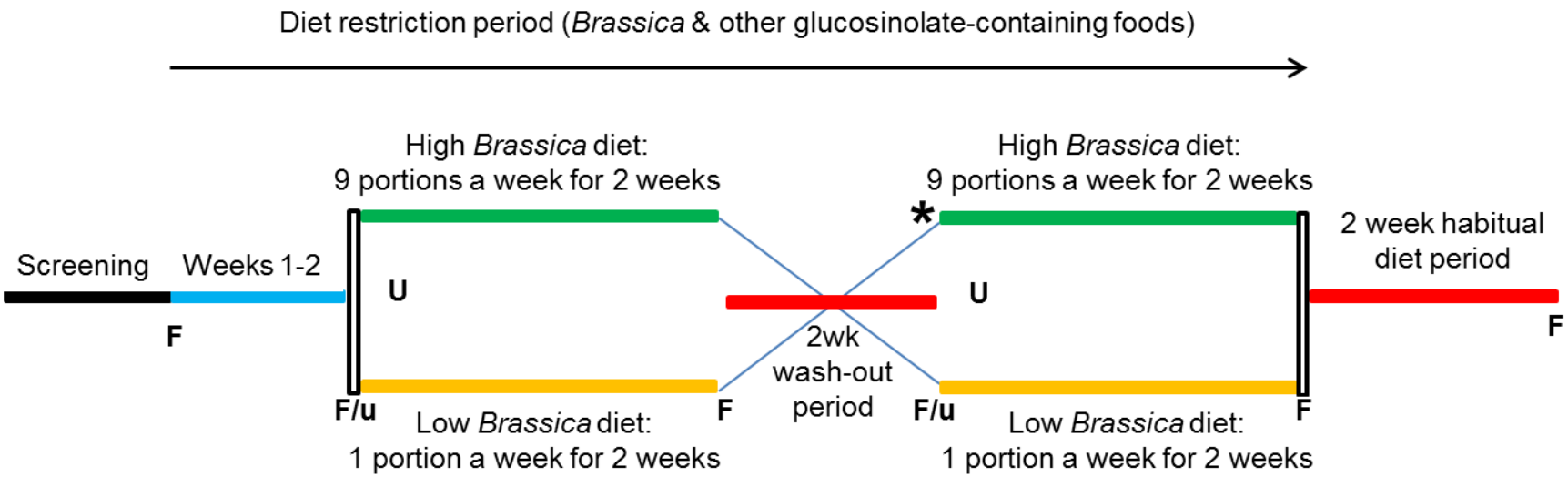


Figure 2.2: Design for the EBL human dietary intervention study. Randomised, two phase crossover, human dietary intervention study: *n* = 10. Following a successful eligibility screening appointment, participants produced a faecal sample before commencing a *Brassica* and glucosinolate-containing food diet restriction. After 2 weeks, a faecal sample (**F**) and urine (**u**) sample were collected, and the first 2 week intervention phase started (low- or high-*Brassica* diet). All urine was collected for 24 hr (**U**) directly after consuming the first portion of broccoli or broccoli-based soup. Participants were restricted from eating further portions until the urine collection was completed. Upon conclusion of the first intervention phase, a faecal sample was collected, and the 2 week washout period began. A further faecal sample and urine sample were collected on completion of the washout period, and the second 2 week intervention phase started (alternative diet to that consumed in intervention phase 1). All urine was collected for 24 hr directly after consuming the first portion of broccoli or broccoli-based soup. Participants were restricted from eating further portions until the urine collection was completed. Upon conclusion of the second intervention phase, a faecal sample was collected and the diet restriction period ceased. After 2 weeks consuming their habitual diet, participants collected a final faecal sample, completing the study. During each intervention phase, and the habitual diet phase, stool charts and food diaries were completed for a period of seven consecutive days. * At the end of the 2 week wash-out period, 8 participants were granted a short break from the study, for a period of no more than 6 weeks, to accommodate the Christmas period. Participants were required to resume the dietary restrictions for 2 weeks prior to producing an additional faecal sample, and starting the second intervention phase.

Participants were required to restrict their diet of *Brassica* and other foods that contain glucosinolates (Appendix: annex 15) during the active study period, starting two weeks prior to the first intervention phase, and finishing upon completion of the second intervention phase. Six faecal samples were collected per participant, following the instructions provided (Appendix: annex 14), at the following stages in the study: before commencing the diet restriction, immediately prior to the two intervention phases, upon completion of each intervention phase, and two weeks after the diet restriction ceased. Participants that required the prescribed study break were required to produce an additional sample following a 2-week diet restriction period prior to the second intervention phase. Urine samples were collected immediately prior to the two intervention phases, and were used for diet restriction compliance testing. Upon consuming the first portion of broccoli or the broccoli-based soup in each dietary intervention phase, participants were asked to refrain from eating further portions until they had collected all of their urine produced during the following 24 hr period. These urine samples were analysed for excreted glucoraphanin hydrolysis products.

Participants were asked to complete stool charts (Appendix: annex 12) to assess any effects of the diets on gut function, and to record fruit and vegetable consumption in food diaries (Appendix: annex 13). These were completed in consecutive 7-day periods during each intervention phase, and in the 2 week period following completion of the second intervention phase.

2.13.3 Effects of Brassica on human gut lactobacilli study sample analysis

2.13.3.1 Phylogenetic analysis of human faecal microbiotas

Approximate 2 g portions of faecal samples were collected in triplicate from each faecal sample, using sterilised spatulas and tubes, and stored at -80˚C. Bacterial DNA was extracted, visualised, and the DNA concentration was quantified following the method described in section 2.9.1. Bacterial DNA concentration was normalised to 1 ng/µl by dilution with DES (MP Biomedicals, UK) to produce a final volume of 20 µl. Normalised DNA samples were sent to the Centre of Genomic Research (Liverpool, UK) for PCR amplification of the 16S rDNA gene, and paired-end Illumina sequencing (2x 250 bp) on the MiSeq platform. Sequencing data were supplied in FASTQ format with adaptors already trimmed.

Sequencing data were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline and RDP 16S rDNA sequence database [330, 331]. All sequences met the following criteria: read length within 200 and 1,000 bp; an Illumina quality digit >0; and a minimum average quality score of 25 within a 50 bp window. ChimeraSlayer was used to filter trimmed reads for chimeric sequences, RDP classifier (version 2.10) was used for bacterial taxonomy assignment with a confidence value threshold of 50%, and trimmed reads clustered into OTUs at 97% identity level. Shannon, PD whole tree, chao1, and observed species were used to compute alpha diversity and rarefaction plots. Weighted and unweighted UniFrac distances were used to generate beta-diversity PCoA plots, which were visualised using the Emperor tool.

2.13.3.2 Metabolite analysis of human faecal waters

Approximately 100 mg of thawed faecal samples were added to sterile tubes. A phosphate buffer was generated by combining $NaH_2PO_4.H_2O$ (0.54 g), K_2HPO_4 (2.82 g), NaN_3 (100 mg), and TSP (34 mg) with 200 ml D_2O. The faecal waters were generated by adding the phosphate buffer to samples in volumes 12-fold greater than the mass of the faecal sample. Faecal waters were homogenised using a Kimble Kontes pellet pestle motor (Cat. # Z359971, Sigma Aldrich) with sterilised pestles (Cat. # Z359947, Sigma Aldrich). Homogenised faecal waters were centrifuged at 16,200 x g at room temperature for 5 min. The supernatants were passed through a 0.2 µm syringe filter into sterile tubes, and 600 µl was transferred into a 5 mm NMR tube for spectral acquisition, as described in section 2.6.2.

2.13.3.3 Urinary glucoraphanin hydrolysis product analysis

Compliance urine samples were mixed, aliquoted and stored at -80˚C. 24 hr urine collections were returned in pre-weighed containers. The mass of the full containers were recorded, and the volume of urine was calculated by subtracting the mass of the empty containers from the mass of the full containers. The urine samples were mixed, aliquoted, and stored at -80˚C. Aliquoted urine samples were thawed at room temperature, mixed, and passed through a 0.2 µm syringe filter into sterile tubes. A 100 µl aliquot of the filtered urine, 890 µl 0.1% ammonium acetate buffer, and 10 µl BITC were combined in sterile tubes and mixed. After centrifugation (17,000 x g, 4˚C, 10 min), 800 µl of the supernatant was transferred to an autosampler vial for LC-MS/MS analysis as described in section 2.4.1.

2.14 Statistical analysis

Data analysis, including calculation of average values, percentage products, standard deviations, and the student's t-test or one-way ANOVA where applicable, was performed using GraphPad Prism 5 (version 5.04). Results were treated as significant if $p \leq 0.05$.

The bacterial population data contained in chapter 8 underwent multivariate analysis prior to statistical analysis, which was performed by Dr Henri Tapp. A sub-set of 66 bacterial taxa were selected, based on their detection (non-zero proportions) in a minimum of 45 of the 67 faecal samples. A small offset (0.00001) was added to the data to counteract the presence of zero values. The data was log transformed to enable clearer visualisation of the data, and variance scaled, as the data was unevenly distributed. Fourteen principal components were retained based on the Kaiser criterion (factors with an eigenvalue >1). A varimax rotation (orthogonal rotation of the factor axes to simplify interpretation) was applied to the retained components, and the new scores were rescaled such that the corresponding loading were of unit length. The correlations between the first and second rotated factor scores and each of the log-transformed variates were plotted to identify bacterial taxa associated with the first rotated factor score.

Differences between the two diet intervention phases were investigated using sequential analysis of variance. The proportions of bacterial taxa were log-transformed following the addition of a small offset (0.00001). The response variable was the transformed post-high-*Brassica* diet bacterial proportion, and the four explanatory variables were, respectively: the transformed post-low-*Brassica* diet bacterial proportion (continuous), the participant code (*n*-level categorical), the study phase (2-level categorical, first intervention and second intervention), and the dietary treatment order (2-level categorical, high - low, low - high). Carry-over was investigated using the sum of the two post-intervention measurements, and statistical analysis was performed using Matlab. Results were treated as significant if $p \leq 0.05$.

CHAPTER THREE

3 Characterisation of broccoli leachates and bacterial media

3.0 Summary

Broccoli is a rich source of fibres, sugars, vitamins and minerals, such as sulphur. The majority of the sulphur is present in sulphate, glucosinolates, S*-methylcysteine sulphoxide, glutathione, and the amino acids methionine and cysteine. This chapter details the process by which broccoli leachates are produced, characterised, and used to generate a broccoli leachate-containing (BL) medium. A glucose (GL) medium is also produced and characterised. The BL medium was found to contain a range of minerals, 21 amino acids and amino acid derivatives, and relatively high levels of sugars. The BL medium was also shown to be particularly rich in total sulphur, glutamine, glutamate, fructose, and glucose. The GL medium also contains multiple minerals, 19 amino acids and amino acid derivatives, and sugars, but generally at lower levels than seen in the BL medium.*

3.1 Introduction

Two F_1 broccoli hybrids, containing high levels of glucoraphanin, were developed during breeding programmes in which the wild broccoli species (*Brassica villosa*) was crossed with a double haploid broccoli breeding line [335]. These F_1 hybrids contain a 2.5 - 3-fold increase in glucoraphanin compared to standard broccoli cultivars [5]. This increase in glucoraphanin content is likely due to the presence of a *B. villosa Myb28* allele, which appears to increase sulphate assimilation, and channels a greater amount of sulphur into the biosynthesis of methionine-derived glucosinolates, such as glucoraphanin [5]. These high-glucoraphanin hybrids are commercially available as Beneforté® broccoli.

Broccoli is a rich source of sulphur, containing *S*-methylcysteine sulphoxide (SMCSO), sulphate, glutathione, and the sulphur-containing amino acids methionine and cysteine, as well as methionine- and tryptophan-derived glucosinolates. Broccoli also contains relatively high levels of potassium, dietary fibres, and vitamins, such as Vitamin C and E, carotene, folate, pantothenate, and riboflavin [336]. This complex mix of potential bacterial nutrients makes broccoli an attractive option as a substrate for human gut microbiota fermentation studies.

Sulphur is an essential component of life, and is required for the bacterial biosynthesis of methionine and cysteine, as well as sulphur-containing co-enzymes [337]. Several research groups have shown that human gut bacteria are able to hydrolyse glucosinolates (releasing glucose, sulphate, and an aglycone), or modify glucosinolates through biotransformation reactions [45, 142, 338].

The formulation of bacterial media for use in fermentation studies with intestinal bacteria are designed to contain the essential nutrients for bacterial growth. Chemostat nutrient media (CNM) is a medium whose recipe has been used in multiple *in vitro* fermentation studies, in which the carbon source added to the medium is the substrate being investigated [339-343]. This allows the investigator to examine the metabolic effects associated with a single compound, whole food, or specific diet when cultured with a single species of bacteria, or a complex bacterial community.

3.2 Objectives

The objective of the work contained in this chapter was to generate and characterise leachates derived from broccoli, which will be used to supplement a basal bacterial medium. The broccoli leachate-containing medium, and a medium supplemented with glucose, were analysed for glucosinolate, mineral, amino acid, and sugar content.

3.3 Materials and methods

Broccoli leachates were generated using commercially sourced Beneforté® broccoli as described in section 2.2, and the basal bacterial medium (CNM) used is detailed in section 2.3. LC-MS/MS (section 2.4) was used to confirm that processing of the broccoli had significantly reduced broccoli myrosinase activity. A desulphoglucosinolate extraction in conjunction with HPLC, was used to investigate the levels of glucosinolates contained within the broccoli leachates, as described in section 2.5. ^{1}H NMR spectroscopy was used to identify the constituent parts of the broccoli leachates and the bacterial medium as explained in section 2.6.2. Samples were centrifuged and the supernatant filtered, prior to being mixed with a 0.4 mM phosphate buffer (section 2.6.1). Sodium 3-(Trimethylsilyl)-propionate-d_4 was used as a chemical shift reference, and metabolite quantification was carried out using the Chenomx NMR suite software. The broccoli leachates were combined with CNM to produce a broccoli leachate-containing (BL) medium, and glucose was added to CNM to generate a glucose (GL) medium. These media were commercially analysed for mineral content by Eurofins, and ^{1}H NMR spectroscopy was used to determine the concentrations of the amino acids and sugars within both media.

3.4 Results

3.4.1 Producing a broccoli leachate for use in culturing human faecal microbiotas

A broccoli leachate was chosen to act as the main substrate in the basal bacterial medium, rather than glucoraphanin alone, as the mix of plant compounds would better reflect the potential *in vivo* intestinal milieu following the consumption of broccoli. The BL media will be standardised to contain 30 µmoles of glucoraphanin. Beneforté® broccoli was selected as the starting substrate due to the increased levels of glucoraphanin compared to standard broccoli. Prior to making the broccoli leachates, it was necessary to investigate what processing would be required to significantly reduce the activity of the plant myrosinase enzyme. As broccoli is commonly steamed from fresh before human consumption, fresh Beneforté® broccoli was purchased and steamed for different durations, prior to lyophilisation. This was performed to identify the time necessary to significantly reduce myrosinase activity without negatively affecting the levels of glucoraphanin. When fresh broccoli was steamed for a minimum of 3 minutes, myrosinase activity was significantly reduced compared to unsteamed broccoli (**Figure 3.1**), and the levels of glucoraphanin remained high (**Figure 3.2**).

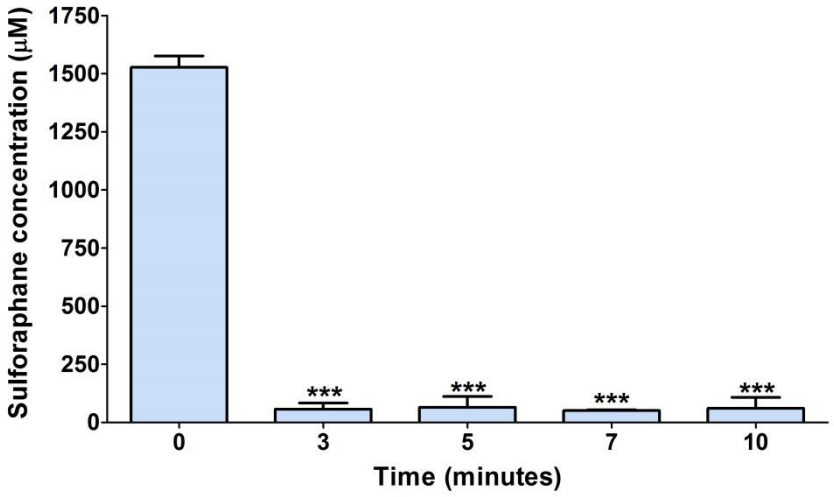


Figure 3.1: Effect of steaming on Beneforté® broccoli myrosinase activity. Fresh Beneforté® broccoli was steamed for different durations (0, 3, 5, 7 and 10 minutes) prior to lyophilisation and grinding. The powdered broccoli was rehydrated in purified water for 30 minutes, and the supernatant was collected and analysed by LC-MS/MS. Data shown = mean ± SD of three biological replicates. Data was statistically analysed using one-way analysis of variance (ANOVA) followed by the Dunnett multiple comparisons test. ***$p \leq 0.001$ vs unsteamed broccoli control.

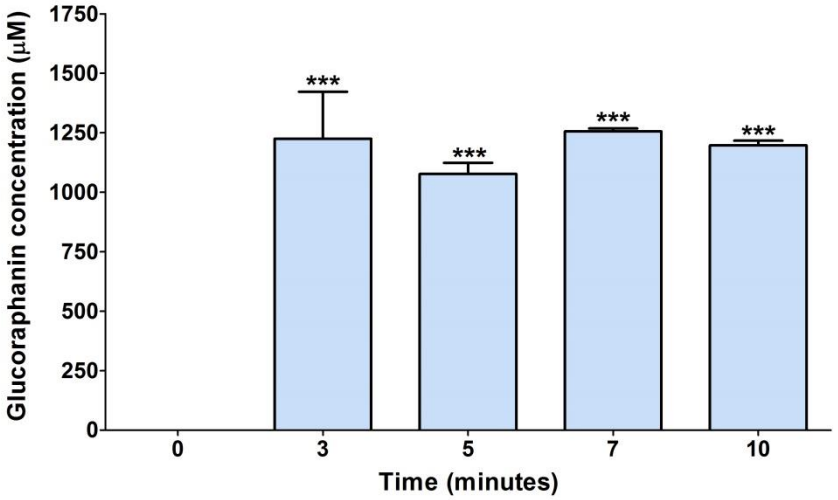


Figure 3.2: Effect of steaming on glucoraphanin content in Beneforté® broccoli. Fresh Beneforté® broccoli was steamed for different durations (0, 3, 5, 7 and 10 minutes) prior to lyophilisation. The powdered broccoli was rehydrated in purified water for 30 minutes, and the supernatant was collected and analysed by HPLC. Data shown = mean ± SD of three biological replicates. Data was statistically analysed using one-way ANOVA followed by the Dunnett multiple comparisons test. ***$p \leq 0.001$ vs unsteamed broccoli control.

From these data, a steaming duration of 3 minutes was deemed sufficient to prepare the fresh Beneforté® broccoli, prior to generating the broccoli leachates for use in the culturing of human faecal bacteria.

3.4.2 Identifying the constituents of the broccoli leachates

3.4.2.1 Glucosinolate content in the broccoli leachates

The broccoli leachates were concentrated through the use of evaporation techniques, and filtered before undergoing analysis to examine the glucosinolate profile (**Figure 3.3**). The glucoraphanin content in the broccoli leachates were between 1517.24 µmoles and 1845.44 µmoles. Lesser amounts of the glucosinolates glucoiberin (15.73 µmoles to 538.82 µmoles) and 4-methoxyindolylmethyl (4-MIND) (1.6 µmoles to 456.43 µmoles) were also present.

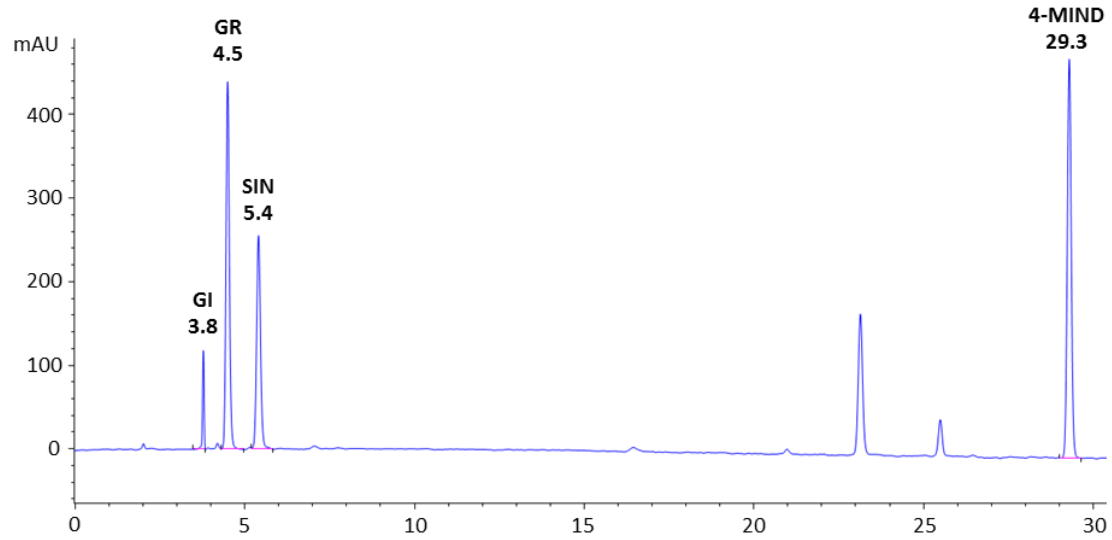


Figure 3.3: Glucosinolate profile of a broccoli leachate. Broccoli leachates were passed through a sephadex column to extract the desulphoglucosinolates, prior to HPLC analysis. The figure shows a representative HPLC chromatogram of a broccoli leachate, indicating which glucosinolates were present in the broccoli leachate. Sinigrin was used as an internal standard. RT = retention time. GI = glucoiberin (RT: 3.8); GR = glucoraphanin (RT: 4.5); 4-MIND = 4-methoxyindolylmethyl (RT: 29.3); SIN = sinigrin (RT: 5.4).

The broccoli leachates were used as stock solutions to generate the BL media, which were standardised to 30 µmoles of glucoraphanin. Although the levels of glucoiberin and 4-MIND varied between batches of BL media, the levels of glucoraphanin remained constant. The differences observed in the levels of glucosinolates in the broccoli leachates were likely due to seasonal variation and the different growing locations of the broccoli.

3.4.2.2 Proton nuclear magnetic resonance spectroscopy analysis of the broccoli leachates

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was used to identify the different constituents present in the broccoli leachates. The spectra from the lower end of the parts per million (ppm) spectrum (0 - 3.2 ppm) showed a range of amino acids, as well as lactate, citrate, choline, SMCSO, and glucoraphanin (**Figure 3.4**).

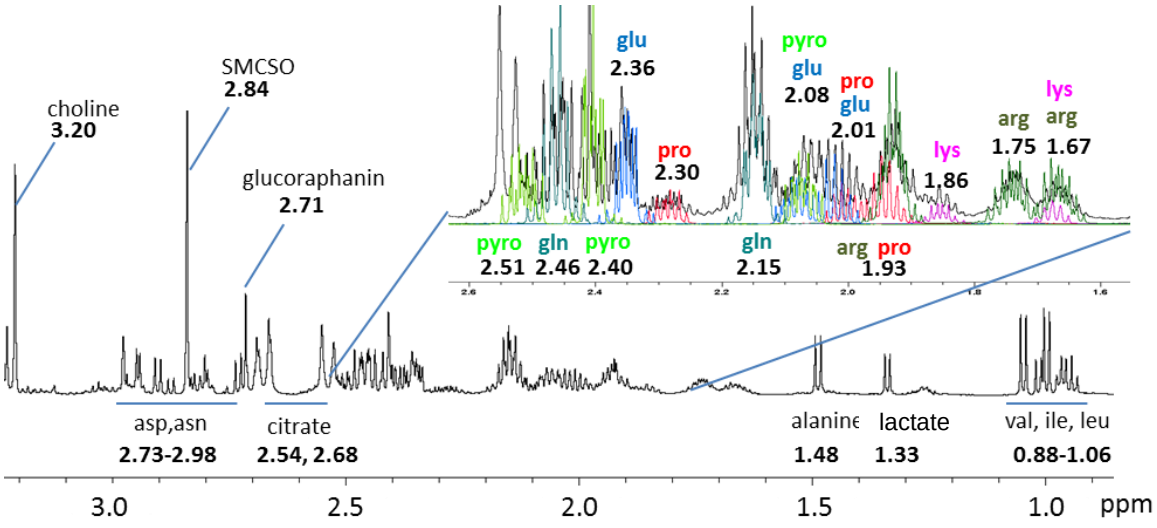


Figure 3.4: The broccoli leachates contain various amino acids. ^{1}H NMR analysis identifies various amino acids alongside several other compounds at the lower range of the ppm spectrum. arg: arginine; asn: asparagine; asp: aspartate; gln: glutamine; glu: glutamate; ile: isoleucine; leu: leucine; lys: lysine; pro: proline; pyro: pyroglutamate; SMCSO: *S*-methylcysteine sulphoxide; val: valine; ppm: parts per million.

Between 3 and 6 ppm, the sugars glucose and sucrose are identified, along with the glucosinolates, glucoraphanin and glucoiberin (**Figure 3.5**). The mass of peaks seen at 3.3 - 4.3 ppm are likely due to a polymer originating from the container used to store the broccoli leachates. Although this area masks peaks representing glucose, other peaks are present to enable the quantification of glucose in the broccoli leachates.

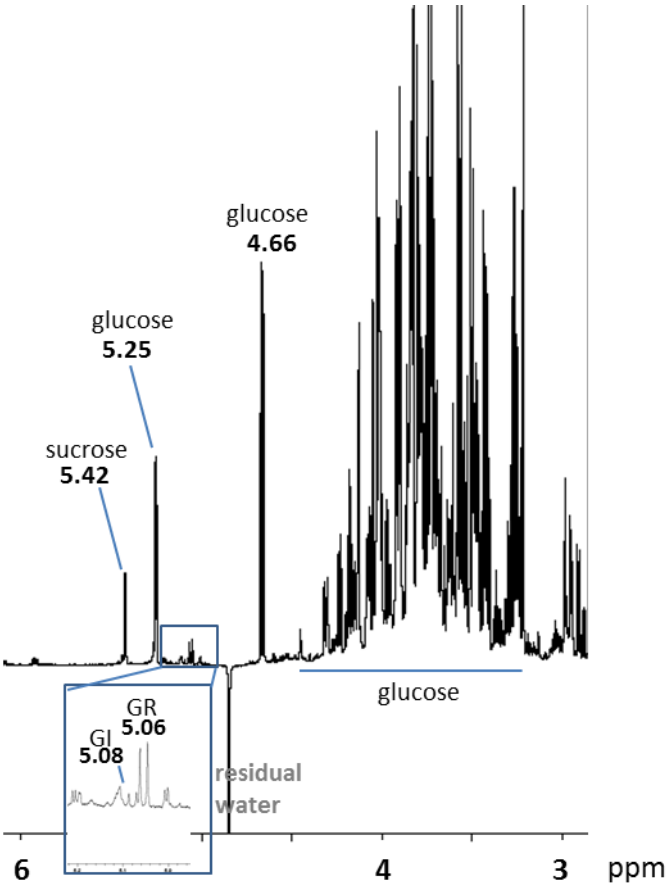


Figure 3.5: The leachates contain sugars derived from broccoli. ^{1}H NMR analysis identifies the sugars, glucose and sucrose, and the glucosinolates, glucoraphanin and glucoiberin between 3 - 6 ppm in the broccoli leachates. GI: glucoiberin; GR: glucoraphanin; ppm: parts per million.

At the higher end of the ppm range (5.9 – 9 ppm) a number of nucleosides and nucleotides were observed, as well as the amino acids tryptophan, tyrosine and phenylalanine. Formate, fumarate, and the indolic 4-MIND glucosinolate were also identified within this region of the ^{1}H NMR spectra (**Figure 3.6**).

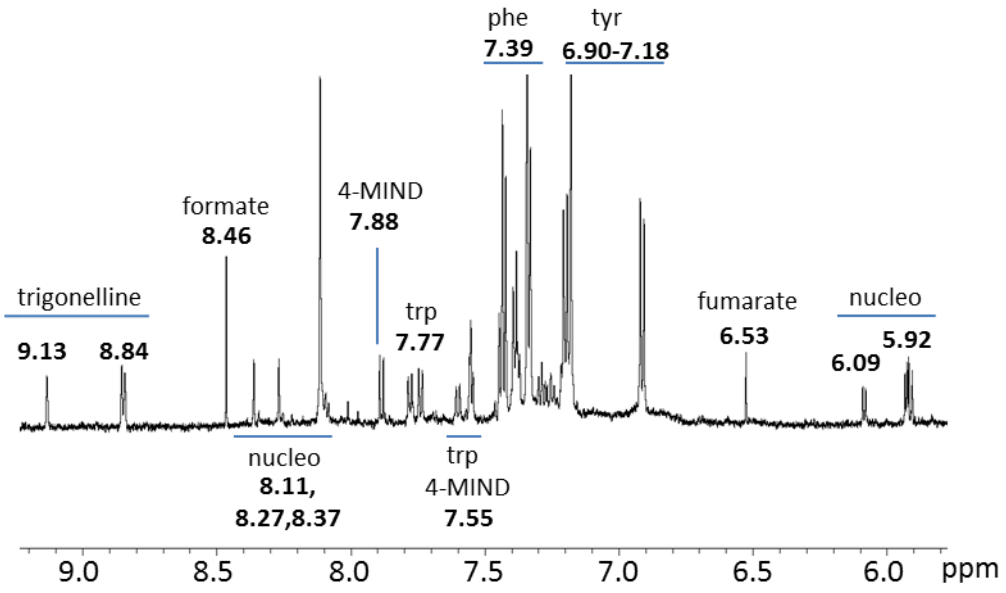


Figure 3.6: The broccoli leachates contain a number of nucleosides and nucleotides. Nucleosides, nucleotides, amino acids, and other compounds including 4-MIND are identified at the upper parts per million (ppm) range. 4-MIND: 4-methoxyindolylmethyl glucosinolate; nucleo: nucleosides/tides; phe: phenylalanine; trp: tryptophan; tyr: tyrosine.

Analysis using ^{1}H NMR showed that the broccoli leachates were a complex mix of amino acids, sugars, glucosinolates, and other compounds that may be able to be used as substrates by the human faecal bacteria during culturing.

3.4.2.3 Proton nuclear magnetic resonance spectroscopy analysis of the chemostat nutrient media

Analysis of the basal bacterial growth medium CNM, using ^{1}H NMR identifies a range of amino acids, lactate, acetate, succinate, betaine, and the presence of lipids and steroids between 0 – 3.6 ppm (**Figure 3.7**). The peaks assigned to lipids and steroids likely originate from the presence of Tween 80 and bile salts, respectively, in CNM.

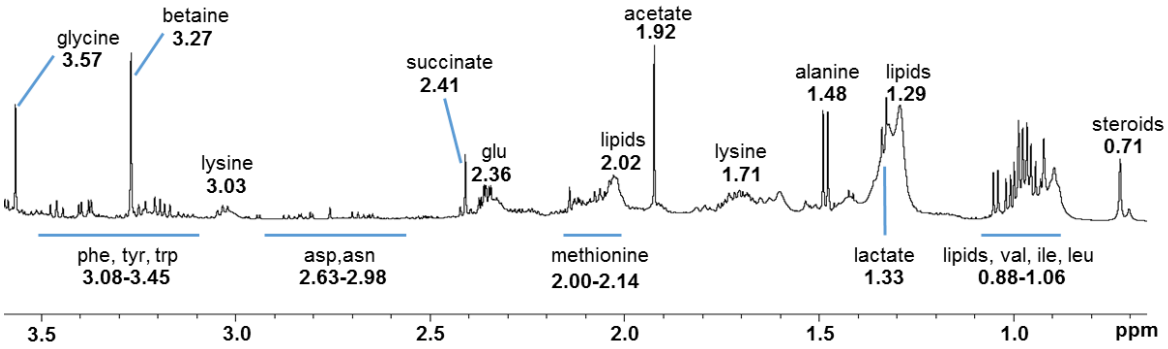


Figure 3.7: The chemostat nutrient media contains a range of amino acids. ^{1}H NMR analysis identifies a number of amino acids and other compounds at the lower parts per million (ppm) range within the basal medium (CNM). asn: asparagine; asp: aspartate; glu: glutamate; ile: isoleucine; leu: leucine; phe: phenylalanine; trp: tryptophan; tyr: tyrosine; val: valine.

Towards the upper range (5.5 – 9 ppm), additional amino acids were identified, as well as nucleosides and nucleotides, uracil, formate, nicotinate and nicotinamide (**Figure 3.8**). As observed in the broccoli leachates, CNM contained a range of amino acids as well as other compounds that are available to bacteria as nutrients.

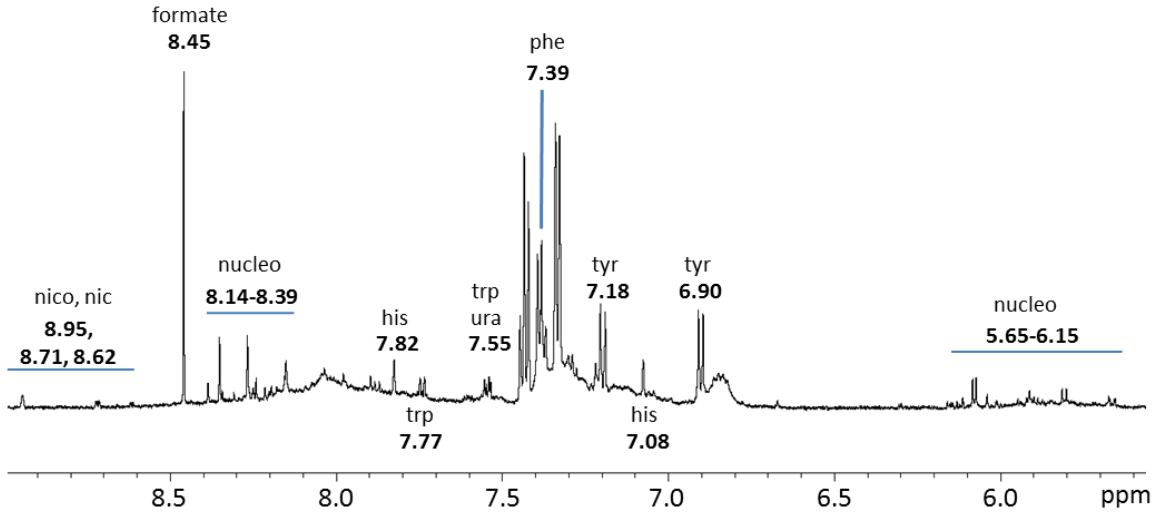


Figure 3.8: The chemostat nutrient media contains a number of nucleosides and nucleotides. Several nucleosides/tides, amino acids, and other compounds are detected using ^{1}H NMR at the upper parts per million (ppm) range for the basal medium. his: histidine; phe: phenylalanine; nic: nicotinamide; nico: nicotinate; nucleo: nucleosides/tides; trp: tryptophan; tyr: tyrosine; ura: uracil.

3.4.3 Analysis of media for use in a batch fermentation model

The broccoli leachate was added to CNM to produce a BL medium containing 30 µmoles of glucoraphanin. Standard commercial broccoli contains approximately 1 µmole of glucoraphanin per gram fresh weight prior to cooking [5]. The BL media was standardised to 30 µmoles as it was considered to be physiologically relevant to the amount of glucoraphanin that may reach the colon. This allowed for the loss of glucoraphanin through chopping and cooking a small portion of broccoli, as well as hydrolysis/absorption in the upper gastrointestinal tract. As the hydrolysis of 1 mole of glucoraphanin releases 1 mole of glucose, a control medium was produced by supplementing CNM to generate a 30 µmole glucose (GL) medium. The mineral content of these media was analysed commercially by Eurofins. The experimental volume of each medium will be 50 ml, and **Table 3.1** displays the amounts of minerals present in 50 ml of both the BL and GL media.

Table 3.1: Mineral analysis of 50 ml of the BL and GL media.

Mineral mass per 50 ml	*BL medium*	*GL medium*
Sodium (g)	0.061 (± 0.01)	0.063 (± 0.01)
Potassium (g)	0.071 (± 0.01)	0.011 (± 0)
Calcium (g)	0.005 (± 0)	<0.0003
Magnesium (g)	0.003 (± 0)	<0.0003
Phosphorus (g)	0.011 (± 0)	0.002 (± 0)
Iron (mg)	0.064 (± 0.01)	<0.05
Zinc (mg)	0.122	<0.1
Total sulphur (mg)	27.48 (± 3.22)	7.43 (± 0.67)

With the exception of sodium, which is present at similar levels within the two media, increased amounts of the other minerals were found in the BL medium compared to the GL medium. In particular, the BL medium contained a higher amount of potassium, and total sulphur than the GL medium. The sulphur contained within the three glucosinolates and SMCSO in the BL medium accounted for 3.97 mg of the total sulphur (**Table 3.2**). The majority of the remaining sulphur in the BL medium may have been incorporated into sulphate, which was not measured.

Table 3.2: Sulphur mass contributed by glucosinolates and SMCSO in 50 ml of the BL medium.

	Glucoraphanin	*Glucoiberin*	*4-MIND*	*SMCSO*
Molecular weight	436.50	422.47	478.50	151.19
No˚ sulphur molecules	3	3	2	1
µmoles solute in 50 ml	30.05	3.7	7.41	8.06
Mass of sulphur (mg)	2.89	0.36	0.46	0.26

Analysis using ^{1}H NMR spectroscopy indicated that supplementation of CNM with the broccoli leachate increased the concentrations of multiple amino acids and their derivatives in the BL medium, compared to the GL medium (**Table 3.3**).

Table 3.3: Amino acid and amino acid derivative analysis of 50 ml of the BL and GL media.

Amino acid (mM)	*BL medium*	*GL medium*
Alanine	1.26 (± 0.32)	0.76 (± 0.19)
Arginine	1.13 (± 0.86)	0.16 (± 0.06)
Asparagine	0.75 (± 0.08)	0.15 (± 0.05)
Aspartate	1.15 (± 0.40)	0.21 (± 0.06)
Cystine	0.23 (± 0.02)	0.26 (± 0.34)
Glutamate	2.78 (± 0.92)	0.87 (± 0.11)
Glutamine	4.21 (± 1.81)	0.02 (± 0.01)
Glycine	0.51 (± 0.15)	0.62 (± 0.23)
Histidine	0.03 (± 0)	0.03 (± 0.03)
Isoleucine	0.64 (± 0.21)	0.29 (± 0.10)
Leucine	0.65 (± 0.07)	0.51 (± 0.18)
Lysine	0.45 (± 0.15)	0.19 (± 0.04)
Methionine	0.08 (± 0.03)	0.09 (± 0.01)
Phenylalanine	0.45 (± 0.12)	0.22 (± 0.04)
Proline	1.29 (± 0.66)	0
Pyroglutamate	1.50 (± 1.01)	0.27 (± 0.05)
Serine	1.54 (± 0.75)	0
Threonine	0.68 (± 0.19)	0.20 (± 0.03)
Tryptophan	0.09 (± 0.01)	0.04 (± 0)
Tyrosine	0.23 (± 0.11)	0.08 (± 0.01)
Valine	1.21 (± 0.46)	0.41 (± 0.13)

The proline and serine detected in the BL medium originates from the broccoli leachate, whilst glutamate, glutamine, and pyroglutamate are present at notably higher concentrations in the BL medium, compared to the GL medium. These data indicate that the sulphur-containing amino acids, cystine (derived from oxidised cysteine) and methionine, are supplied to both media through CNM.

Table 3.4: Sugar content in 50 ml of the BL and GL media

Sugars (mM)	*BL medium*	*GL medium*
Fructose	10.44 (± 3.30)	0
Glucose	8.92 (± 1.87)	0.73 (± 0.04)
Sucrose	1.15 (± 0.33)	0
Trehalose	0.22 (± 0.01)	0.24 (± 0.06)

The BL medium contained a larger range, and higher concentration, of sugars than the GL medium, with fructose, glucose, and sucrose originating from the broccoli leachate (**Table 3.4**). Trehalose, present in both media, would likely have originated from the yeast extract used to generate CNM. These analyses indicate that the BL medium was a richer source of minerals, amino acids, and sugars than the GL medium.

3.5 Discussion

3.5.1 Reduction of broccoli myrosinase activity

Beneforté® broccoli was used to generate broccoli leachates containing high levels of glucoraphanin. It was necessary to inactivate, or significantly reduce, the activity of the broccoli myrosinase enzyme to prevent glucosinolate hydrolysis. It was determined that a steaming duration of 3 minutes was sufficient to significantly reduce the activity of the myrosinase enzyme (**Figure 3.1**), whilst minimising glucosinolate loss through excessive heat treatment or leaching (**Figure 3.2**). There was little difference in the levels of sulforaphane or glucoraphanin in the broccoli steamed for any of the times tested, but steaming for a shorter duration may preserve the volatile sulphur-containing compound, SMCSO.

3.5.2 Broccoli leachate characterisation

The concentrated broccoli leachates were analysed for glucosinolate content, and they were found to contain glucoraphanin, glucoiberin, and 4-MIND glucosinolate (**Figure 3.3**). These glucosinolates were detected in all the broccoli leachates produced, but were present at varying levels. This was likely due to the broccoli being grown in different locations, with different soils and weather conditions, as well as seasonal variations. The main focus of the experiments will be on the metabolic fate of glucoraphanin, therefore the BL media will be standardised for glucoraphanin content.

^{1}H NMR spectroscopy of the broccoli leachates indicated the presence of SMCSO (**Figure 3.4**). Broccoli is known to also contain sulphur in the form of methionine, cysteine, and glutathione [5], however these compounds were not detected using ^{1}H NMR spectroscopy. This may have been due to the degradation of these compounds during steaming or the broccoli leachate processing steps, which entailed an extended evaporation procedure at 60˚C, at a pressure of 72 mbar. Cooking foods can lead to the release of sulphur-containing volatile compounds, with the breakdown of cysteine, methionine, and derivatives, such as glutathione, considered to be the prime source for simple organic sulphur compounds [344, 345]. In addition, it has been shown that the thermal degradation of glutathione produces several products including pyroglutamate, therefore the breakdown of glutathione may have accounted for a portion of the pyroglutamate detected in the BL media [346]. Sulphate accounts for a large proportion of the sulphur present in broccoli, and the leachate characterisation would have been strengthened through performing sulphate analysis.

The broccoli leachates were found to contain sugars (**Figure 3.5**), and a variety of amino acids (**Figure 3.4** and **Figure 3.6**). In addition, choline, citrate, lactate, formate, fumarate, and trigonelline were also detected (**Figure 3.4** and **Figure 3.6**). The broccoli

leachates contain a large number of compounds that can be used as a nutrient source for human faecal bacteria.

3.5.3 Chemostat nutrient media characterisation

As with the broccoli leachates, CNM contains a variety of amino acids, formate, and lactate (**Figure 3.7** and **Figure 3.8**). In addition, CNM also contains acetate, succinate, betaine, uracil, nicotinate, and nicotinamide (**Figure 3.7** and **Figure 3.8**). Nicotinate and nicotinamide can be used by bacteria as precursors for the production of the co-enzymes NAD^+ and $NADP^+$ [347]. Lipids and steroids were also detected in the CNM (**Figure 3.7**), and are available for hydrolysis or biotransformation by gut bacteria [348].

3.5.4 Characterisation of BL and GL media

The BL and GL media were produced to a volume of 50 ml and analysed for mineral, amino acid, and sugar content. The BL medium was found to be a richer source of multiple minerals, various amino acids, and total sugars, than the GL medium. The most notable difference between the media, detected by mineral analysis, was that there was approximately 3.5-fold greater amounts of total sulphur present in the BL medium (27.48 ± 3.22), compared to that found in the GL medium (7.43 ± 0.67) (**Table 3.1**). The sulphur contained within the GL medium would also be present in the BL medium at approximately the same mass. This is because sulphur would have been present in the CNM, which was used to make both media types. The remaining sulphur (~20.05 mg) would have originated from the broccoli leachate.

The glucosinolates and SMCSO present in the BL medium accounted for 3.97 mg (19.8%) of the total sulphur derived from the broccoli leachate in the BL medium (**Table 3.2**), leaving approximately 16.08 mg unaccounted for. Detailed total sulphur analysis

of the F_1 broccoli hybrids 1199 and 1639 (commercialised as Beneforté®), performed by Traka *et al*, indicated that approximately half of the total sulphur within the broccoli was present as sulphate (29.4%), methionine (10.6%), cysteine (8.7%), and glutathione (1.8%) [5]. This accounted for 50.5% of the total sulphur within the broccoli, with the remaining sulphur incorporated into SMCSO (13.6%) and total glucosinolates (28.9%), and 7.2% unaccounted for. If the total sulphur proportions of sulphate, methionine, cysteine, glutathione, and the missing sulphur present in the broccoli tested were similar in the BL medium, it would leave 4.51 mg unaccounted for.

It is a limitation of the broccoli leachate characterisation process that sulphate was not quantified, as this could have been performed using validated anion-exchange chromatography techniques [349]. The total sulphur analysis performed by Traka *et al* was accomplished through the analysis of fresh broccoli florets, whereas the broccoli leachate was generated using steamed broccoli florets. It has been reported that mild heating can lead to the formation of sulphur-containing volatiles, which may be formed from methionine, cysteine, and glutathione [350, 351]. These compounds are responsible for the odours associated with cooked broccoli, and SMCSO is particularly prone to thermal degradation [151]. Therefore, part of the missing total sulphur in the BL media may be attributed to the loss of sulphur-containing volatiles generated during the heating processes necessary to produce the broccoli leachate. The effects of processing the broccoli leachate could have been investigated. Analysis of samples before and after steaming the broccoli and the evaporation technique, may have allowed a greater understanding of the fate of the sulphur-containing compounds.

^{1}H NMR spectroscopy indicated that the GL medium contained a wide range of amino acids, though at lower concentrations than detected in the BL medium (**Table 3.3**). The BL medium contained notably higher concentrations of glutamine, glutamate, and

pyroglutamate, compared to the GL medium. As well as being used by bacteria in protein synthesis, glutamine acts as a nitrogen donor in various biosynthetic reactions, and has a role as a signalling molecule in the regulation of bacterial nitrogen metabolism [352].

Glutamate is also involved in a wide range of bacterial metabolic processes, and plays an important role in bacterial stress responses [353, 354]. Pyroglutamate can be formed from glutamine or glutamate, or hydrolysed to produce glutamate, and is also an intermediate metabolite in glutathione degradation [355, 356]. It is possible that the processing steps, necessary to generate the broccoli leachates, promoted glutathione degradation, as indicated by the relatively high concentrations of pyroglutamate and glutamate within the BL medium. Proline and serine were present in the BL medium, but absent from the GL medium, indicating that these amino acids originated from the broccoli leachate. Proline is derived from glutamate, whilst serine is a precursor in the synthesis of multiple amino acids by bacteria, and can be degraded to form pyruvate, an important molecule in bacterial metabolic processes [357].

The BL medium was found to contain high concentrations of fructose and glucose, with lesser amounts of sucrose and trehalose (**Table 3.4**). In comparison, the GL medium contained the glucose that was added to the CNM to generate the GL medium, and a similar concentration of trehalose to that observed in the BL medium. Much of the trehalose detected in both media would have likely originated from the yeast extract, which was used to make CNM. The high levels of sugars, amino acids and minerals, such as sulphur and potassium, found in the BL medium, makes this medium a rich source of nutrients and utilisable metabolites for human faecal bacteria.

3.6 Conclusions

Beneforté® broccoli was heat-treated and further processed to generate broccoli leachates for use in culturing human faecal bacteria. The broccoli leachates were analysed and found to contain three glucosinolates, a range of amino acids, sugars, and other compounds that could be utilised by bacteria. The broccoli leachates were used to supplement the basal bacterial medium, CNM. Therefore, CNM was also analysed and was found to contain multiple amino acids, lipids, steroids, and other compounds, including the metabolically important nicotinamide and nicotinate. Broccoli leachates were added to CNM to produce a BL medium standardised to contain 30 µmoles of glucoraphanin. A GL medium was produced by supplementing CNM with 30 µmoles of glucose. Both of these media were then characterised for mineral, amino acid, and sugar content. In comparison to the GL medium, the BL medium was found to be a particularly rich source of total sulphur, glutamine, glutamate, pyroglutamate, and sugars. The next chapter will detail the use of the BL medium to investigate the metabolic fate of glucosinolates, when cultured with human faecal microbiotas in a static batch fermentation model.

CHAPTER FOUR

4 Dietary broccoli and the gut microbiota: Investigations into the metabolic fate of glucosinolates

4.0 Summary

The majority of glucosinolates from cooked Brassica *vegetables reach the colon intact, where they can be metabolised by the gut microbiota. It has previously been shown that some bacteria are able to directly hydrolyse glucosinolates, whilst other bacteria convert glucosinolates to the reduced analogue via a bacterial-mediated reduction reaction of the sulphoxide group at the terminal R group. This chapter investigates the metabolic fate of glucosinolates in a broccoli leachate when cultured with human faecal microbiotas. The main bacterial activity observed was the reduction of glucoraphanin and glucoiberin, to glucoerucin and glucoiberverin, respectively. Although LC-MS/MS analysis does not indicate a notable degree of glucosinolate hydrolysis, a portion of the glucosinolates are unaccounted for and may have been metabolised to unknown products.*

4.1 Introduction

Chapter 3 described the production and characterisation of a broccoli leachate-containing (BL) media. This chapter describes the metabolic fate of glucosinolates contained within this media, when cultured with human faecal microbiotas.

Links between the diet, gut microbiota, and health-promoting effects have led to an increased interest in identifying functional foods that promote the growth of beneficial bacteria. As discussed in chapter 3, cooking can inactivate the plant myrosinase found in *Brassica* vegetables. Therefore, we rely entirely on the myrosinase-like activity of the gut microbiota to hydrolyse glucosinolates, forming the bioactive breakdown products. Investigations into the microbial metabolism of glucosinolates have included the use of *in vitro* models (isolated bacterial strains or bacterial communities) [90, 123, 125, 358],

rodent models [98, 135, 338], and human intervention studies [84, 134, 142]. Research has shown that single strains of human gut bacteria from a range of phyla have the ability to hydrolyse glucosinolates [90, 123, 125, 358], that bacterial glucosinolate metabolism occurs *in vivo* with a potential role for enterohepatic recycling [91, 98], and that there are inter-individual differences in glucosinolate metabolism, as evidenced by variation in urinary ITC excretion [111, 124, 359].

Recent studies have determined that human gut bacteria are able to convert glucosinolates via the reduction of a sulphoxide group [45, 142, 144]. This bacterial-mediated reduction reaction has been shown to convert glucoraphanin to glucoerucin, and glucoiberin to glucoiberverin. It has been postulated that the reduction of glucosinolates may be a mechanism employed by some bacteria to overcome steric hindrance, caused by the presence of the sulphoxide groups rendering the β-thioglucosidic bonds inaccessible to bacterial myrosinase-like enzymes [45]. It was shown that *Escherichia coli* VL8 did not exhibit the ability to hydrolyse glucosinolates until after it had converted them to their reduced analogues [45]. This indicates that there may be multiple mechanisms by which the human gut microbiota can hydrolyse glucosinolates.

4.2 Objectives

The hypothesis of the research presented in this chapter is that glucosinolates in a broccoli leachate-containing (BL) media would be metabolised by human faecal bacteria when cultured *in vitro* under anaerobic conditions.

4.3 Materials and methods

The faecal material used in this work originated from five healthy human participants (**Table 4.1**). All participants consented to have their biological samples used for these experiments as part of 'The conversion of encapsulated glucoraphanin, gut microbiota phylogeny and genotype' (ENGAGE) human intervention study (ClinicalTrials.gov: NCT01927666). The study was managed by Dr J. F. Doleman at the Institute of Food Research. A broccoli leachate was generated using commercially sourced Beneforté® broccoli as described in section 2.2, and the basal bacterial medium used is detailed in section 2.3. The human faecal bacteria were cultured in the BL media and GL media, as described in detail in section 2.8.3, and summarised in **Figure 4.1**. Glucosinolate levels within the BL media were measured prior to inoculation, and after each of four consecutive 12 hr incubations with human faecal bacteria. A glucosinolate extraction was performed prior to HPLC analysis as described in sections 2.5.1 and 2.5.2, respectively. Identification of glucosinolate hydrolysis products contained within the BL media was performed using LC-MS/MS (section 2.4).

Table 4.1: Age, gender, and Body Mass Index (BMI) of the faecal donors.

Faecal donors	*Age (years)*	*Gender*	*BMI (kg/m^2)*	*Smoker*
Subject 1	44	Male	24.1	N
Subject 2	24	Female	23.3	N
Subject 3	52	Female	20.9	N
Subject 4	50	Female	38.4	N
Subject 5	61	Female	20.5	N

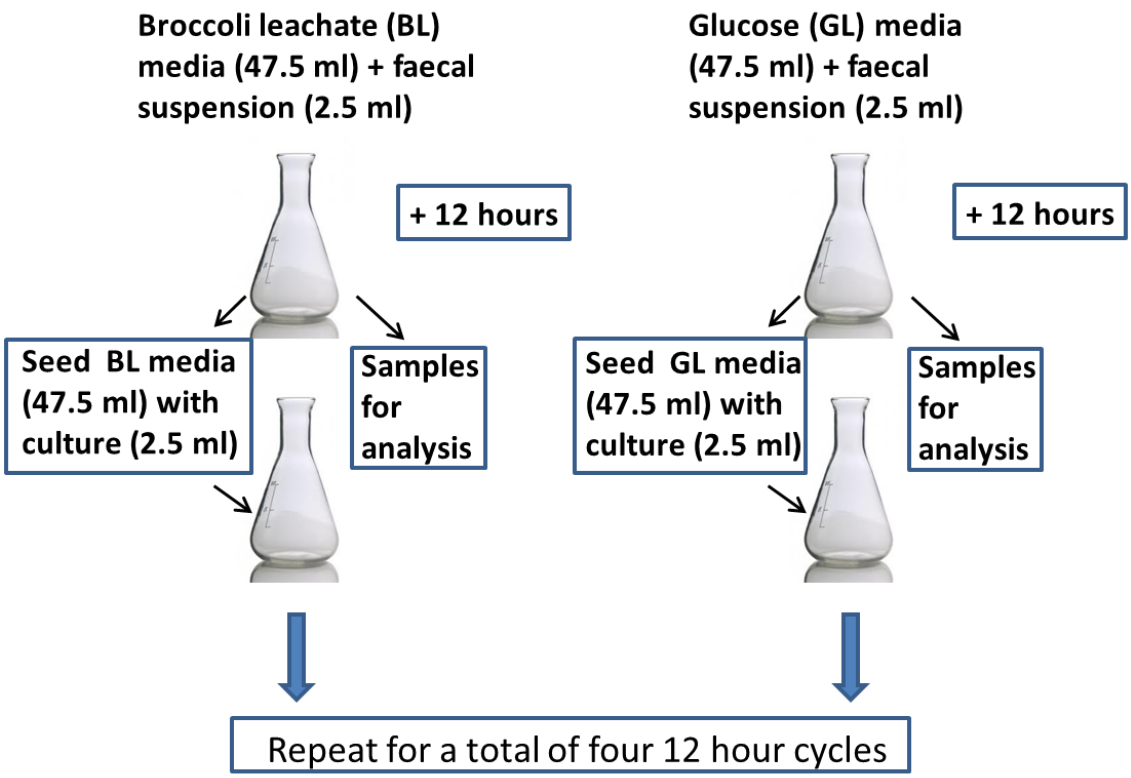


Figure 4.1: Schematic of experimental design. Broccoli leachate (BL) media (47.5 ml) and glucose (GL) media (47.5 ml) were seeded with a faecal suspension (2.5 ml) and cultured for 12 hr at 37˚C, in anaerobic conditions. After a 12 hr incubation period, samples (2 ml) were taken for glucosinolate and isothiocyanate (ITC) analysis, and a 2.5 ml aliquot was used to seed the respective fresh media. This was repeated for four cycles of bacterial enrichment.

4.4 Results

4.4.1 Evaluating glucoraphanin stability in the bacterial medium

Prior to receiving the faecal samples that would be used for the experiments that are the focus of this chapter, small-scale experiments were performed to evaluate the optimal time for each cycle of bacterial incubation, and whether the glucoraphanin would remain stable in the chemostat nutrient media (CNM). Repeated experiments using the BL medium and human faecal samples indicated that glucoraphanin was stable in the media (**Figure 4.2**).

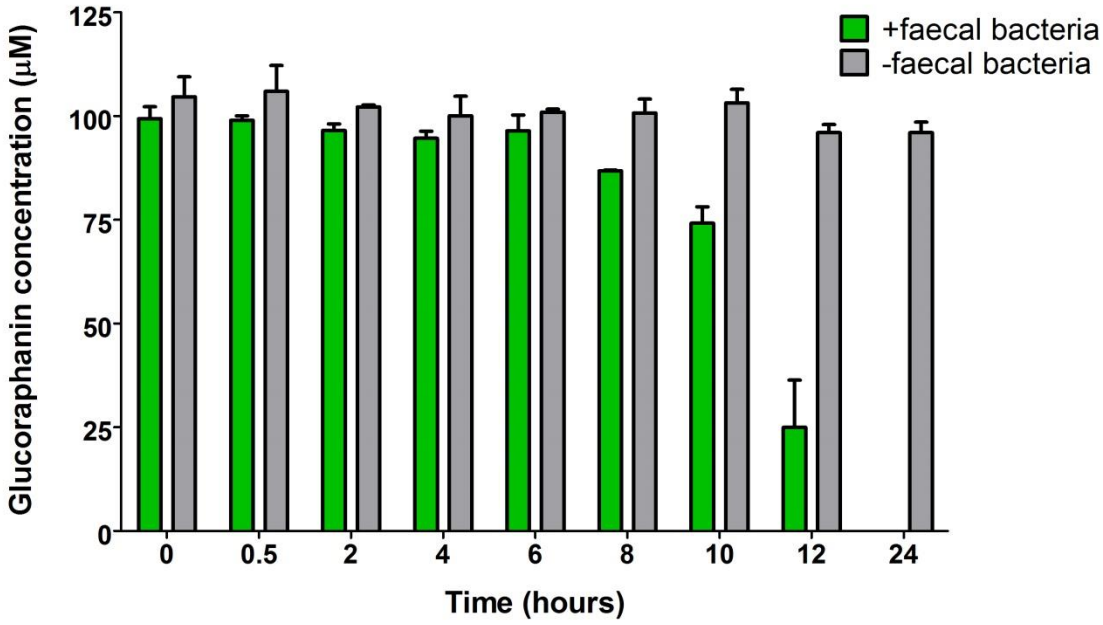


Figure 4.2: Glucoraphanin is stable in an aqueous media over a period of 24 hours. A human faecal microbiota was cultured in a broccoli leachate-containing media for 24 hours at 37˚C, under anaerobic conditions. Glucoraphanin was converted to desulphoglucosinolates prior to HPLC analysis. Data shown = mean ± SD of three technical replicates.

The levels of glucoraphanin in the samples that were inoculated with faecal bacteria started to decrease after 6 hours, and the glucoraphanin was completely depleted by 24 hours. However, the glucoraphanin levels remained constant throughout the entire 24 hour period in the control samples that had 0.22 µm filtered water added in place of the faecal bacteria. It was concluded that 12 hours was an appropriate length of time for each cycle, and that glucoraphanin was stable in the BL media.

4.4.2 Analysis of glucosinolates in human faecal bacteria cultures

Five independent human faecal microbiotas were cultured in a BL medium to assess how the human gut bacteria may contribute to the metabolic fate of glucosinolates. Samples were taken to determine whether these microbial communities were metabolising the glucosinolates derived from the broccoli. HPLC analysis indicated

that, of five independent experiments, four cultured faecal bacterial communities (subjects 1, 3, 4, and 5) exhibited the ability, to varying degrees, to biotransform glucoraphanin, converting it to glucoerucin, the reduced analogue of glucoraphanin. **Figure 4.3** shows the level of glucoraphanin conversion to glucoerucin performed by the faecal microbiota of subject 3.

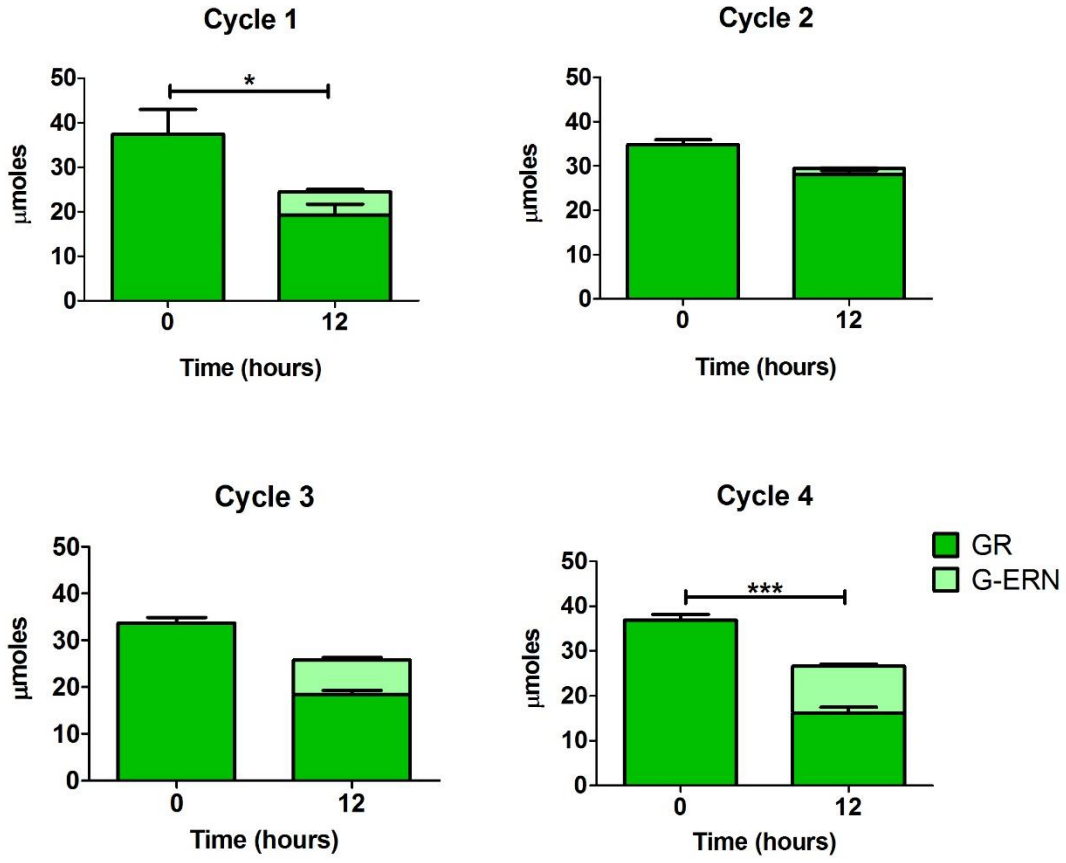


Figure 4.3: Conversion of glucoraphanin to glucoerucin when cultured with human faecal bacteria. Human faecal microbiotas were cultured separately with a broccoli leachate-containing media for four 12 hr cycles at 37˚C, under anaerobic conditions. The figure shows the conversion of glucoraphanin to glucoerucin by the faecal microbiota of subject 3, which is representative of the observations of the microbiotas of subjects 1, 3, 4, and 5. The y-axis refers to the number of µmoles in the total culture volume. Glucosinolates were converted to desulphoglucosinolates prior to HPLC analysis. GR; Glucoraphanin: G-ERN; Glucoerucin. Data shown = mean ± SD of four technical replicates. The data was statistically analysed using paired Student's t-tests (two-tailed). *p<0.05; ***p<0.001 zero hr vs 12 hr.

Although the conversion of glucoraphanin to glucoerucin accounts for a large proportion of the glucoraphanin that disappears during each 12 hour cycle, a small portion of glucoraphanin is still unaccounted for. Of the five faecal microbiotas tested, the cultured microbiota of subject 2 did not convert glucoraphanin to glucoerucin (**Figure 4.4**).

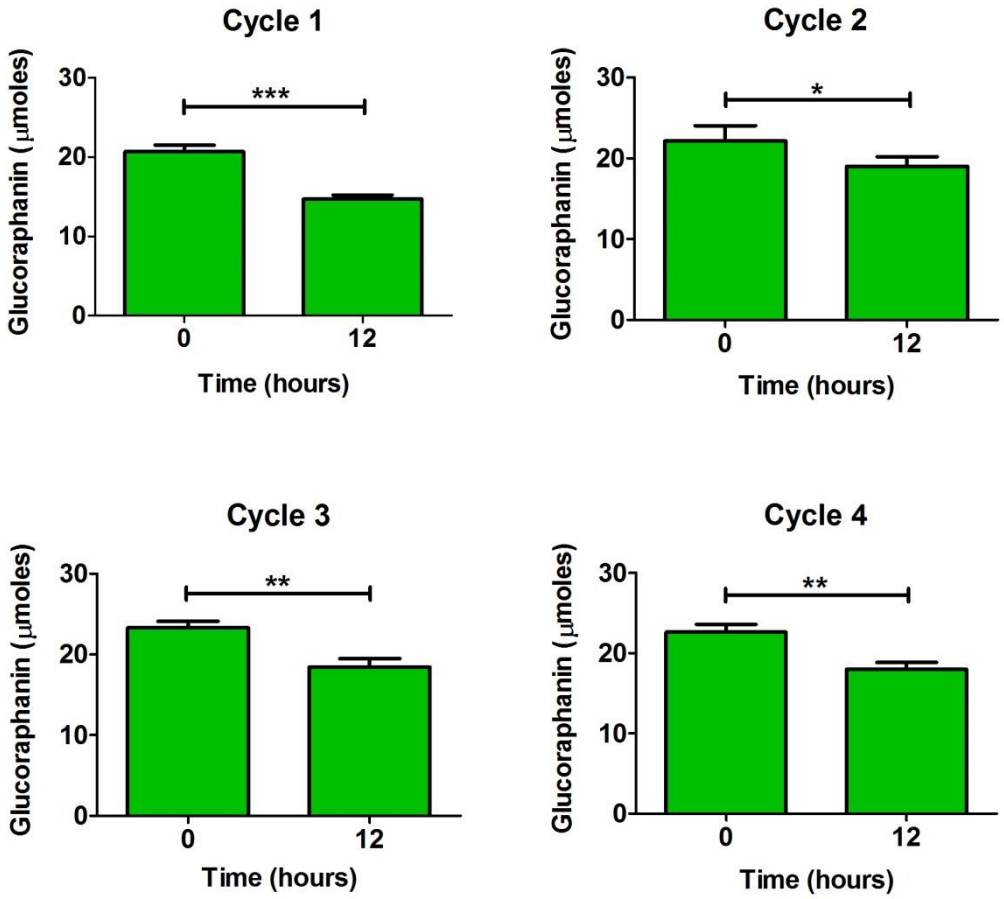


Figure 4.4: Changes in glucoraphanin levels when cultured with human faecal bacteria. Human faecal microbiotas were cultured with a broccoli leachate-containing media for four 12 hr cycles at 37˚C, under anaerobic conditions. The graph details the results obtained from the cultured microbiota of subject 2. The y-axis refers to the number of μmoles in the total culture volume. Glucosinolates were converted to desulphoglucosinolates prior to HPLC analysis. Data shown = mean ± SD of four technical replicates. The data was statistically analysed using paired Student's t-tests (two-tailed). *p<0.05; **p<0.01; ***p<0.001 zero hr vs 12 hr.

The bacterial community of subject 2 does not seem to have the ability to reduce glucoraphanin to glucoerucin, but as with the other four faecal microbiotas tested, a decrease in glucoraphanin levels were observed across each 12 hour cycle. Glucoraphanin was the most prevalent glucosinolate in the culture medium, but the glucosinolates glucoiberin and 4-MIND were also present and available to the cultured faecal bacteria. As previously mentioned in section 3.4.2.1, the BL media was standardised for glucoraphanin in each experiment, but varied in the levels of glucoiberin and 4-MIND. When culturing the five independent faecal microbiotas, the levels of glucoiberin in the starting media (50 ml) ranged from 4.39 µmoles to 19.07 µmoles, but were consistent within each separate experiment. Glucoiberin was converted to the reduced analogue glucoiberverin by three of the faecal microbiotas tested (subjects 1, 3, and 5). **Figure 4.5** illustrates the reduction of glucoiberin that occurred whilst culturing the microbiota of subject 5 in the BL media.

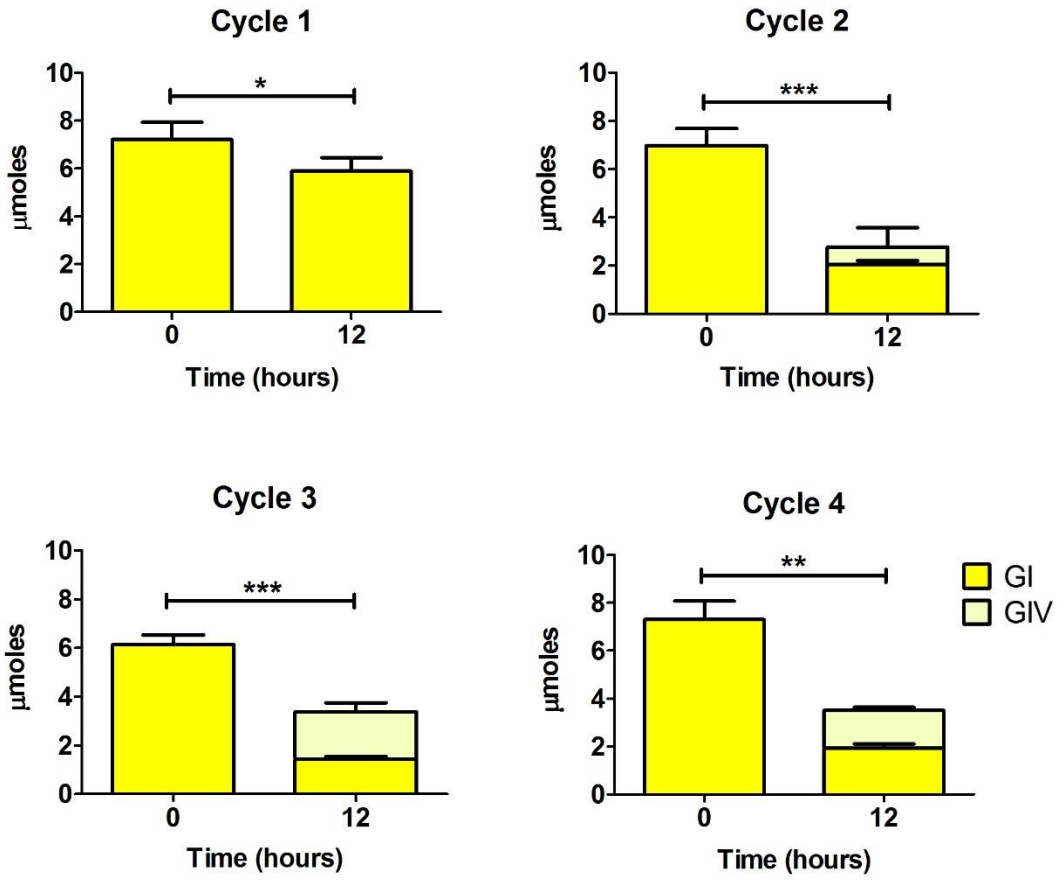


Figure 4.5: Reduction of glucoiberin to glucoiberverin when cultured with human faecal bacteria. Human faecal microbiotas were cultured separately with a broccoli leachate-containing media for four 12 hr cycles at 37˚C, under anaerobic conditions. This figure shows the conversion of glucoiberin to glucoiberverin by the faecal microbiota of subject 5, which is representative of the observations of two other microbiotas tested (subjects 1, 3 and 5). The y-axis refers to the number of µmoles in the total culture volume. Glucosinolates were converted to desulphoglucosinolates prior to HPLC analysis. GI; Glucoiberin: GIV; Glucoiberverin. Data shown = mean ± SD of four technical replicates. The data was statistically analysed using paired Student's t-tests (two-tailed). *p<0.05; **p<0.01; ***p<0.001 zero hr vs 12 hr.

The bacterial-mediated reduction of glucoiberin occurred in the cultured microbiotas of subjects 1, 3, and 5, in which the reduction of glucoraphanin had been previously observed. Of the two remaining cultured faecal microbiotas (subjects 2 and 4) where glucoiberverin was not detected, these microbiotas exhibited the least (subject 4) or no (subject 2) reduction activity on glucoraphanin.

Figure 4.6 displays data indicating a lack of reductase activity on glucoiberin by the faecal microbiota of subject 2, which is representative of the activity observed for the cultured faecal microbiota of subject 4.

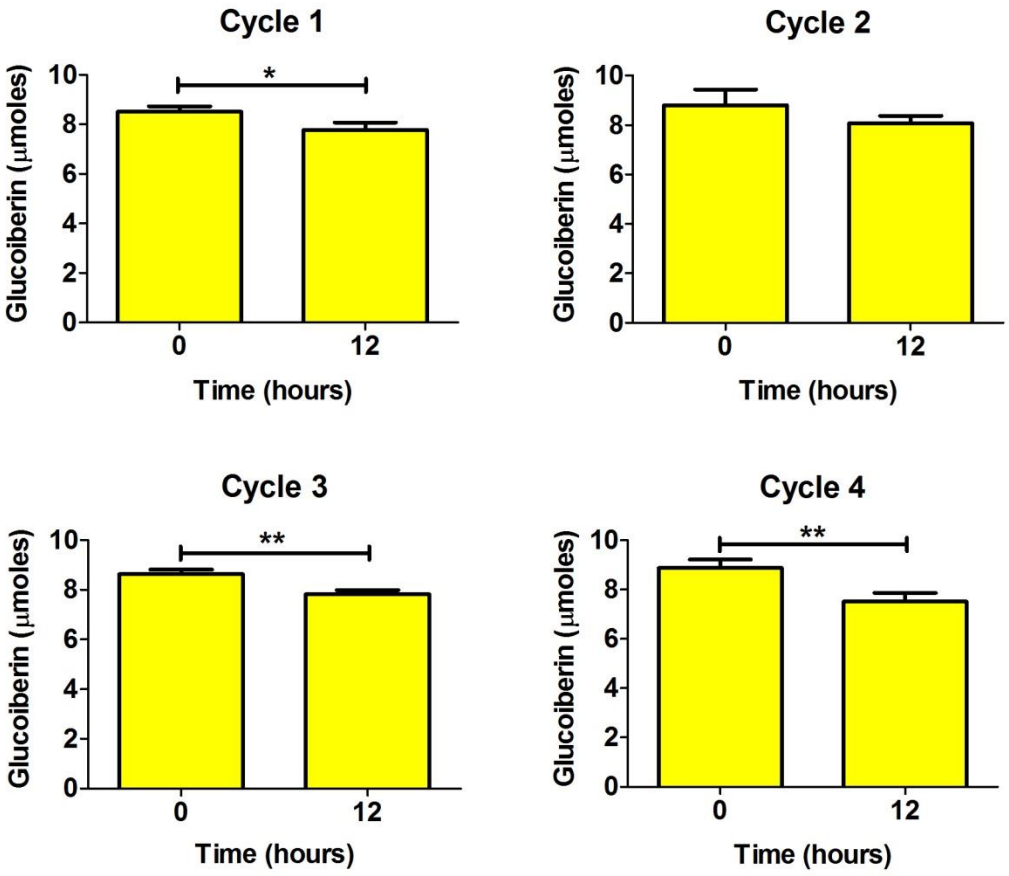


Figure 4.6: Changes in glucoiberin levels when cultured with human faecal bacteria. Human faecal microbiotas were cultured with a broccoli leachate-containing media for four 12 hr cycles at 37˚C, under anaerobic conditions. This figure shows that glucoiberin was not converted to glucoiberverin by the faecal microbiota of subject 2, which is representative of the observations for the microbiotas of subjects 2 and 4. The y-axis refers to the number of µmoles in the total culture volume. Glucosinolates were converted to desulphoglucosinolates prior to HPLC analysis Data shown = mean ± SD of four technical replicates. The data was statistically analysed using paired Student's t-tests (two-tailed). *p<0.05; **p<0.01 zero hr vs 12 hr.

As with the glucoiberin content, the levels of 4-MIND varied (0.48 µmoles to 7.15 µmoles) in the starting media (50 ml), but were stable within each experiment. A statistically significant decrease in the levels of 4-MIND in the BL media was observed

when each of the five microbiotas were cultured, although no reduction occurred as this particular glucosinolate does not have an oxide group available for enzymatic reduction. **Figure 4.7** displays data from the cultured microbiota of subject 5, which is representative of the data for all five cultured microbiotas.

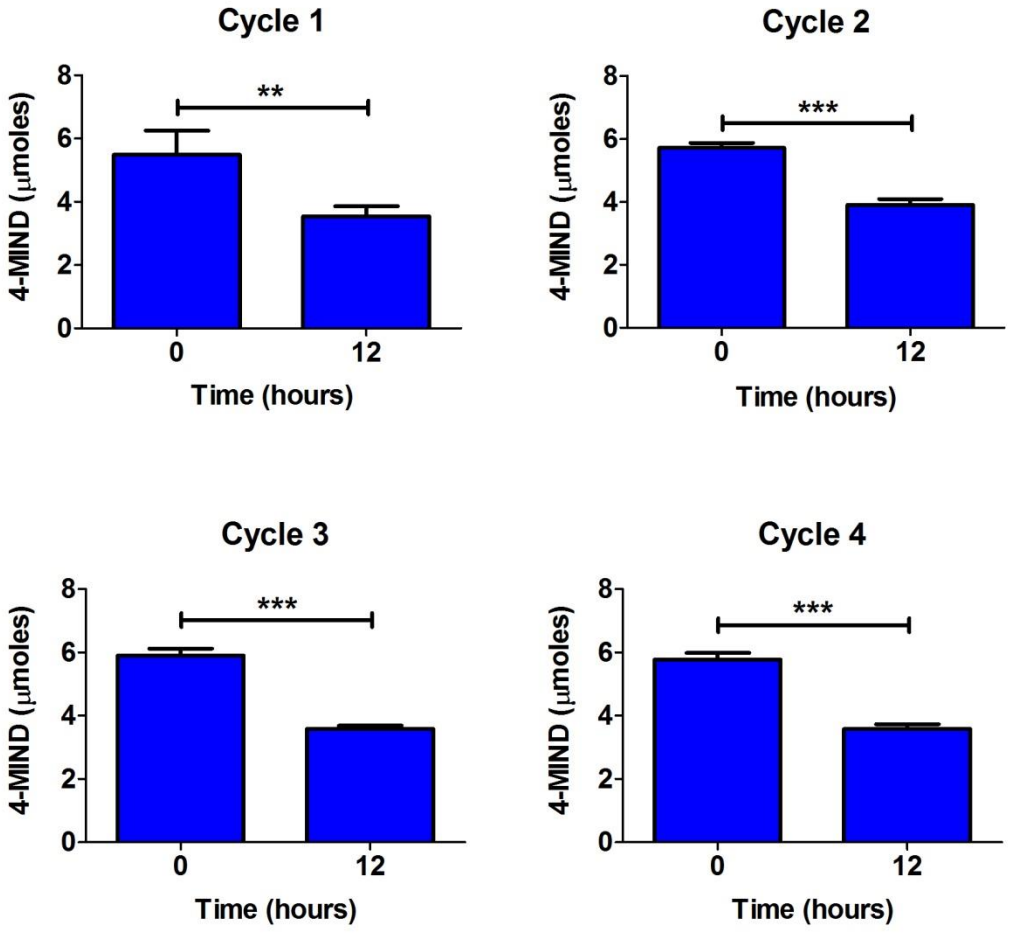


Figure 4.7: Changes in 4-MIND levels when cultured with human faecal bacteria. Human faecal microbiotas were cultured with a broccoli leachate-containing media for four 12 hr cycles at 37˚C, under anaerobic conditions. This figure shows the decrease in levels of 4-MIND when cultured with the faecal microbiota of subject 5, which is representative of the observations for all five cultured microbiotas. The y-axis refers to the number of μmoles in the total culture volume. Glucosinolates were converted to desulphoglucosinolates prior to HPLC analysis. Data shown = mean ± SD of four technical replicates. The data was statistically analysed using paired Student's t-tests (two-tailed). ** p<0.01; *** p<0.001 zero hr vs 12 hr.

Table 4.2 summarises the metabolic fate of glucoraphanin, glucoiberin, and 4-MIND when human faecal microbiotas were cultured in the BL media. The amount of each glucosinolate remaining in the BL media, converted to the reduced analogue, or unaccounted for during each of the four 12 hr cycles was averaged over each experiment and displayed as a percentage of the starting levels of each glucosinolate.

Table 4.2: Metabolic fate of the glucosinolates in the BL media cultured with human faecal microbiotas, averaged across all four 12 hr cycles.

Source of	***Glucoraphanin***			***Glucoiberin***			***4-MIND***		
microbiota	**% remaining**	**% converted**	**% missing**	**% remaining**	**% converted**	**% missing**	**% remaining**	**% converted**	**% missing**
Subject 1	55.1	31.1	13.8	55.4	1.1	43.5	64.8	0	35.2
Subject 2	78.9	0	21.1	89.6	0	10.4	68.4	0	31.6
Subject 3	57.7	15.1	27.2	55.9	8.8	35.3	60.5	0	39.5
Subject 4	81.2	5.1	13.7	84.6	0	15.4	78.7	0	21.3
Subject 5	56.7	20.2	23.1	40.2	15.9	43.9	64.0	0	36.0

4.4.3 Analysis of glucoraphanin hydrolysis products in human faecal bacteria cultures

Samples were taken for LC-MS/MS analysis of glucoraphanin hydrolysis products before the BL media was inoculated with human faecal microbiotas, and following twelve hours of anaerobic growth, for each of four cycles. Although some of the decreases in glucoraphanin levels were due to its conversion to glucoerucin, a portion of glucoraphanin was still unaccounted for within each experiment. LC-MS/MS was used to identify whether the missing glucoraphanin had been hydrolysed to form glucosinolate breakdown products by the bacterial communities. Low levels of the ITC sulforaphane (SF), derived from glucoraphanin, was present in the starting media, and SF levels decreased within each 12 hr cycle, during all five experiments (**Figure 4.8**).

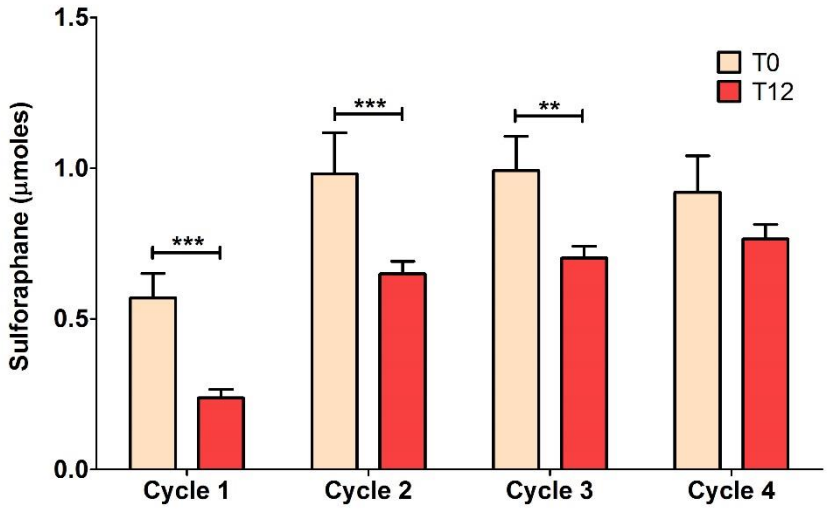


Figure 4.8: Levels of sulforaphane in the BL media when cultured with a human faecal microbiota. Human faecal microbiotas were cultured with a broccoli leachate-containing (BL) media for four 12 hr cycles at 37˚C, under anaerobic conditions. This figure shows the µmoles of sulforaphane in the BL media when cultured with the faecal microbiota of subject 5, which is representative of the observations of all five microbiotas tested. The y-axis refers to the number of µmoles in the total culture volume. Glucoraphanin hydrolysis products were measured using LC-MS/MS. T0; prior to inoculation: T12; 12 hours post-inoculation. Data shown = mean ± SD of four technical replicates. The data were statistically analysed using paired Student's t-tests (two-tailed). ** p<0.01; *** p<0.001 zero hr vs 12 hr.

Glucoraphanin hydrolysis, whether non-enzymatically or due to bacterial myrosinase activity, may have led to the production of conjugated SF products as well as free SF, and these are also identifiable through the use of LC-MS/MS. SF nitrile was found to be the most abundant glucoraphanin hydrolysis product in the starting media (**Figure 4.9**).

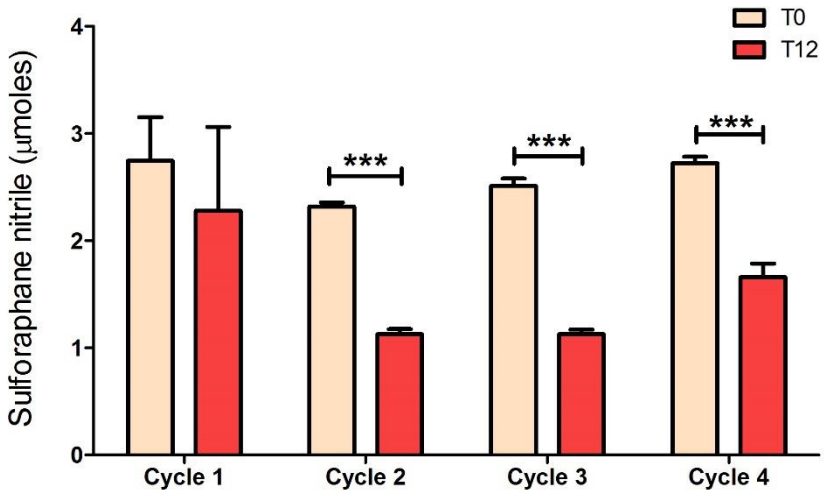


Figure 4.9: Presence of sulforaphane nitrile in the BL media when cultured with a human faecal microbiota. Human faecal microbiotas were cultured with a broccoli leachate-containing (BL) media for four 12 hr cycles at 37˚C, under anaerobic conditions. This figure shows the µmoles of sulforaphane nitrile in the BL media when cultured with the faecal microbiota of subject 5, which is representative of the observations of all five microbiotas tested. The y-axis refers to the number of µmoles in the total culture volume. Glucoraphanin hydrolysis products were measured using LC-MS/MS. T0; prior to inoculation: T12; 12 hours post-inoculation. Data shown = mean ± SD of four technical replicates. The data were statistically analysed using paired Student's t-tests (two-tailed). ****p*<0.001 zero hr vs 12 hr.

As with free SF, SF nitrile was present in the starting media and decreased within each 12 hour cycle. The conjugated ITC products, SF-cysteine, SF-cysteine-glycine, SF-glutathione, SF-*N*-acetylcysteine and erucin-*N*-acetylcysteine were identified at trace levels (**Figure 4.10** and **Figure 4.11**).

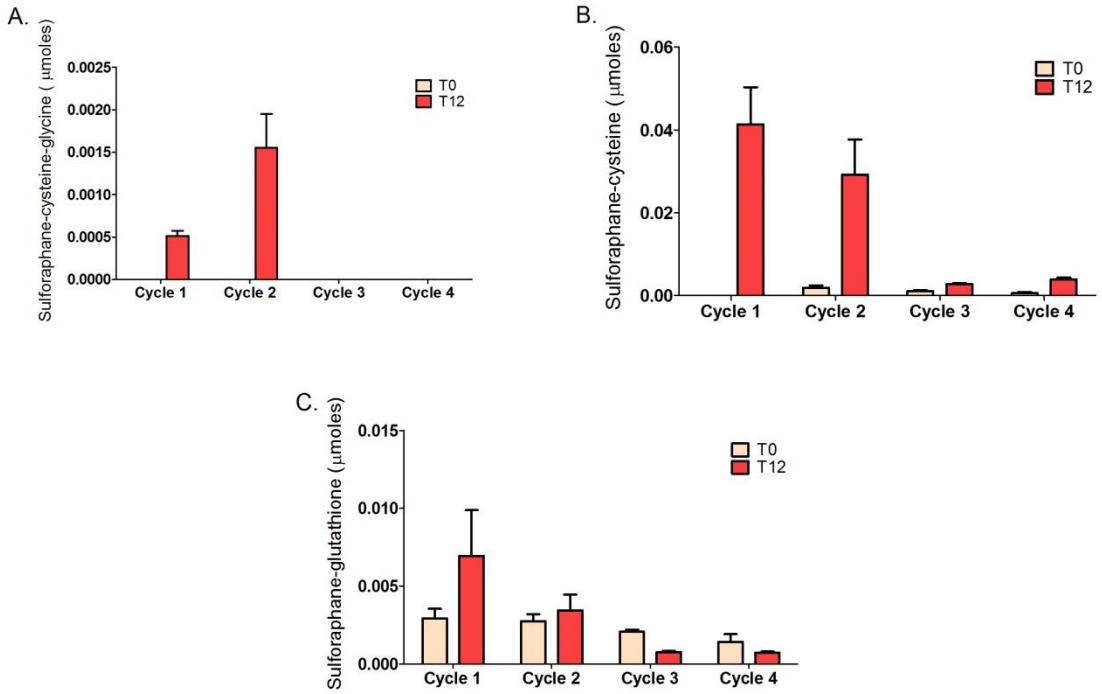


Figure 4.10: Levels of sulforaphane conjugates in the BL media when cultured with a human faecal microbiota. Human faecal microbiotas were cultured with a broccoli leachate-containing (BL) media for four 12 hr cycles at 37˚C, under anaerobic conditions. This figure shows the presence of sulforaphane conjugates at low levels in the BL media when cultured with the faecal microbiota of subject 5, which is representative of the observations of all five microbiotas tested. The y-axis refers to the number of µmoles in the total culture volume. Glucoraphanin hydrolysis products were measured using LC-MS/MS. A. SF-cysteine-glycine; B. SF-cysteine; C. SF-glutathione. T0; prior to inoculation: T12; 12 hours post-inoculation. Data shown = mean ± SD of four technical replicates. The data were statistically analysed using paired Student's t-tests (two-tailed). ***p<0.001 zero hr vs 12 hr.

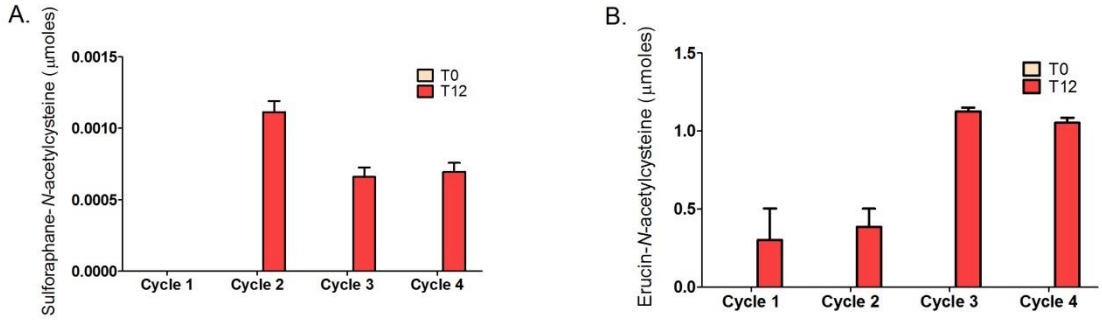


Figure 4.11: Levels of isothiocyanate *N*-acetylcysteine conjugates in the BL media when cultured with a human faecal microbiota. Human faecal microbiotas were cultured with a broccoli leachate-containing (BL) media for four 12 hr cycles at 37˚C, under anaerobic conditions. This figure shows the presence of ITC *N*-acetylcysteine conjugates at low levels in the BL media when cultured with the faecal microbiota of subject 5, which is representative of the observations of all five microbiotas tested. The y-axis refers to the number of µmoles in the total culture volume. Glucosinolate hydrolysis products were measured using LC-MS/MS. A. SF-*N*-acetylcysteine; B. Erucin-*N*-acetylcysteine. T0; prior to inoculation: T12; 12 hours post-inoculation. Data shown = mean ± SD of four technical replicates. The data were statistically analysed using paired Student's t-tests (two-tailed). ****p*<0.001 zero hr vs 12 hr.

Combining the HPLC and LC-MS/MS data of glucoraphanin, glucoerucin, and their related hydrolysis products over each 12 hour culturing period, does not account for the entire amount of glucoraphanin and associated breakdown products present in the starting media. The average percentage of glucoraphanin and its related products unaccounted for by the end of each cycle in each experiment was 26.65% (SD 12.69%). Interestingly, the average percentage unaccounted for in each 12 hour culturing period for the experiments in which glucoraphanin was biotransformed to glucoerucin is 31.06% (SD 10.94%), whilst only 13.39% (SD 7.33%) was unaccounted for in the one experiment where glucoraphanin was not reduced to glucoerucin. Performing a Student's t-test (unpaired, 2-tailed) on the percentage of missing starting material in the cultured microbiota where reductase activity was absent, against those experiments where reductase activity occurred shows that there is a statistically significant difference ($p = 0.01$).

4.5 Discussion

4.5.1 Stability of glucoraphanin in the aqueous medium

The initial objective of this experiment was to establish whether glucoraphanin would remain stable in the BL media under the proposed experimental conditions. The stability of glucoraphanin in CNM was confirmed across a 24 hr period, at 37˚C under anaerobic conditions. The glucoraphanin levels in the BL media remained constant in the absence of faecal bacteria (**Figure 4.2**). The samples cultured with faecal bacteria exhibited a decrease in glucoraphanin after 6 hours, and the glucoraphanin was fully depleted in the media after 24 hours. These data indicated that a period of 12 hours would be suitable to investigate the metabolic fate of glucosinolates when cultured with human gut bacteria, and that any change to the levels of glucoraphanin could be attributed to bacterial metabolic activity.

4.5.2 Reduction of glucosinolates

When human faecal microbiotas were cultured in a BL media, decreases in the levels of glucoraphanin were recorded for each experiment. A concomitant appearance of glucoerucin was observed in four of the five experiments (**Figure 4.3**). Glucoraphanin can be converted to glucoerucin via the reduction of the sulphoxide present on the *R* group of glucoraphanin (**Figure 4.12**). As it had already been shown that glucoraphanin was stable under these experimental conditions, it can be inferred that the reduction of glucoraphanin was bacterial-mediated. The reduction of glucosinolates by a human faecal microbiota [142], and bacteria isolated from human faecal material [45] has previously been reported.

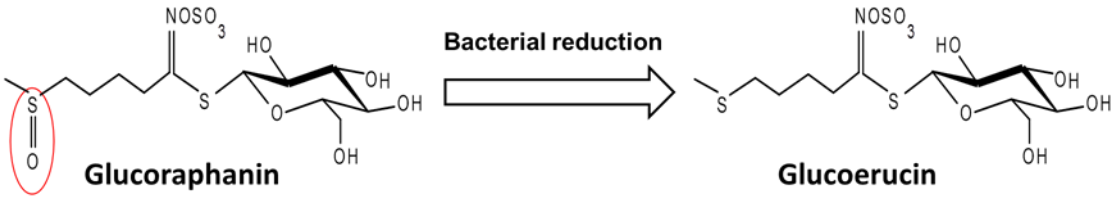


Figure 4.12: Schematic indicating the bacterial reduction of glucoraphanin. The sulphoxide (circled) on the *R* group of glucoraphanin can be reduced by human intestinal bacteria to produce the reduced analogue, glucoerucin.

The BL media contained lesser amounts of glucoiberin and 4-MIND, compared to glucoraphanin levels. Glucoiberin was also observed to be converted to glucoiberverin via the reduction of the sulphoxide group (**Figure 4.5**). The reduction of both glucoraphanin and glucoiberin occurred in experiments using the faecal microbiotas of subjects 1, 3, and 5, suggesting that they were modified by the same bacterial reaction, as reported in other studies [45, 144]. Furthermore, this implies that the cultured microbiota in which the reduction reaction was not observed for either glucosinolate, may have lacked the bacteria able to perform a reduction reaction. The amount of 4-MIND decreased by an average of 32.7% during 12 hr incubation cycles using the five faecal microbiotas (**Figure 4.7**). This could not be due to a reduction reaction as this glucosinolate lacks an oxide group, but a portion of 4-MIND may have been metabolised by bacteria contained within the faecal microbiota to unidentified metabolites.

4.5.3 Glucoraphanin hydrolysis products

Low levels of glucoraphanin hydrolysis products were present in the starting media, which may have been the result of non-enzymatic glucosinolate hydrolysis [46, 360], the action of the broccoli myrosinase enzyme prior to steaming, or because the myrosinase enzyme was not completely abolished during the preparation of the broccoli leachate. The amount of sulforaphane (**Figure 4.8**) and sulforaphane nitrile (**Figure 4.9**) in the BL media decreased within each 12 hour culturing period. The loss of these compounds may have been due to the inherent instability of ITCs in an aqueous medium [125], or due to bacterial metabolism. Furthermore, some of the glucoraphanin and glucoiberin were unaccounted for during the experimental periods, and it is unknown whether these were hydrolysed by the cultured microbiotas, and subsequently metabolised further to unidentified products. The hydrolysis of glucoerucin, or the reduction of sulforaphane or sulforaphane nitrile, can produce erucin or erucin nitrile. These are volatile compounds, which may accumulate in the headspace of the sample vessel. Additional information regarding the missing glucoraphanin may have been gained through the analysis of samples for volatile ITC products. GC-MS can analyse the gaseous space to identify volatile and semi-volatile compounds using thermal desorption. Therefore, the conclusions derived from this experimental work could have been strengthened through the use of GC-MS to determine whether erucin and erucin nitrile were present in the cultured BL media.

4.6 Conclusions

Using a static batch fermentation model, differential metabolism of glucosinolates by independent human faecal microbiotas was observed. It was determined that the sulphoxide group, which forms part of the structure of both glucoraphanin and glucoiberin, could be reduced by bacterial communities originating from the human gut to form glucoerucin and glucoiberverin, respectively. However, this bacterial reductase activity was not observed in one of the five microbiotas tested. This may indicate that bacteria that possessed the ability to reduce glucosinolates were not present in that sample. The total amount of the three glucosinolates present in the BL media (glucoraphanin, glucoiberin, and 4-MIND) could not be fully accounted for at the end of each of the experiments, indicating the possible formation of unidentified metabolites. To complement these results, the next chapter will detail how the community composition of the faecal microbiotas used in these experiments was modified by growth in the BL media.

CHAPTER FIVE

5 Dietary broccoli and the gut microbiota: Bacterial community modulation through repeated exposure to a broccoli leachate

5.0 Summary

The diet has modulatory effects on the composition of the human gut microbiota. Bacterial fermentation of dietary carbohydrates can lead to the production of metabolic end-products, such as short-chain fatty acids, which are beneficial to human health. These acidic compounds can alter the pH of the colonic microenvironment, giving acid-tolerant bacteria a competitive advantage. This chapter investigates how microbial growth in a medium supplemented with a broccoli leachate, may modulate the composition of human faecal microbiotas, compared to a medium containing glucose. Bacterial communities cultured in a broccoli leachate-containing (BL) media were enriched for lactic acid bacteria (LAB), particularly Lactobacillus*, compared to communities cultured in a glucose (GL) media. LAB enrichment was associated with a decrease in pH, which would likely have given this group of bacteria a competitive advantage.*

5.1 Introduction

The previous chapter focused on how human faecal microbiotas influenced the metabolic fate of glucosinolates, when cultured in a BL media. This chapter details how the community compositions of the faecal microbiotas were modified through a repeated exposure to either a BL media or a GL media.

The structure of the human gut microbiota is influenced by multiple factors, such as the birthing method [187], age [230, 361], host genotype [362-364], antibiotic treatment [365-367], environmental factors [368], and diet [238, 254, 259, 369, 370]. As such, a large variation in the gut microbiota composition has been observed between individuals [180, 202]. The enterotype hypothesis proposes that the human gut

microbiota can be stratified into three distinct clusters according to the relative abundance of *Bacteroides*, *Prevotella*, or *Ruminococcus* [226]. It has been suggested that the enterotype clustering may be primarily driven by the ratio of *Prevotella* to *Bacteroides* [239, 371]. Although the analysis of several datasets have indicated the presence of these enterotypes [181, 202], other studies were unable to confirm these bacterial clusters [368, 372].

The diet is considered to be one of the most influential chronic factors determining the composition of the human gut microbiota [239, 373, 374]. It has been suggested that an individual's microbial profile is strongly associated with their long-term dietary habits [239]. Studies indicate that a long-term diet rich in fibre correlates with an increased abundance of *Prevotella*, whilst a long-term diet containing high levels of protein and fat is associated with higher levels of *Bacteroides* [238, 239]. Short-term dietary interventions are able to produce rapid changes to the composition of the gut microbiota, but the community reverts to the original composition within days of the intervention ending [239, 254]. This illustrates the elasticity and the range of functional capabilities within the microbiota. As the nutrient profile of the intestinal environment changes, the composition of the bacterial community alters to maximise nutrient utilisation.

In some cases it may be more relevant to group the gut microbiota by metabolic function rather than composition [181, 202, 226]. Lactic acid bacteria (LAB) have limited biosynthetic capabilities, but are efficient metabolisers of carbohydrates [375]. The major fermentation product is lactic acid, which can be further metabolised to produce short-chain fatty acids (SCFAs) [376]. Although SCFAs are bacterial waste products, these compounds have been shown to have beneficial properties on human health [377].

The human colon has a pH gradient ranging from mildly acidic within the proximal colon, to a neutral pH at the distal colon [378, 379]. It is understood that the proximal colon is the favoured site of substrate fermentation, and the production of weakly acidic bacterial fermentation products lowers the luminal pH [380]. Many Firmicutes, including LAB, have evolved to be tolerant of the acidic microenvironments that are generated at the sites of substrate fermentation [270, 381, 382]. Acid-tolerance can give these bacteria a competitive advantage over bacteria, such as *Bacteroides* and strains of *Escherichia coli*, whose growth have been shown to be inhibited at a physiologically relevant pH of 5.5 [270].

5.2 Objectives

The hypothesis for the research contained within this chapter, was that the community composition of the human faecal microbiotas would be altered through the bacterial metabolism of compounds contained within a broccoli leachate media.

5.3 Materials and methods

The samples tested in this work originated from the experiment detailed in section 2.8.3, and summarised in **Figure 5.1**. Details of the faecal donors are displayed in **Table 5.1**, and the samples analysed were obtained at the same time-points as the samples discussed in chapter 4. The bacterial DNA was extracted, using a bead-beating technique, following the method in section 2.9.1, with the effectiveness of the extraction technique determined by gel electrophoresis, and the DNA yield and purity examined using a NanoDrop spectrophotometer (section 2.9.1.1). 16S rDNA was

amplified by PCR, and sequenced commercially using a 454 pyrosequencing platform, as detailed in section 2.9.2. Bioinformatic analysis was performed on the sequencing output files using the QIIME 1.9.0 pipeline, with RDP as the reference sequence database (section 2.9.3).

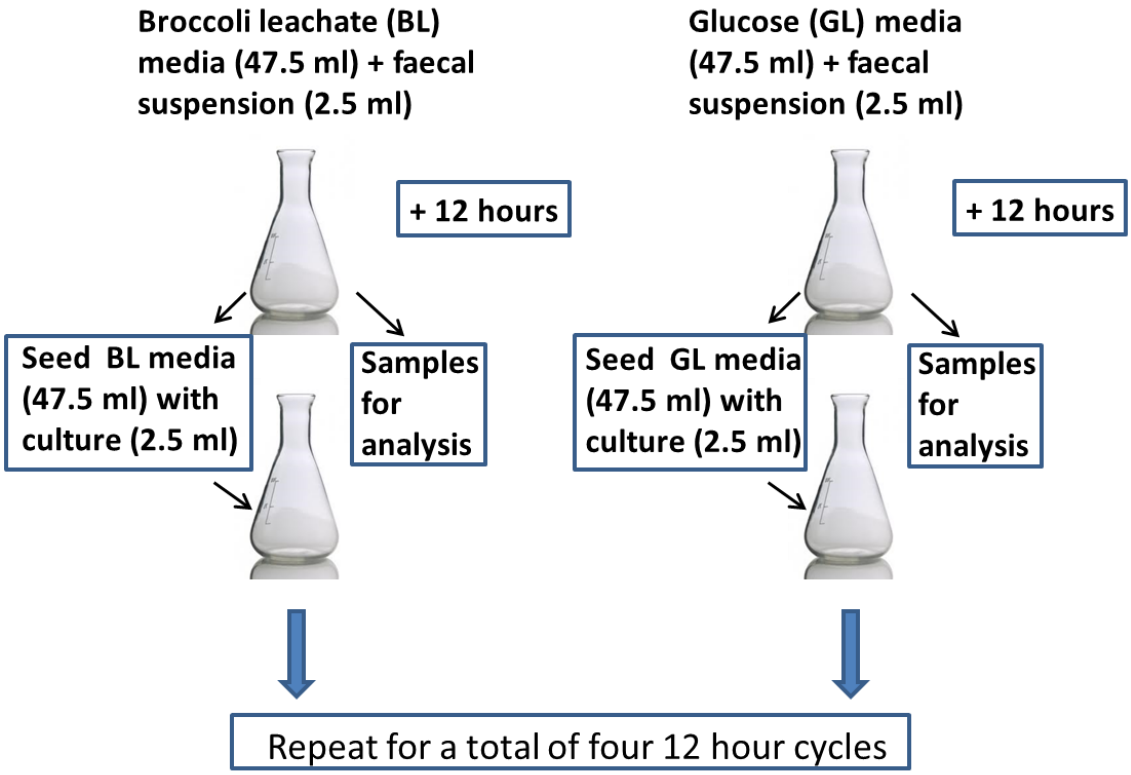


Figure 5.1: Schematic of experimental design. Broccoli leachate (BL) media (47.5 ml) and glucose (GL) media (47.5 ml) were seeded with a faecal suspension (2.5 ml) and cultured for 12 hr at 37˚C, under anaerobic conditions. Samples of the cultured media were taken for sequencing analysis of the variable V4 and V5 regions within the 16S rDNA gene. An aliquot of the cultured media was used to seed the respective fresh media. This was repeated for four cycles of bacterial enrichment.

Table 5.1: Age, gender, Body Mass Index (BMI), and smoking status of the faecal donors.

Faecal donors	*Age (years)*	*Gender*	*BMI (kg/m^2)*	*Smoker*
Subject 1	44	Male	24.1	N
Subject 2	24	Female	23.3	N
Subject 3	52	Female	20.9	N
Subject 4	50	Female	38.4	N
Subject 5	61	Female	20.5	N

5.4 Results

5.4.1 Extracting bacterial DNA

Bacterial DNA was extracted from the samples collected from the batch fermentation model experiments (section 2.8.3) using a FastDNA SPIN Kit for Soil, following a method adapted from Maukonen *et al* (section 2.9.1) [328]. The extraction of bacterial DNA was confirmed using agarose gel electrophoresis (**Figure 5.2**) and a NanoDrop spectrophotometer as described in section 2.9.1.1.

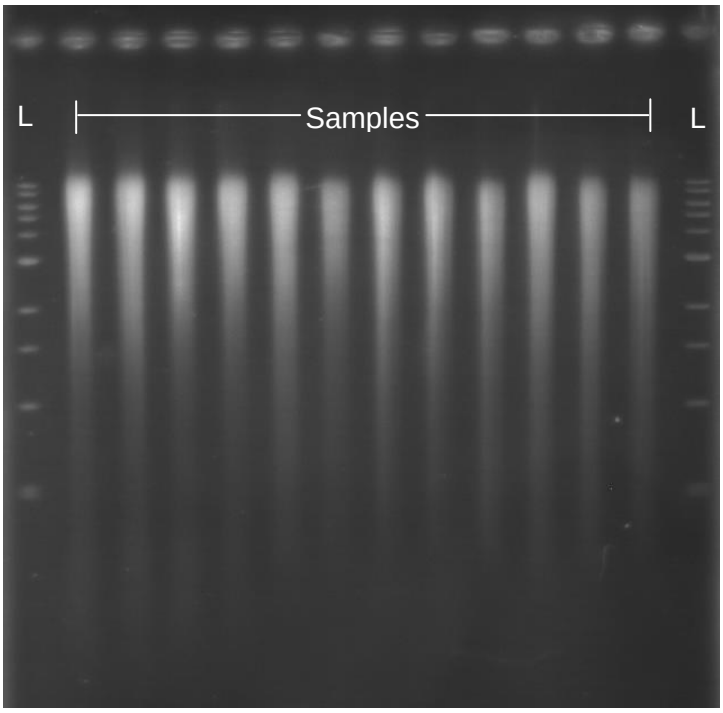


Figure 5.2: Representative image of a 1% agarose gel containing bacterial DNA extracted from cultured faecal microbiotas. Human faecal microbiotas were cultured with a broccoli leachate-containing media and a glucose media for four 12 hr cycles at 37˚C, under anaerobic conditions. Bacterial DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals), run on a 1% agarose gel, and visualised, after ethidium bromide staining, under UV trans-illumination. The image is representative of the visualised agarose gels of all of the bacterial DNA extracted. L = Bioline Hyperladder I (10 Kb).

5.4.2 Phylogenetic analysis of human faecal microbiotas

The bacterial DNA obtained from the cultured faecal microbiotas and the original faecal samples had the V4 and V5 variable regions of the 16S rDNA gene amplified commercially using PCR to generate amplicons. The amplicons were then sequenced commercially using 454 pyrosequencing, and analysed using the QIIME pipeline (section 2.9.2 and 2.9.3). Faecal samples from 5 healthy humans, aged 24 - 61 years with a mean age of 46 years and an average BMI of 25.4 kg/m^2, were collected and sequenced. This produced 453,810 high-quality reads, with an average of 5,403 ± 3,019 reads per subject, which clustered into 10,503 operational taxonomic units (OTUs) at 97% identity.

5.4.2.1 Microbiota composition

The relative abundance of bacterial taxa within each sample is represented as a proportion of each taxonomic unit within the microbiota. Differences in the relative abundance of reads assigned to taxonomic groups within the microbiotas were observed, and are displayed at both the phylum and genus levels (**Figure 5.3** and **Figure 5.4,** respectively).

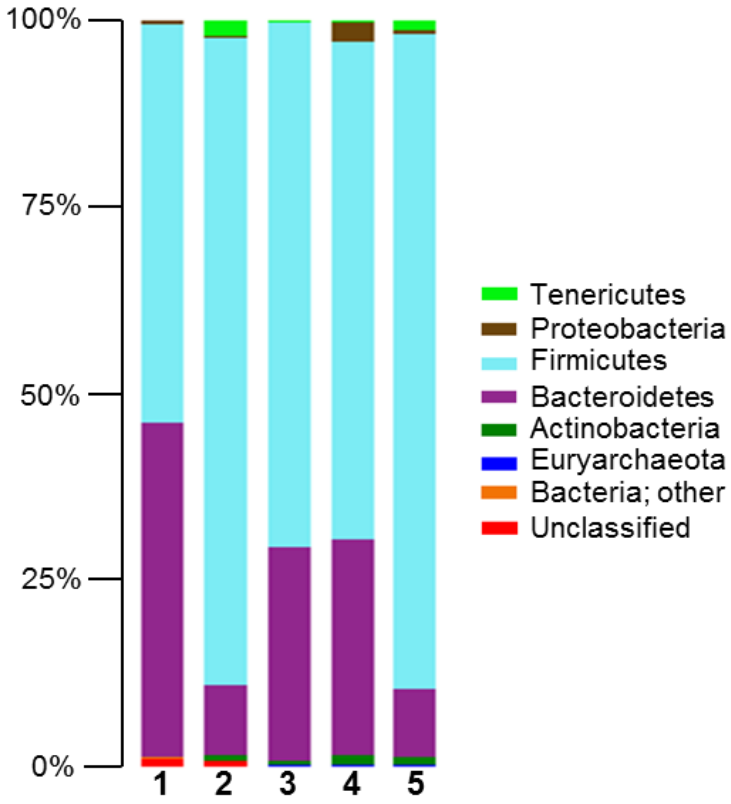


Figure 5.3: Firmicutes are highly represented in the human faecal microbiotas. Bacterial DNA from faecal samples obtained from 5 healthy human subjects (numbered 1 - 5) was extracted, and the V4 and V5 regions of the 16S rDNA gene were sequenced. Bioinformatic analysis was performed using QIIME 1.9.0, and the bar chart displays the proportion of OTUs per faecal microbiota classified at the phylum level.

Across the five microbiotas, the Firmicutes were highly represented at an average proportion of 73% (SD 14.5%) along with Bacteroidetes at 24.1% (SD 15%), together accounting for an average of 97.2% (SD 1.3%) of the bacteria present in the faecal

communities. The standard deviations indicate that although there is variability in the proportions of Firmicutes and Bacteroidetes among the five microbiotas, the proportion of both phyla combined is relatively consistent. Other phyla present are Tenericutes (0.8%; SD 0.9%), Actinobacteria (0.8%; SD 0.4%), Proteobacteria (0.7%; SD 0.9%), and Euryarchaeota (0.04%; SD 0.1%), with 0.4% of phylum OTUs unclassified.

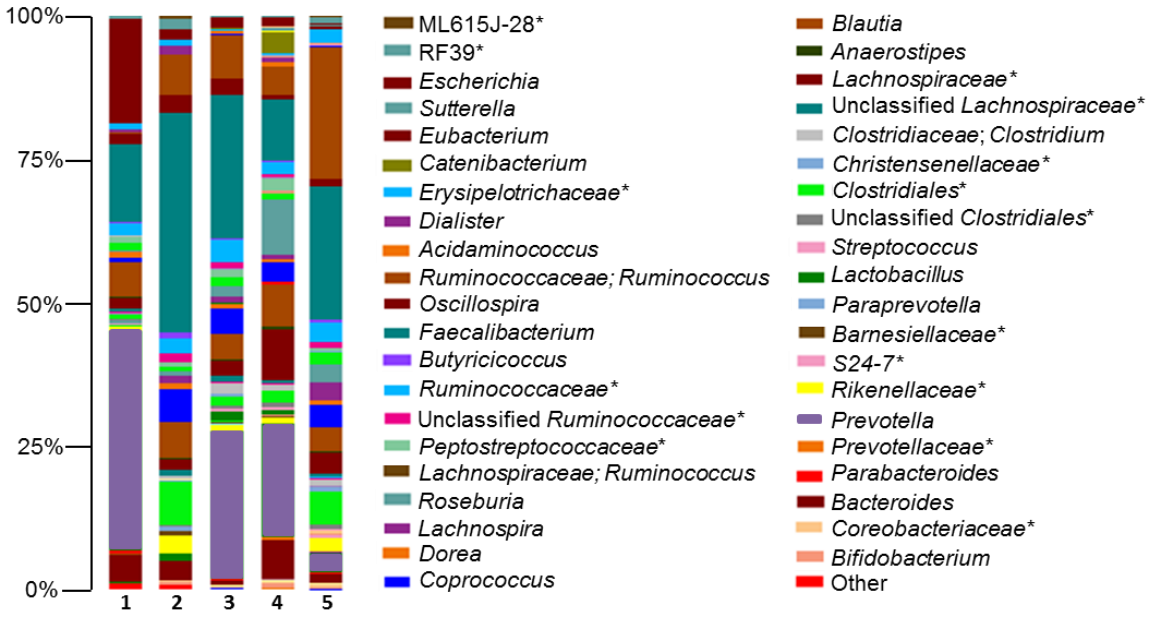


Figure 5.4: The genera *Faecalibacterium* and *Prevotella* are most abundant amongst the human faecal microbiotas. Bacterial DNA from faecal samples obtained from 5 healthy human subjects (numbered 1 - 5) was extracted, and the V4 and V5 regions of the 16S rDNA gene were sequenced. Bioinformatic analysis was performed using QIIME 1.9.0, and the bar chart displays the proportion of OTUs per faecal microbiota. The key reads from top left to bottom right, and consists of genera with a relative abundance ≥0.5% in at least one faecal microbiota. * represents unclassified OTU reported at a higher taxonomic level.

The genera most represented amongst the faecal microbiotas were *Faecalibacterium* (22.2%; SD 9.8%), *Prevotella* (17.7%; SD 13.8%), *Ruminococcus* of the *Ruminococcaceae* family (8.6%; SD 7.6%), *Blautia* (5.7%; SD 1.2%), and *Eubacterium* (4.4%; SD 6.9%). Interestingly, the data indicates that subjects 2 and 5 may share a highly similar microbiota, characterised by a smaller proportion of *Prevotella* (1 - 4%) and increased abundance of *Clostridiales*, compared to subjects 1, 3, and 4. These

inter-individual similarities appear to be present at the phylum level, with reduced levels of Bacteroidetes, and an increased abundance of Firmicutes and Tenericutes in subjects 2 and 5, compared to the other microbiotas sequenced (**Figure 5.3**).

5.4.2.2 Faecal microbiota alpha diversity

Bacterial diversity within the microbiotas (alpha diversity) was measured using four metrics: Observed Species, Phylogenetic Distance (PD), Chao1, and the Shannon Index. The Observed Species metric produces a count of the number of unique OTUs within each sample, whilst PD requires a phylogenetic tree to be generated from the samples, and uses the branch lengths that separate the taxa within a community to estimate diversity [383, 384]. Chao1 estimates species richness within a sample, and the Shannon Index accounts for both species richness and distribution (evenness), via quantification of the entropy associated with the OTU abundances [385]. Alpha rarefaction plots depicting the diversity within each of the faecal microbiotas show that the number of unique OTUs, species richness, and phylogenetic diversity within all faecal samples increase as the number of sequences obtained for each sample increases (**Figure 5.5** and **Figure 5.6**). The faecal microbiota of subject 5 contains the largest number of unique OTUs (609.5 in 3,010 sequences) and highest level of species richness (1,927.4 in 3,010 sequences) (**Figure 5.5 A**). The lowest quantity of unique OTUs (412.4 in 3,010 sequences) and degree of species richness (1,149.9 in 3,010 sequences) was observed in the faecal microbiota of subject 1, with similar numbers obtained from the microbiota of subject 3 (428.1 and 1,312.7 in 3,010 sequences, respectively) (**Figure 5.5**). The faecal microbiotas of subjects 2 and 4 have a comparable number of unique OTUs (526.2 and 492.7 in 3,010 sequences, respectively) and measure of species richness (1,633.3 and 1,513.1 in 3,010 sequences, respectively).

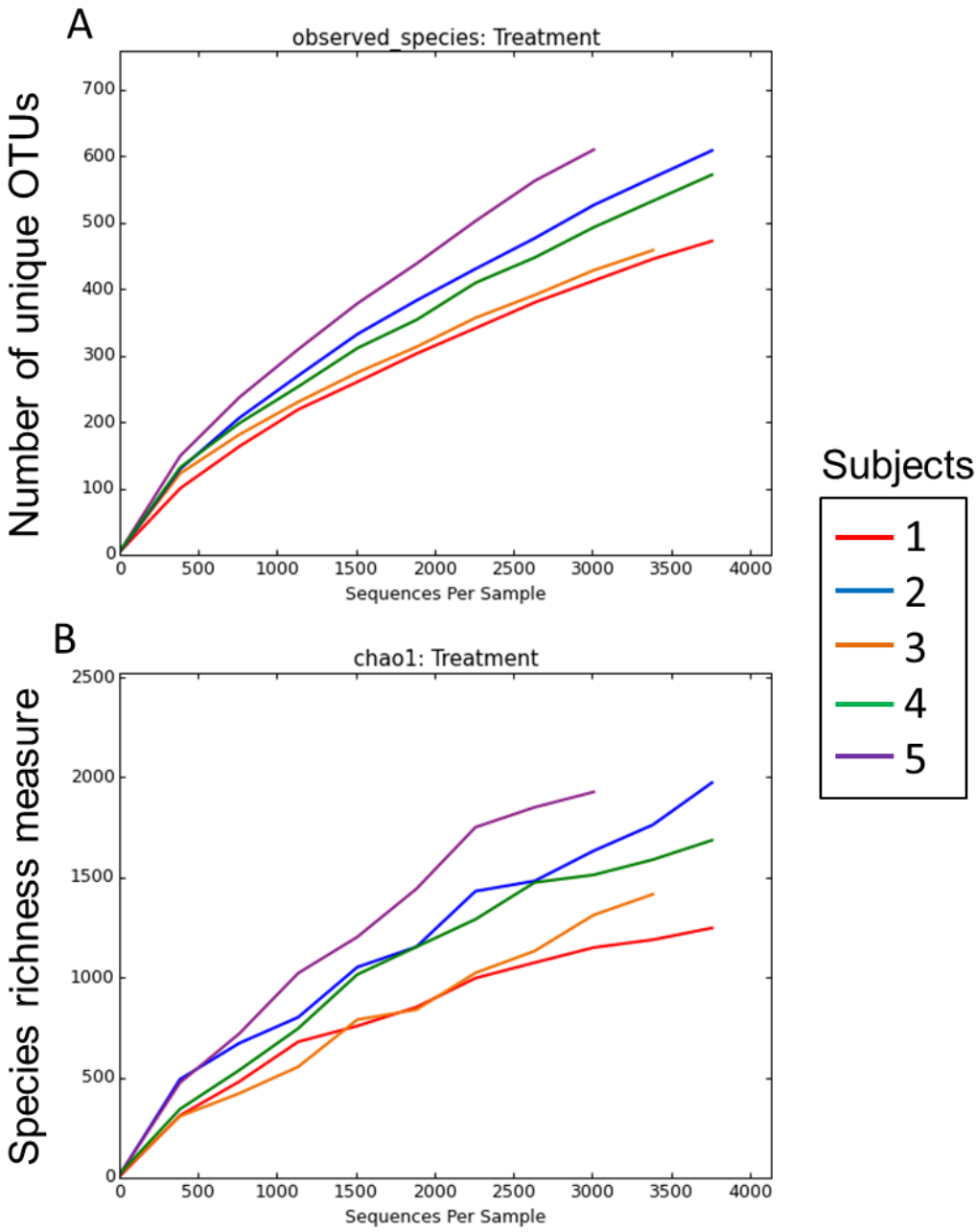


Figure 5.5: Faecal microbiota of subject 5 contains the greatest number of unique OTUs and highest level of species richness. Alpha diversity rarefaction plots of (A) number of unique OTUs, and (B) species richness within the human faecal microbiota samples (subjects 1 - 5). The y-axis is a measure of diversity within each community, whilst the x-axis represents the number of sequences used in the diversity calculation. Rarefaction plots were generated using QIIME 1.9.0.

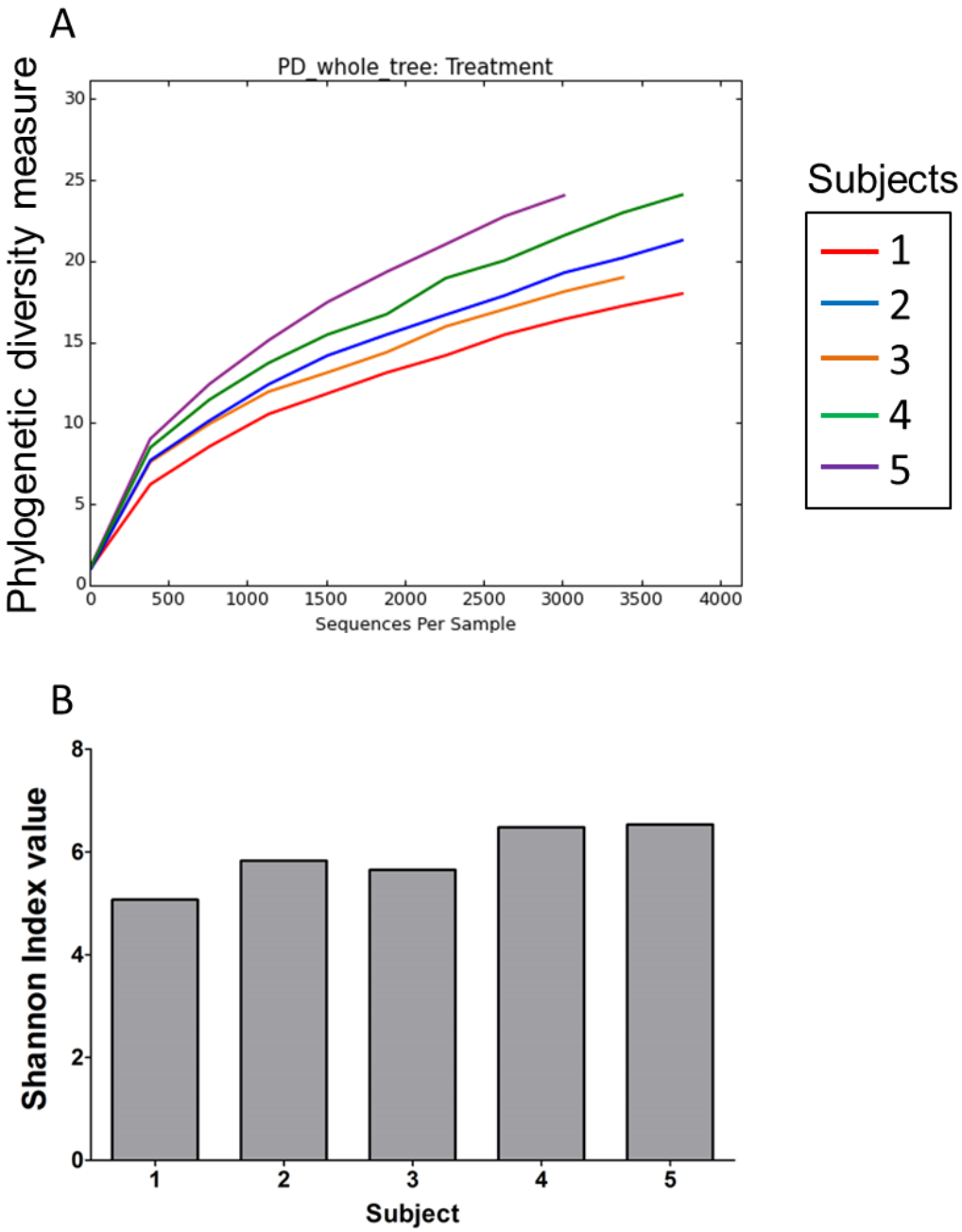


Figure 5.6: Greater phylogenetic diversity within the faecal microbiota of subject 5, and increased species richness and evenness in subjects 4 and 5. Alpha diversity rarefaction measures of (A) phylogenetic diversity and (B) species richness and evenness within the human faecal microbiota samples (subjects 1 - 5). (A) The y-axis is a measure of phylogenetic diversity within each community, whilst the x-axis represents the number of sequences used in the diversity calculation. (B) The y-axis represents the Shannon Index value associated with species richness and evenness at 3,010 samples per sequence. The microbiotas of the five subjects are plotted along the x-axis. The rarefaction plot was generated using QIIME 1.9.0, and the bar graph was produced using GraphPad Prism 5.

The greatest phylogenetic diversity is found in the faecal microbiota of subject 5 (24.0 in 3,010 sequences), with the microbiota of subject 1 exhibiting the least phylogenetic diversity (16.4 in 3,010 sequences) (**Figure 5.6 A**). The results from the analysis performed using the Shannon Index gives a similar score to the faecal microbiotas of subjects 4 and 5 (6.48 and 6.54, respectively), with subject 1 receiving the lowest score (5.07) (**Figure 5.6 B**).

5.4.2.3 Faecal microbiota beta diversity

Bacterial diversity between the microbiotas (beta diversity) was measured using QIIME and the UniFrac metric, with the results displayed as a principal coordinates analysis (PCoA) plot generated using the Emperor tool. The taxa present in each of the samples are mapped onto a phylogenetic tree, and the distance between the samples is calculated using their UniFrac measure to generate a PCoA plot to visualise the dissimilarities between microbiotas. The unweighted UniFrac calculation is based on which taxa are present and shared between microbiotas, and does not take the relative abundance of each of the taxa within the communities into consideration. Calculating the phylogenetic distance between the five faecal microbiotas using the unweighted UniFrac metric indicated that the microbiotas of the five subjects did not share a large range of taxa (**Figure 5.7**).

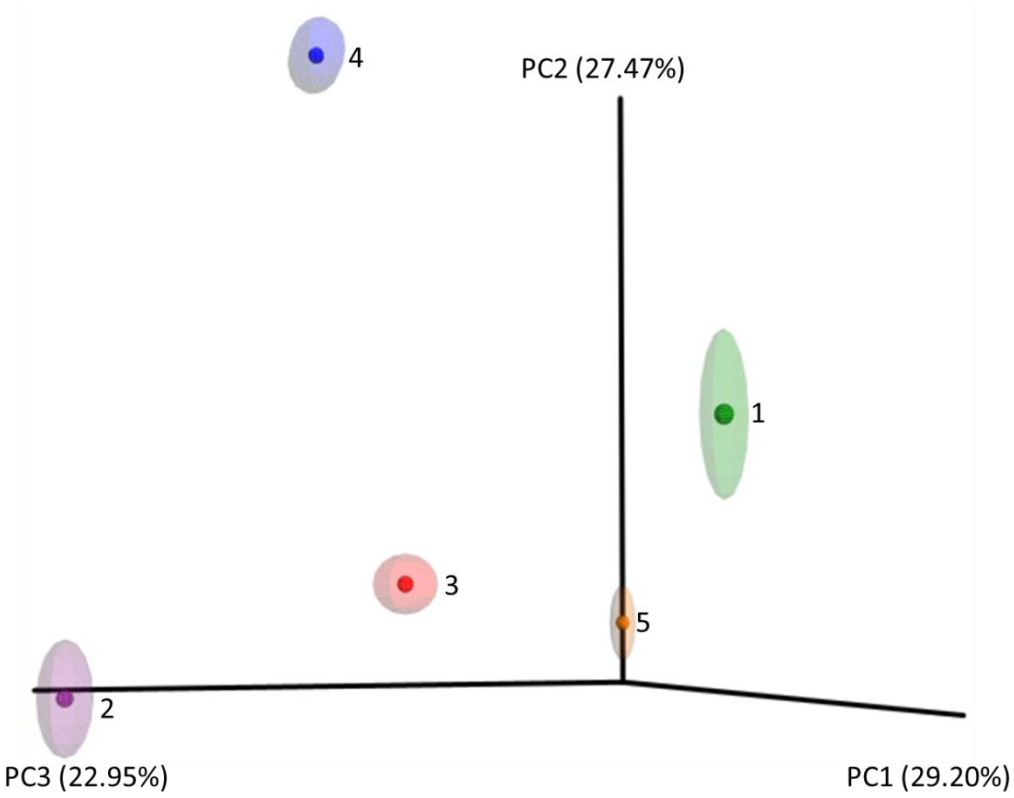


Figure 5.7: Faecal microbiotas were not phylogenetically similar. Unweighted beta diversity analysis of faecal microbiotas (subjects 1 - 5) using the UniFrac metric, visualised as a 3D PCoA plot. Beta diversity analysis performed using QIIME 1.9.0, and plotted using Emperor.

The weighted UniFrac calculation factors in the relative abundance of each of the taxa within the communities, alongside which taxa are shared amongst the samples. Calculating the phylogenetic distance between the five faecal microbiotas using the weighted UniFrac metric suggests that the microbiotas of subjects 2 and 5 are most similar to one another (**Figure 5.8**). The similarity between the microbiotas of subjects 2 and 5 concurs with the observations made based on the bar chart displaying the proportions of genera present in the faecal microbiotas (**Figure 5.4**).

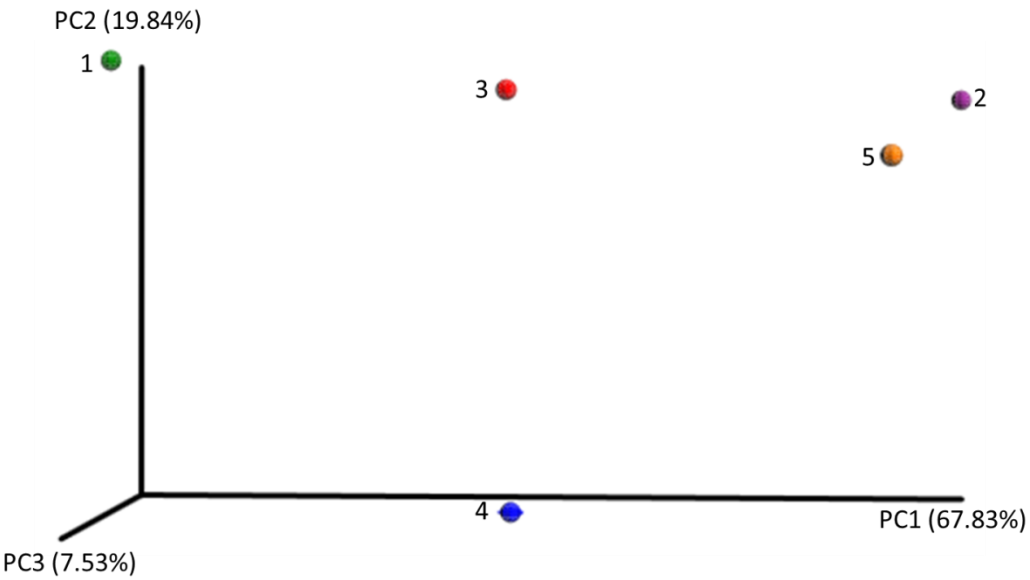


Figure 5.8: Faecal microbiotas of subjects 2 and 5 are phylogenetically similar when relative abundance of taxa is considered. Weighted beta diversity analysis of faecal microbiotas (subjects 1 - 5) using the UniFrac metric, visualised as a 3D PCoA plot. Beta diversity analysis performed using QIIME 1.9.0, and plotted using Emperor.

5.4.3 Phylogenetic analysis after culturing of the microbiotas

The five faecal microbiotas were used in independent experiments to test whether culturing in a broccoli leachate-containing (BL) media would modify the microbiota composition, compared to a glucose (GL) media control (detailed in section 2.8, and summarised in **Figure 5.1**). All experiments were performed with four replicates, under anaerobic conditions at 37˚C. The broccoli leachates were tested for sterility, both before and during each experiment, to confirm that any bacterial growth observed originated from the human faecal suspension. The faecal bacteria were cultured for 12 hours in both media before samples were taken for phylogenetic analysis, and aliquots were used to seed the respective fresh media. This procedure was repeated for four 12 hour cycles. Phylogenetic analysis was performed on samples taken from both cultured media types after the completion of cycle 1 and cycle 4, to identify the effects of repeated exposure to the broccoli leachate on bacterial community composition.

5.4.3.1 Variation amongst biological replicates

Phylogenetic analysis of four biological replicates for both the cultured BL media and the GL media, across all five independent experiments, indicated a high level of similarity in the community composition between the experimentally linked replicates. **Figure 5.9** shows the similarity between replicates for the cultured microbiota of subject 1 at the genus level.

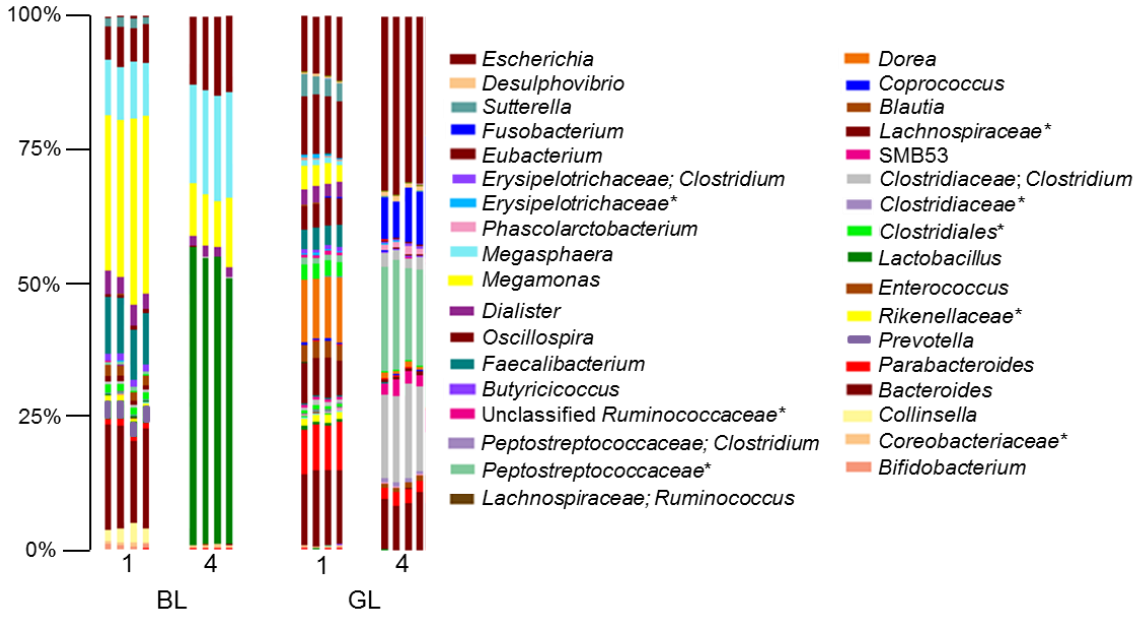


Figure 5.9: Minimal variation between four replicates at the genus level under both conditions at two time-points. Five human faecal samples were cultured independently in a broccoli-leachate-containing (BL) media and a glucose (GL) media. Bacterial DNA was extracted, and the variable V4 and V5 regions within the 16S rDNA gene were sequenced. Bioinformatic analysis was performed using QIIME 1.9.0. The bar chart displays the proportion of OTUs per cultured faecal microbiota of subject 1 after the first cycle (1) and the last cycle (4). These data are representative of the level of similarity observed between the replicates of each of the five cultured microbiotas. The key reads from top left to bottom right, and consists of genera with a relative abundance ≥0.5% in at least one sample. * represents unclassified OTU reported at a higher taxonomic level.

The strong similarity between replicates at the genus level allowed each set to be grouped and averaged to form a single dataset for the subsequent analyses.

5.4.3.2 Modification of microbiotas through culturing in a BL and GL media

The community compositions of the faecal microbiotas were differentially modified when cultured in a BL media and a GL media. **Figure 5.10** shows the changes at the phylum level when the microbiota of subject 1 was cultured in the BL and GL media.

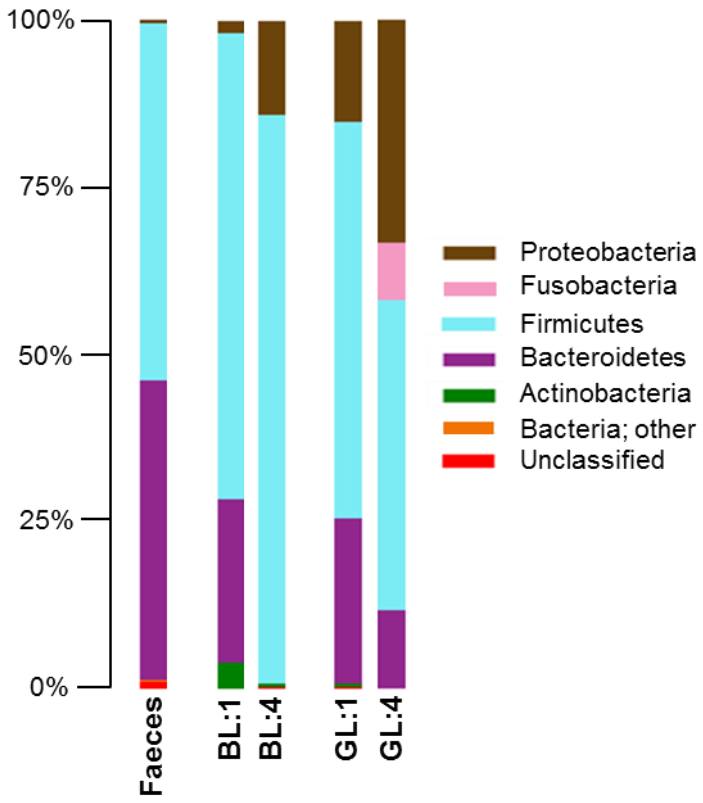


Figure 5.10: The faecal microbiota was differentially modified at the phylum level when cultured in two media types. Five human faecal samples were cultured independently in a broccoli-leachate-containing media (BL) and a glucose media (GL). Bacterial DNA was extracted, and the 16S rDNA gene V4 and V5 regions were sequenced. Bioinformatic analysis was performed using QIIME 1.9.0. The bar chart displays the proportion of OTUs per cultured faecal microbiota of subject 1 after the first cycle (1) and the last cycle (4), and is representative of all five cultured microbiotas. The key consists of phyla with a relative abundance ≥0.5% in at least one sample.

Across the five cultured microbiotas, the average proportion of Bacteroidetes decreased after cycle 1, from 24.1% (SD 15%) to 18.4% (SD 2.9%) in the BL media and 21.8% (SD 4.3%) in the GL media. After cycle 4 Bacteroidetes were present at a proportion of 0.1% (SD 0.04%) in the BL media, and 8.6% (SD 1.2%) in the GL media. Conversely, an increase in the proportion of Proteobacteria, averaging 0.7% in the faecal microbiotas, was observed in the BL media and GL media at cycle 1 (5.6% (SD 3.4%) vs 19.6% (SD 8.8%), respectively) and cycle 4 (26.8% (SD 11.4%) vs 38.2% (SD 2.5%), respectively). The proportion of Firmicutes, which were present at an average of 73% in the faecal microbiotas, were modified differently, and this was dependent on the media. The proportion of Firmicutes remained relatively stable in the BL media (72% (SD 11.4%) after cycle 4), whilst a decrease in the proportion of this phylum was seen when cultured in the GL media (50.4% (SD 1.6%). On average, Actinobacteria proportionally increased from 0.8% (SD 0.4%) in the faecal microbiotas to 12.3% (SD 1.6%) in the BL media after cycle 1, before decreasing to 1% (SD 0.4%) after cycle 4. In the GL media, the average proportion of Fusobacteria increased to 1.8% (SD 0.7%) of the cultured microbiota after cycle 4, from <0.1% in the faecal starting material, and was absent from the cultured BL media. The cultured microbiotas were investigated for modification of the community at the genus level to gain a deeper understanding of the effect of the broccoli leachate (**Figure 5.11**).

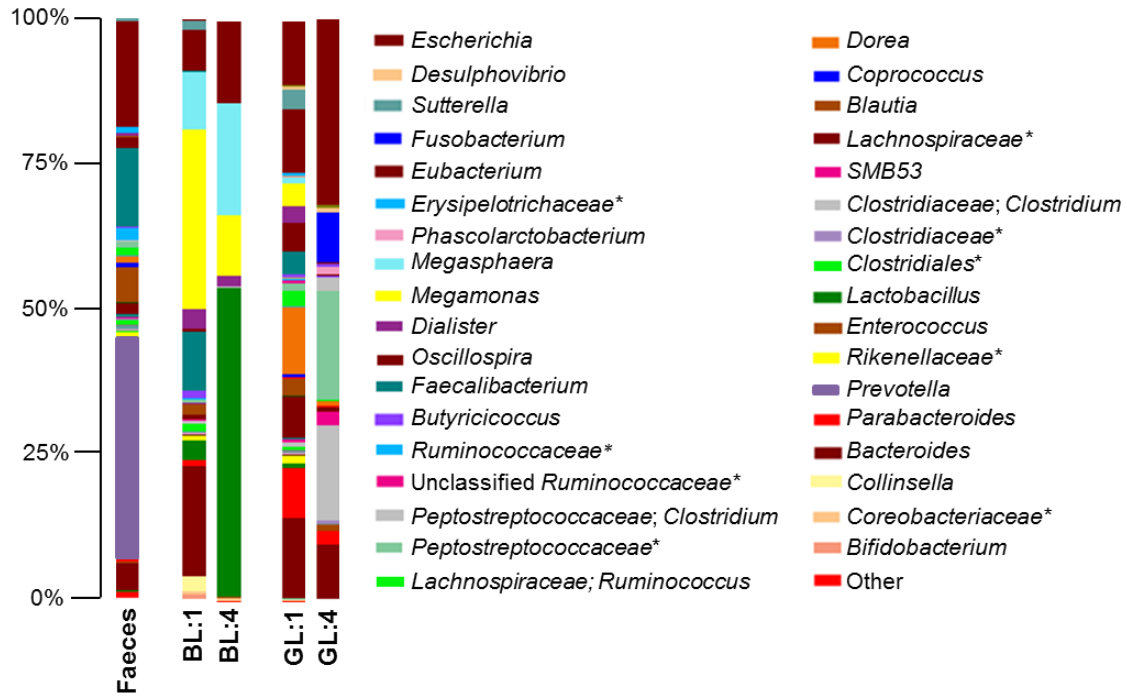


Figure 5.11: The proportion of the genus *Lactobacillus* increases after culturing faecal microbiotas in the media containing a broccoli leachate. Five human faecal samples were cultured independently in a broccoli-leachate-containing (BL) media and a glucose (GL) media. Bacterial DNA was extracted, and the V4 and V5 regions of the 16S rDNA gene were sequenced. Bioinformatic analysis was performed using QIIME 1.9.0. The bar chart displays the proportion of OTUs per cultured faecal microbiota of subject 1 after the first cycle (1) and the last cycle (4). This figure is representative of the observations that lactobacilli proportionally increased in the BL media for four of the five cultured microbiotas (subjects 1 - 3 and 5). The key reads from top left to bottom right, and consists of genera with a relative abundance ≥0.5% in at least one sample. * represents unclassified OTU reported at a higher taxonomic level.

At the genus level, the greatest effect observed was an increase in *Lactobacillus* in the cultured BL media. The average proportion of lactobacilli present in the initial faecal samples was 0.5% (SD 0.7%). Culturing these faecal microbiotas in the BL media, caused the average proportion of lactobacilli to increase to 10.2% (SD 20.6%) after cycle 1, and 42.0% (SD 31.4%) after cycle 4. As a similar bloom of lactobacilli were not seen in the GL media (cycle 1, 1% (SD 2%); cycle 4, 0.2% (SD 0.3%)), this indicated that the increased growth was due to constituents of the broccoli leachate, rather than the experimental conditions or basal media.

In experiments with four of the five human faecal microbiotas (subjects 1 - 3, and 5) cultured in the BL media, the proportion of the genus *Lactobacillus* increased to between 22% and 94% of the microbial community at the end of cycle 4. The only experiment in which lactobacilli were present in the GL media after cycle 4 (0.8%), was when the media was cultured with the microbiota of subject 3. In the experiment using the microbiota of subject 4, the proportion of lactobacilli cultured in the BL media increased to 4.3% at the end of cycle 4, compared to 0.8% in the faecal material. However, this bacterial community was dominated by another genus of the lactic acid bacteria, *Streptococcus* (**Figure 5.12**).

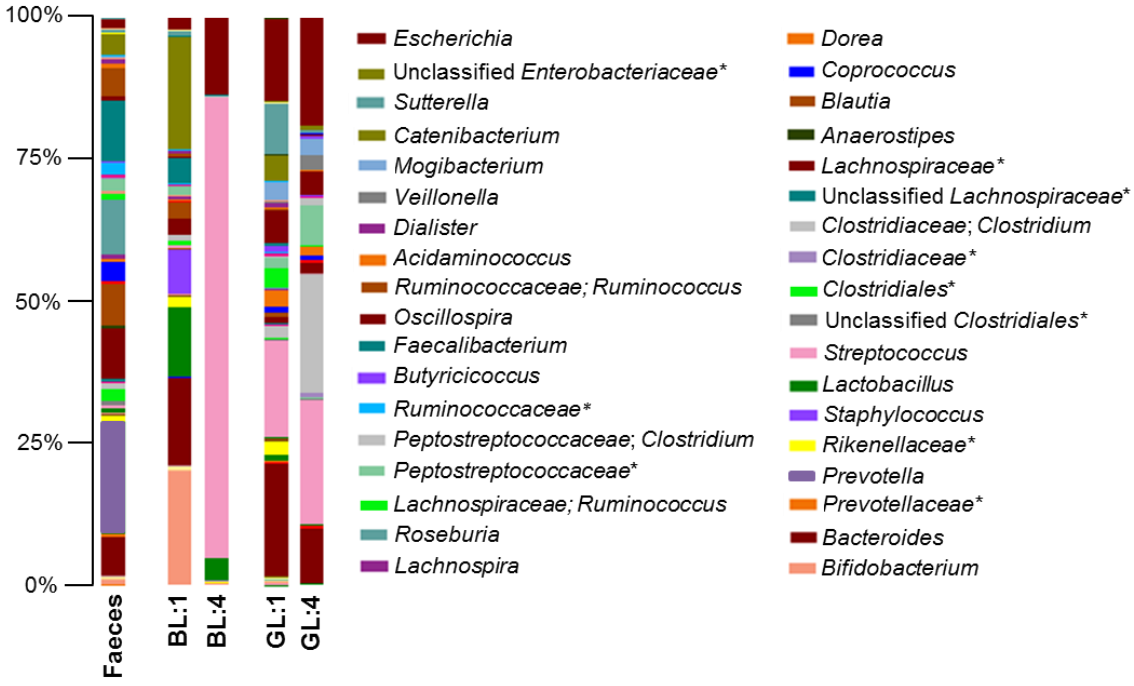


Figure 5.12: The faecal microbiota cultured in the broccoli leachate-containing media is highly represented by the genus *Streptococcus*. Five human faecal samples were cultured in a broccoli-leachate-containing (BL) media and a glucose (GL) media. Bacterial DNA was extracted, and the V4 and V5 regions of the 16S rDNA gene were sequenced. Bioinformatic analysis was performed using QIIME 1.9.0, the bar chart displays the proportion of OTUs per cultured faecal microbiota of subject 4 after the first cycle (1) and the last cycle (4). The key reads from top left to bottom right, and consists of genera with a relative abundance ≥0.5% in at least one sample. * represents unclassified OTU reported at a higher taxonomic level.

At the end of the experiment, the faecal microbiota of subject 4 cultured in the BL media largely consisted of the genus *Streptococcus* (77.4% (SD 18.3%)), with *Lactobacillus* present at 4.3% of the microbiota. Unlike previously, when lactobacilli were enriched, members of *Streptococcus* were also present at a relatively high proportion in the GL media at this time-point (21.9% (SD 0.5%)). This may indicate that the basal media and/or experimental conditions favoured the growth of streptococci, and that this effect was enhanced by the presence of the broccoli leachate. Other notable changes to the microbiota composition that were seen repeatedly, concerned the genera *Bacteroides* and *Escherichia*. The average proportion of the *Bacteroides* that were observed in the faecal samples was 3.4% (SD 2.2%). Growth in the BL media caused a reduction of all Bacteroidetes by cycle 4, with the average proportion of *Bacteroides* decreasing to 0.03% (SD 0.04%). However, an increase in *Bacteroides* at cycle 4 was observed when the faecal microbiotas were cultured in the GL media (9.4% (SD 2.8%)). Coupled with the information obtained regarding changes at the phylum level (**Figure 5.10**), this would suggest that growth in the presence of the broccoli leachate favoured members of the Firmicutes.

The proportions of the genus *Escherichia* were found to increase regardless of whether they were grown in the BL or GL media. On average, *Escherichia* was proportionally represented at 0.3 (SD 0.6%) of the initial faecal material, and increased to 25.4% (SD 2.4%) in the BL media and 31.2% (SD 7.9%) in the GL media, by the end of the fourth growth cycle. This indicates that the growth of *Escherichia* species was likely due to the experimental model and/or basal media, rather than a specific effect of the broccoli leachate. This reflects the ease at which *Escherichia* species grow in a laboratory environment under the conditions tested here.

5.4.3.3 Microbial diversity within cultured microbiotas

Diversity analysis was performed on the cultured faecal microbiotas to determine how within-sample diversity differed between cultured media types and the initial community composition. As discussed previously (section 5.4.2.2), alpha rarefaction methods were employed to investigate diversity within the microbial communities, using the Observed Species, PD, Chao1, and Shannon Index metrics. Fewer unique OTUs and a reduction in species richness were observed in samples cultured in both media types, compared to the starting faecal material (**Figure 5.13**). Analysis of the faecal material, at 3,796 sequences per sample, recorded 446 unique OTUs and a species richness measure of 1,358.1. When cultured for four cycles in the GL media, the number of unique OTUs decreased to 140.9 ± 8.9, with fewer present in the cultured BL media (31.7 ± 1.9). Effects of culturing on species richness within the microbiota followed the same trend at cycle 4, with a lower richness found in the BL media cultured community (78.9 ± 11.6) compared to the community grown in the GL media (333.9 ± 25.6).

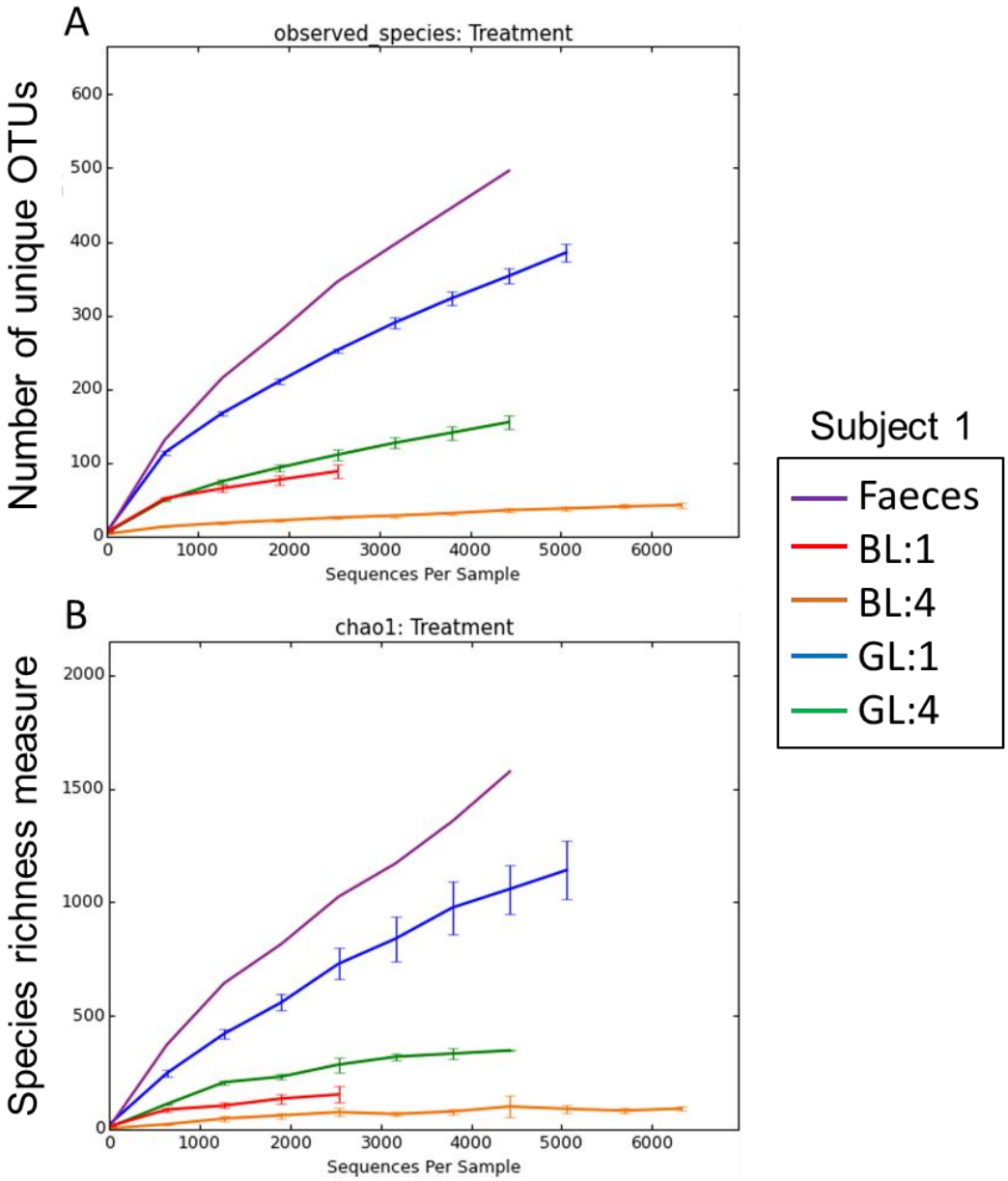


Figure 5.13: A lower number of unique OTUs, and reduced species richness is found in the microbiota cultured with the broccoli leachate media. Alpha diversity rarefaction plots of (A) number of unique OTUs, and (B) species richness within the faecal microbiota of subject 1 cultured in a broccoli leachate-containing (BL) media or glucose (GL) media. Data plotted represents diversity within the communities after the first cycle (1) and the last cycle (4). (A) The y-axis is a measure of diversity within each community, whilst the x-axis represents the number of sequences used in the diversity calculation. Rarefaction plots generated using QIIME 1.9.0. Data = mean ± SD of four technical replicates for the cultured samples, whilst the data for the uncultured faecal sample is from a single sample.

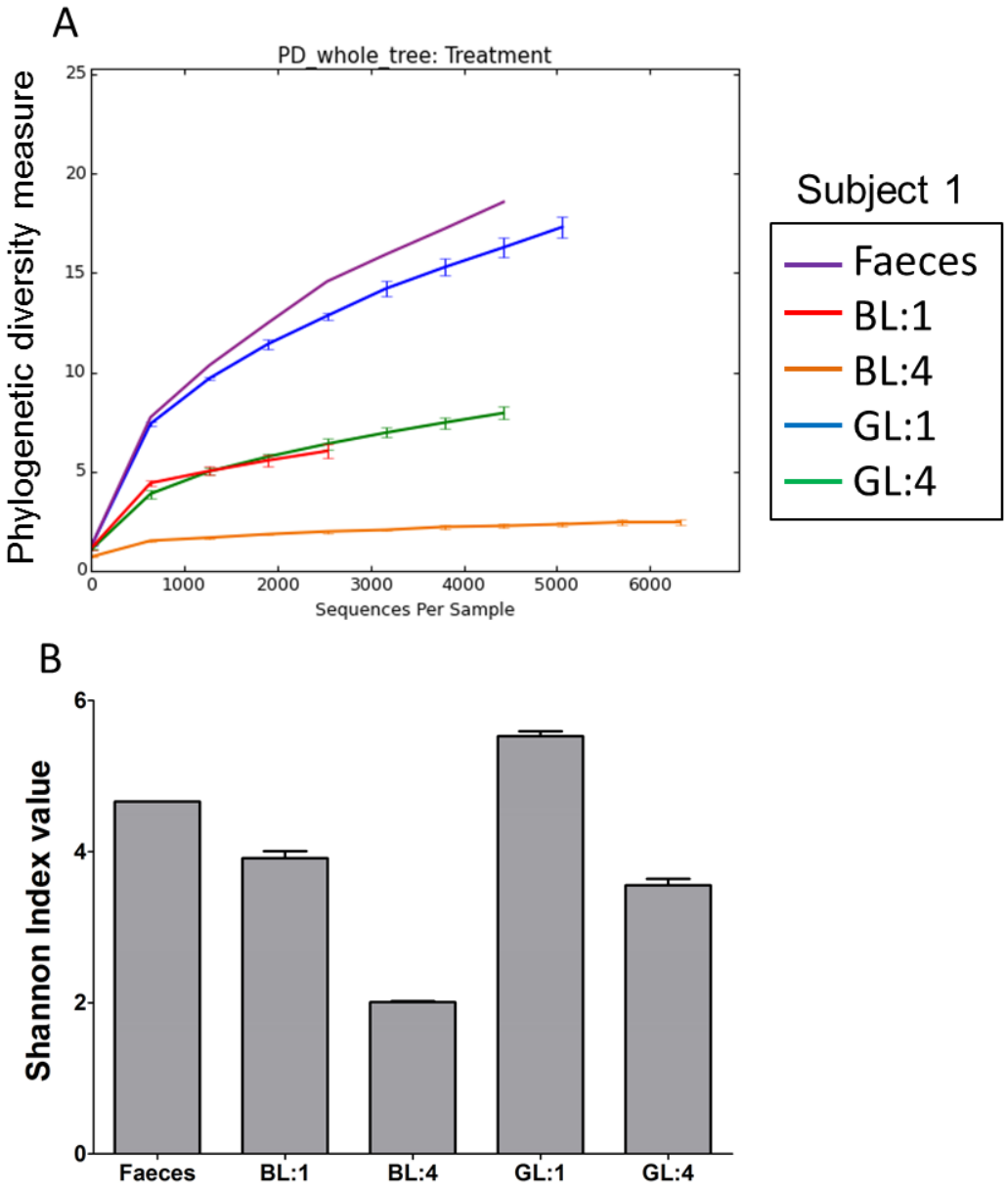


Figure 5.14: Faecal microbiota cultured with a broccoli leachate exhibits a reduced phylogenetic diversity and lower species richness and evenness. Alpha diversity rarefaction plots of (A) phylogenetic diversity, and (B) species richness and evenness within the faecal microbiota of subject 1 cultured in a broccoli leachate-containing (BL) media or glucose (GL) media. Data plotted represents diversity within the communities after the first cycle (1) and the last cycle (4), and is representative of the five cultured microbiotas. (A) The y-axis is a measure of phylogenetic diversity within each community, whilst the x-axis represents the number of sequences used in the diversity calculation. (B) The y-axis represents the Shannon Index value associated with species richness and evenness at 2,534 samples per sequence. The initial faecal microbiota and cultured microbiotas of subject 1 are plotted along the x-axis. The rarefaction plot was generated using QIIME 1.9.0, and the bar graph was produced using

GraphPad Prism 5. Data = mean ± SD of four technical replicates for the cultured samples, whilst the data for the uncultured faecal microbiota is from a single sample.

Both media had similar effects on the phylogenetic diversity, and combined species richness and evenness, within the cultured microbiotas (**Figure 5.14**). A decrease in phylogenetic diversity was observed for microbiotas cultured in BL media (2.2 ± 0.11) and GL media (7.5 ± 0.3) after cycle 4, compared to the original faecal microbiota (17.2). When the relative microbial abundance is considered in the calculation for species richness (Shannon Index), the microbiota cultured in the BL media retains a lower diversity measure than the microbiota grown in the GL media (cycle 4: 2.04 ± 0.04 vs 3.58 ± 0.5), and the uncultured faecal microbiota (4.73). Analysis using all four metrics (**Figure 5.13** and **Figure 5.14**) indicates that modification of the microbiotas occurs rapidly, with large changes seen after the first 12 hours of culturing (cycle 1). A combination of the Chao1 and Shannon Index analyses implies that the first cycle of growth in the GL media produced a more even distribution of bacterial genera within the cultured microbiota. This hypothesis appears to be corroborated by the bar chart displaying the proportion of each genus within the bacterial community (**Figure 5.11**).

Unweighted beta diversity analysis indicated a clear divergence in community composition between microbiotas cultured in the two media types (**Figure 5.15**). Twelve hours growth in the BL media or GL media was sufficient to modify the initial faecal community to form two distinct microbiotas.

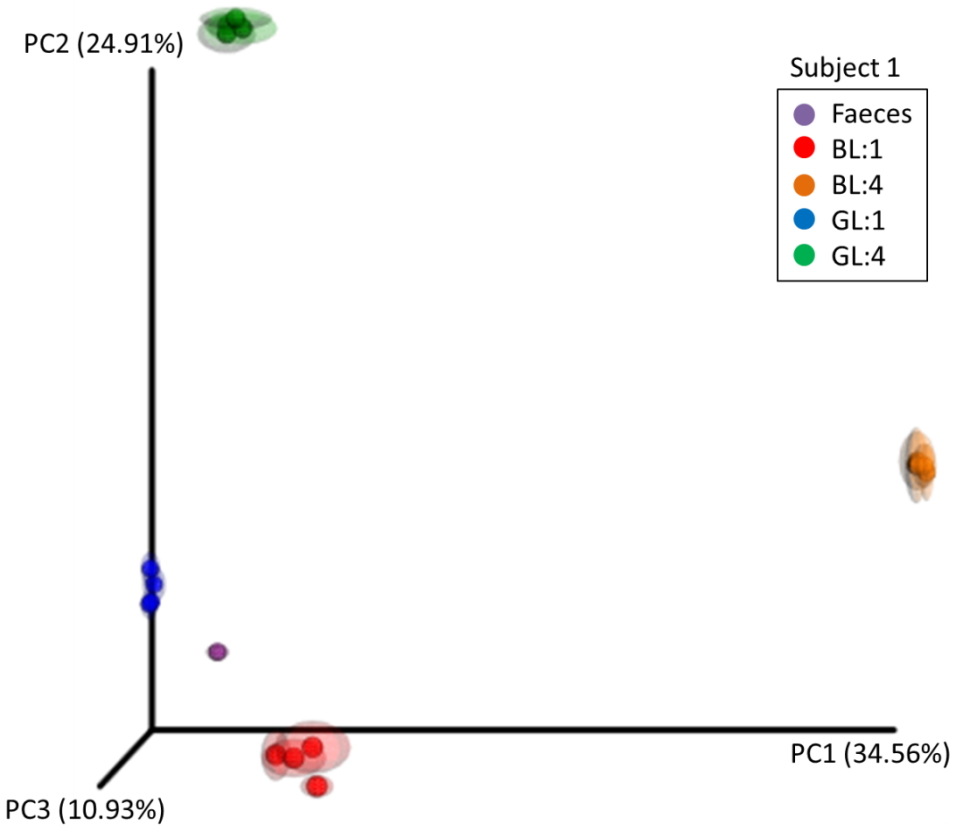


Figure 5.15: Media-dependent modulation of faecal microbiotas. Unweighted beta diversity analysis of the faecal microbiota of subject 1, cultured in a broccoli leachate-containing (BL) media and glucose (GL) media, after the first cycle (1) and the last cycle (4). This is representative of the five cultured microbiotas. Beta diversity calculated using UniFrac distances through QIIME 1.9.0, and visualised as a 3D PCoA plot using Emperor.

Weighted beta diversity analysis was performed to examine whether differences in the relative abundance of taxa would affect the level of diversity between the cultured microbiotas (**Figure 5.16**). The weighted analysis suggested a greater divergence of the cultured microbiotas from the original faecal sample following the first twelve hours of growth. The inclusion of relative abundance into the beta diversity calculation did not report a notably altered level of diversity between the cultured microbiotas at the end of the experiment, comparable to the unweighted analysis.

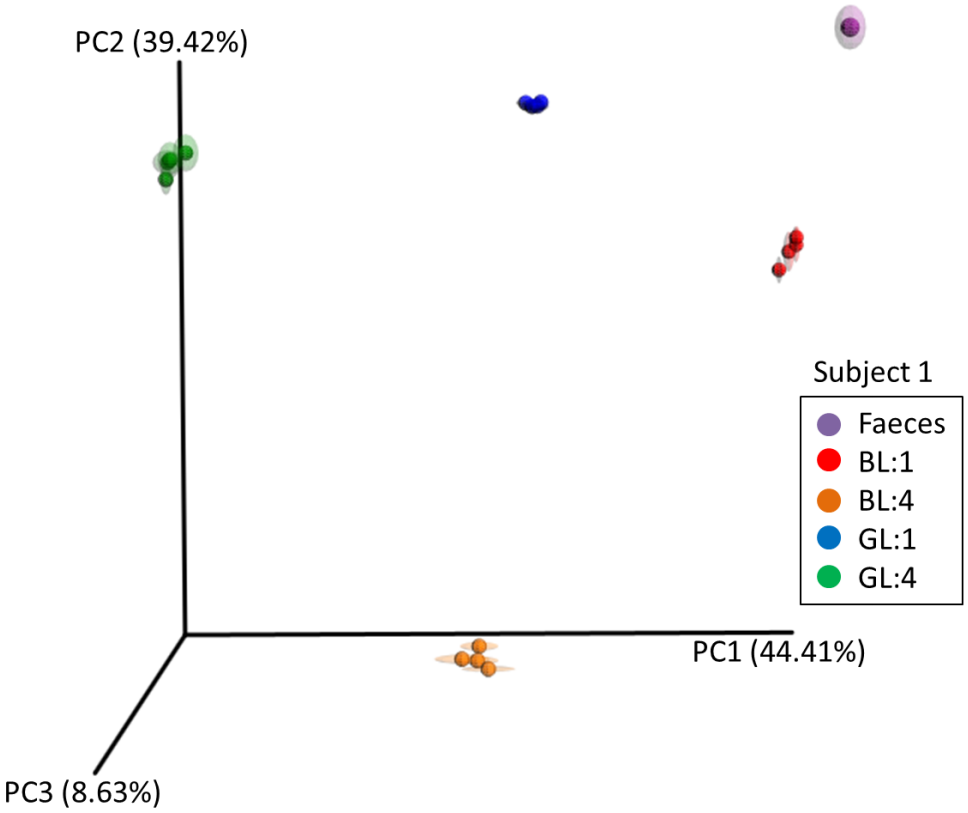


Figure 5.16: Diversity between cultured faecal microbiotas is not abolished when relative abundance is considered. Weighted beta diversity analysis of the faecal microbiota of subject 1, cultured in a broccoli leachate-containing (BL) media and glucose (GL) media, after the first cycle (1) and the last cycle (4). This is representative of the five cultured microbiotas. Beta diversity calculated using UniFrac distances through QIIME 1.9.0, and visualised as a 3D PCoA plot using Emperor.

The effects on beta diversity were greater on the microbiotas cultured in the BL media, highlighting the strong selection pressure that the broccoli leachate exerted on the microbial communities.

5.4.4 Changes in pH during culturing with human faecal bacteria

Members of the *Lactobacillus* and *Streptococcus* are known producers of lactic acid. It was hypothesised that the production of lactic acid may have reduced the pH of the medium, and inhibited the growth of bacteria sensitive to acidic conditions. In respect of this, the pH values of the faecal microbiota cultures were recorded at the end of the first and fourth cycles (**Figure 5.17**).

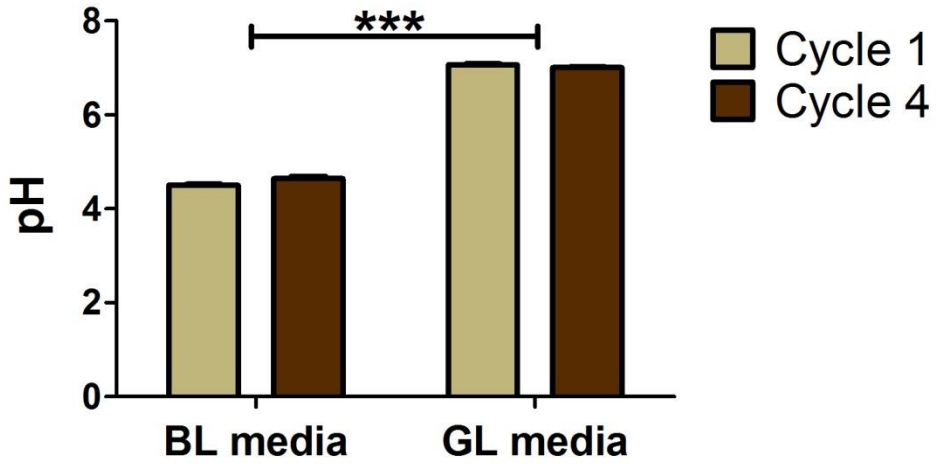


Figure 5.17: The pH of both media types after the first and fourth 12 hr culturing periods with faecal bacteria. Human faecal microbiotas were cultured with a broccoli leachate-containing (BL) media and a glucose (GL) media for four 12 hr cycles at 37˚C, in anaerobic conditions. The pH values of both cultured media types were recorded after the first (cycle 1) and fourth (cycle 4) 12 hr incubation period. The figure shows the pH values of the cultured microbiota of subject 5, and is representative of the four microbiotas tested (subjects 2 – 5). Data shown = mean ± SD of four technical replicates. The data was statistically analysed using two-way ANOVA followed by Bonferroni multiple comparisons test. ****p*<0.001 BL media vs GL media.

The above graph (**Figure 5.17**) is representative of the changes in pH observed in the culturing experiments using the faecal microbiotas of subjects 2 – 5, the pH of the cultured microbiota of subject 1 was not measured. Both the BL media and the GL media had a pH of 7.0 ± 0.02 prior to inoculation with human faecal bacteria. Combining the data from the experiments using the microbiotas of subjects 2 - 5, the pH of the BL media dropped from 7.0 to 4.52 ± 0.22 at the end of cycle 1, and was an average of 4.87 ± 0.43 at the end of cycle 4. Conversely, the pH of the cultured GL media remained relatively stable throughout the experiments; cycle 1 = 7.14 ± 0.13, and cycle 4 = 7.02 ± 0.21.

5.5 Discussion

5.5.1 Optimising bacterial DNA extraction

The protocol for the FastDNA SPIN kit for soil DNA extraction kit was modified based on the current literature. Zoetendal *et al* reported the presence of an extra amplicon following a period of bead-beating for 3 minutes, suggesting the presence of bacteria that were difficult to lyse [236]. Recently, it was identified that an increase in the mechanical disruption of cells, through the use of bead-beating, from 30 seconds to 2 minutes increased the detection of all bacterial groups [386]. Faecal samples subjected to 5 minutes bead-beating were associated with a decrease in the detection of bacterial taxa, likely due to the degradation of DNA during this extended time. Therefore, the DNA extraction method used maximised the detection of bacterial taxa, whilst minimising the loss of DNA.

5.5.2 Microbial composition of faecal microbiotas

Phylogenetic analysis of the five faecal microbiotas indicated a dominance of Firmicutes and Bacteroidetes at the phylum level (**Figure 5.3**). This concurs with the findings of other studies using 16S rDNA gene sequencing methods, where Firmicutes and Bacteroidetes have been found to constitute >90% of the distal gut microbiota, at an approximate ratio of 2.8:1 [227, 238, 371, 387]. The microbiotas of subjects 1, 3, and 4 contained the highest proportion of Bacteroidetes. An examination of the community composition at the genus level indicates that this is principally due to a relatively large proportion of *Prevotella* in these microbiotas (**Figure 5.4**). *Prevotella* are known to degrade cellulose and a variety of complex glycans [374]. Decreased levels of *Prevotella* have been reported to correlate with increased energy intake and adiposity, and to be associated with a diet low in carbohydrates [238, 388]. The genus *Faecalibacterium* contains one known species, *F. prausnitzii*, which is reportedly the most abundant bacterium found in the gut microbiota of healthy humans, representing 5 - 15% of the total bacterial community [389, 390]. *Faecalibacterium* were the most prevalent genera amongst the faecal samples tested, and the relative abundances correlated with the proportions observed in previous work. *F. prausnitzii* is a major butyrate producer [391], with anti-inflammatory and immunomodulatory capabilities [392, 393]. Decreased levels of this bacterium has been associated with Crohn's disease [394] and ulcerative colitis [395].

5.5.3 Diversity analysis of faecal microbiotas

The alpha diversity scores, measuring the diversity within faecal microbiotas, indicated increased diversity within the faecal microbiota obtained from subject 5 (**Figure 5.5** and **Figure 5.6**), compared to the faecal microbiotas of subjects 1 - 4. As the Chao1 analysis indicated that the faecal microbiota of subject 5 exhibited increased species

richness versus that of subject 4 (1,927.4 vs 1,513.1) (**Figure 5.5 B**), the Shannon Index score (**Figure 5.6 B**) suggests a more even distribution of bacterial taxa within the microbiota of subject 4. Comparing the Shannon Index score against the Chao1 measurement for subject 3 versus that of subject 2 (5.66 vs 5.83), indicates that the bacterial taxa are more evenly distributed within the microbiota of subject 3, compared to the microbiota of subject 2 (**Figure 5.5 B** and **Figure 5.6 B**). The faecal microbiota of subject 1 was consistently scored as having the lowest diversity, but the alpha diversity measurements for all microbiotas were similar to previously reported scores [396-398]. Unweighted beta diversity analysis indicated that the microbiotas of the five subjects were dissimilar based on the level of shared taxa (**Figure 5.7**). However, the weighted analysis, which also computes the similarity between the relative abundance of taxa between communities, suggested that the microbiotas of subjects 2 and 5 were phylogenetically similar (**Figure 5.8**). It has been proposed that the human gut microbiota can be stratified into clusters known as enterotypes, principally by the levels of *Prevotella* and *Bacteroides* present in the microbiota [226, 239]. There was no evidence that the microbiotas of subjects 2 and 5 correlated with any of the proposed enterotypes.

5.5.4 Effect of broccoli leachate on gut microbiota community

After the first cycle of growth, the cultured microbiotas of both media types differed from the original faecal microbiota composition, and these differences were enhanced by the end of the fourth cycle. At the phylum level, microbiotas cultured in either the BL or GL media exhibited a decrease in Bacteroidetes, and an increase in Proteobacteria (**Figure 5.10**) in all five microbiotas tested. An increase in the proportion of Firmicutes was observed in the microbiotas cultured in the BL media.

The differential effects of the culture medium on the microbiotas can be clearly seen at the genera level (**Figure 5.11**). At the end of four of the five experiments, the microbiota cultured in the BL media was dominated by *Lactobacillus*, whilst this genus was only observed in the GL media at low levels (0.8%) after one experiment. This was observed in four of the five experiments and would seem to indicate that nutrients available in the BL media favoured the growth of members of the *Lactobacillus*. In a recent study performed by Filannino *et al*, the metabolism of phenolic compounds by *Lactobacillus* sp. was investigated using a broccoli puree [399]. All strains tested exhibited good growth, and it was found that two strains of *Lactobacillus* (*L. fermentum* FUA3165 and *L. reuteri* FUA3168) were able to reduce quinic acid, which was postulated to give these organisms an energetic advantage through NAD^+ regeneration [399]. Multiple *in vitro* and *in vivo* studies have indicated that dietary components may have the potential to increase beneficial intestinal lactobacilli [400-408]. In the experiment where a bloom of *Lactobacillus* was not observed, *Streptococcus* was present at the largest proportion (**Figure 5.12**). *Lactobacillus* and *Streptococcus* are from the order *Lactobacillales*, and are considered core members of the LAB. This group of Gram-positive bacteria are found in the small intestine and colon, where they metabolise dietary carbohydrates. Broccoli is rich in galacturonic acid, glucose, galactose, and arabinose, with lesser amounts of mannose, xylose, rhamnose and fucose also present [409]. This source of carbohydrates may have provided *Lactobacillus* and *Streptococcus* with a competitive advantage over other members of the microbiota.

5.5.5 Effect of broccoli leachate on gut microbiota diversity

Alpha diversity analysis, using a range of metrics, indicated that both of the cultured microbiotas exhibited a decrease in diversity compared to the bacterial communities present in the original faecal material (**Figure 5.13** and **Figure 5.14**). It is believed that

approximately 60 - 80% of the intestinal bacteria identified by metagenomic analyses have yet to be cultured, although recent advances in culturomics may help to overcome this deficiency [410-412]. Therefore, the decrease in diversity seen within the cultured microbiotas, compared to that of the initial faecal microbiota, were likely partly due to the experimental conditions not supporting the growth of the less robust members of the microbiota. The strong selection pressure exerted by the broccoli leachate is illustrated in the reduced diversity observed within the microbiota cultured in the BL media, compared to that of the GL media. Beta diversity analysis highlights the similarity between replicates, and the effect of the media type on bacterial community modulation (**Figure 5.15** and **Figure 5.16**). Changes from the initial bacterial community composition occurred within the first 12 hours of growth, and these changes were media-dependent. The divergence between the cultured microbiotas increased following the fourth cycle of growth, although the similarity between the replicates remained high.

5.5.6 Effect of pH on growth environment

A decrease in pH was observed in the cultured BL media (mean final pH of 4.87), whilst the cultured GL media remained at a neutral pH (**Figure 5.17**). The major metabolic end-product of carbohydrate fermentation by LAB, such as *Lactobacillus* or *Streptococcus*, is lactic acid [413]. The production of lactic acid, or other acidic metabolites (e.g. SCFAs), may have been responsible for the increased acidity of the BL media. Culturing *Lactobacillus* strains in a broccoli puree has been shown to cause a decrease in pH from 6.51 – 4.28 [399]. Previous work has shown that the growth of *Bacteroides* species and *Escherichia coli* were negatively affected at a pH of 5.5 [270]. The consistent loss of *Bacteroides* from the microbiota cultured in the BL media may have been influenced by the acidity of the growth medium. Conversely, *Bacteroides* were consistently observed in the cultured GL media, which retained a neutral pH.

Although *Escherichia* were present in the BL media at the completion of the fourth cycle, they were present at a lower proportion than observed in the GL media. Many Firmicutes are tolerant of acidic conditions, and it would be reasonable to expect that these bacteria would have gained a competitive edge in the acidic conditions of the cultured BL media. *In vitro* studies have shown that a difference in pH of 1 unit is sufficient to alter the bacterial community composition and metabolic output [270, 271]. Therefore, the batch fermentation model used for this research has limitations. A pH controlled continuous fermentation model would have allowed for a constant influx of media, mitigating any effects on the bacterial community caused by the carbon source being depleted, and removing the advantage to acid-tolerant bacteria by maintaining a stable pH. These experimental conditions would have better reflected the environment that the human gut microbiota inhabits.

5.6 Conclusions

The results presented in this chapter detail how the compositions of human faecal microbiotas were modulated differentially by growth in a BL media or a GL media. The nutrients available in the BL media favoured the growth of *Lactobacillus* species, which was associated with an increase in the acidity of the culture medium. These acidic conditions would enhance the growth of acid-tolerant bacteria, such as *Lactobacillus*, whilst simultaneously inhibiting the growth of other bacteria within the microbiota. Neither a notable presence of *Lactobacillus*, nor a decrease in pH was observed in the cultured GL medium. The next chapter will detail the use of metabolomic analyses to determine a) whether the change in pH was linked to the production of lactic acid, b) whether SCFAs accumulated in the BL and GL media, and to what degree, and c) how the metabolic profiles may have differed between cultured microbiotas.

CHAPTER SIX

6 Dietary broccoli and the gut microbiota: Production of bacterial metabolites after repeated exposure to a broccoli leachate

6.0 Summary

Bacterial carbohydrate fermentation produces weakly acidic products, such as lactic acid, which can lower the luminal pH of the colon. Lactate can be utilised by bacteria in metabolic processes that generates SCFAs, which are thought to be beneficial to the host. This chapter investigates which bacterial metabolites were produced when human faecal microbiotas were cultured in BL and GL media. The cultured BL media contained high concentrations of lactate, which was largely absent from the cultured GL media. Higher concentrations of numerous SCFAs were present in the cultured BL media, compared to the GL media. When the microbiota of subject 1 was cultured in the BL media, relatively high levels of propionate and valerate were observed, which were associated with relatively low levels of lactate. It is suggested that this is due to the activity of members of the genus Megasphaera*. Comparison of the fermentation intermediates and SCFAs produced by the cultured microbiota of subject 4 is suggestive of a switch in* Streptococcus *fermentation activity, when cultured in the GL media. Metabolite analysis indicates that the observed decrease in pH of the cultured BL media was likely due to lactate produced by the LAB, which may have been further metabolised to generate SCFAs.*

6.1 Introduction

The previous chapter detailed how the community compositions of the faecal microbiotas were modified through repeated exposure to BL and GL media. This chapter will investigate whether the observed decreases in pH of the cultured BL media was associated with the presence of lactate. In addition, samples will be analysed for the presence of SCFAs, and differences between the bacterial metabolic profiles of the cultured human faecal microbiotas will be explored.

The fermentation of carbohydrates by the human gut bacteria leads to decreases in the luminal pH of the colon. This is due to the production of weakly acidic bacterial metabolites, such as lactate and SCFAs (**Figure 6.1**). The lactic acid bacteria (LAB) are a group of bacteria considered beneficial to human health, whose major metabolic end-product is lactate, which is a good substrate for the growth of many intestinal bacteria, and can be utilised to form SCFAs [414-416]. LAB can be classified as heterofermenters (*Weisella*, *Leuconostoc*, and some lactobacilli) or homofermenters (*Streptococcus*, *Pediococcus*, *Lactococcus*, and some lactobacilli) based on the end-products of fermentation [252]. Whilst homofermenters produce lactate as the major, or sole, fermentation end-product, heterofermenters produce equimolar amounts of lactate, ethanol, and CO_2 [252]. Some bacteria can switch fermentation strategies, and this is dependent on nutrient availability [417].

SCFAs are bacterial waste products, which are necessary to balance redox equivalent production in the anaerobic environment of the human gut [281]. The most physiologically prevalent SCFAs in the human gut are acetate, propionate, and butyrate, all of which are potential end-products of lactate metabolism [273, 418-420]. In the human colon and stool, acetate, propionate, and butyrate are found in an approximate molar ratio of 60:20:20 [274-276]. SCFA production is closely associated with diet, and total SCFA concentrations have been found to range between ~70 – 140 mM in the proximal colon, decreasing to ~20 – 70 mM in the distal colon [277]. Following absorption, SCFAs are used as an energy source by the host, and are believed to have multiple beneficial effects in humans. These physiological effects include regulating immune responses [421], maintaining intestinal homeostasis [422-425], functioning as signalling molecules [284, 285, 426, 427], and anti-inflammatory and anti-carcinogenic activity [286, 287, 428-430].

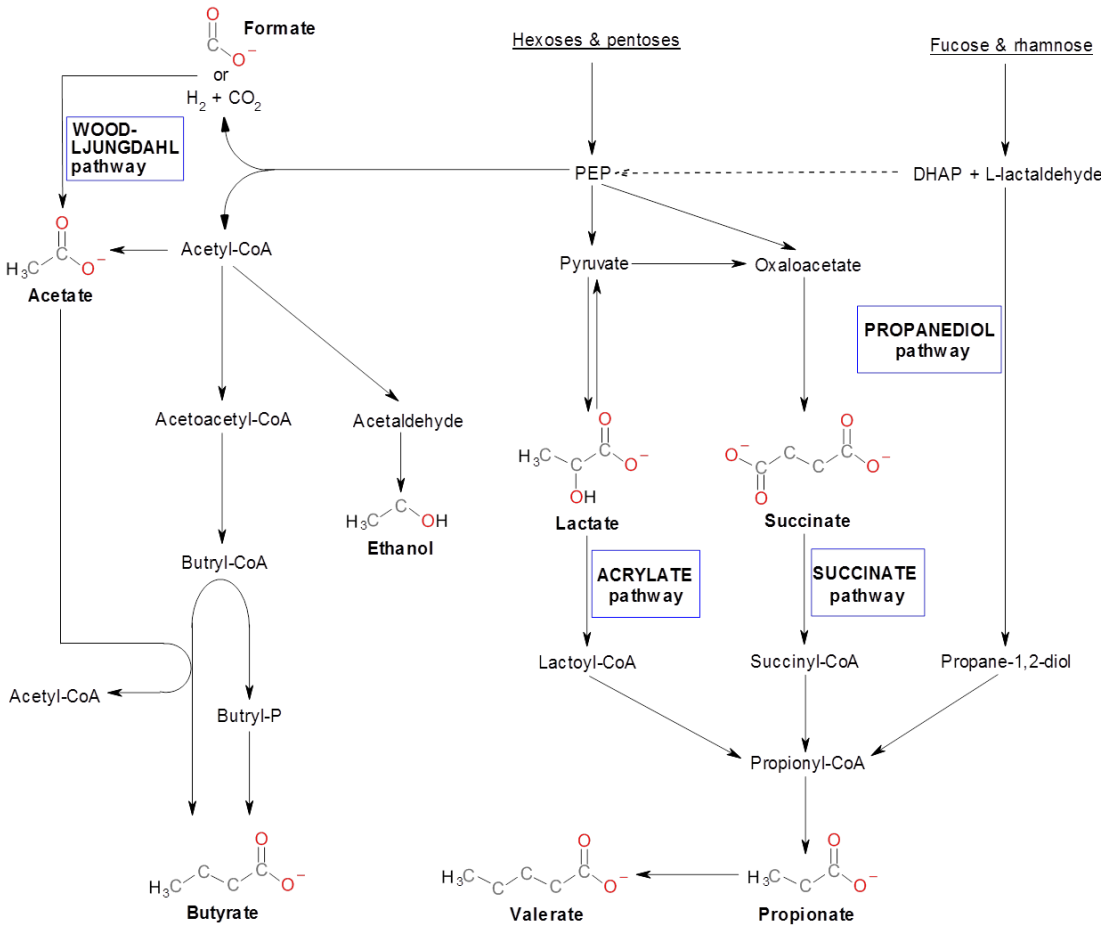


Figure 6.1: Schematic of microbial short-chain fatty acid production. Metabolism of sugars by the human gut microbiota can lead to the production of the short-chain fatty acids (acetate, butyrate, propionate, or valerate), by various metabolic pathways (the acrylate, propanediol, succinate, or Wood-Ljungdahl pathways) (adapted from Louis *et al* [280]). Abbreviations: DHAP = dihydroxyacetone phosphate ; PEP = phosphoenolpyruvate

6.2 Objectives

The hypothesis for the research presented in this chapter was that the presence of acidic compounds, such as lactate, would link the increase in proportions of the genera *Lactobacillus* and *Streptococcus* with the decrease in pH of the BL media. Additionally, it was hypothesised that culturing human faecal microbiota in the BL and GL media would result in different metabolic profiles, due to the increased nutrient availability in the BL media.

6.3 Materials and methods

The samples analysed in this work originated from the experiment detailed in section 2.8, and summarised in **Figure 6.2**. Details of the faecal donors are displayed in **Table 6.1**. The samples were collected at the same time-points as those discussed in chapters 4 and 5. The samples were centrifuged and the supernatant filtered, prior to being mixed with a 0.4 mM phosphate buffer (section 2.6.1). ^{1}H NMR spectroscopy was performed as detailed in section 2.6.2, with sodium 3-(Trimethylsilyl)-propionate-d_4 used as a chemical shift reference. Metabolite quantification was carried out using the Chenomx NMR Suite software.

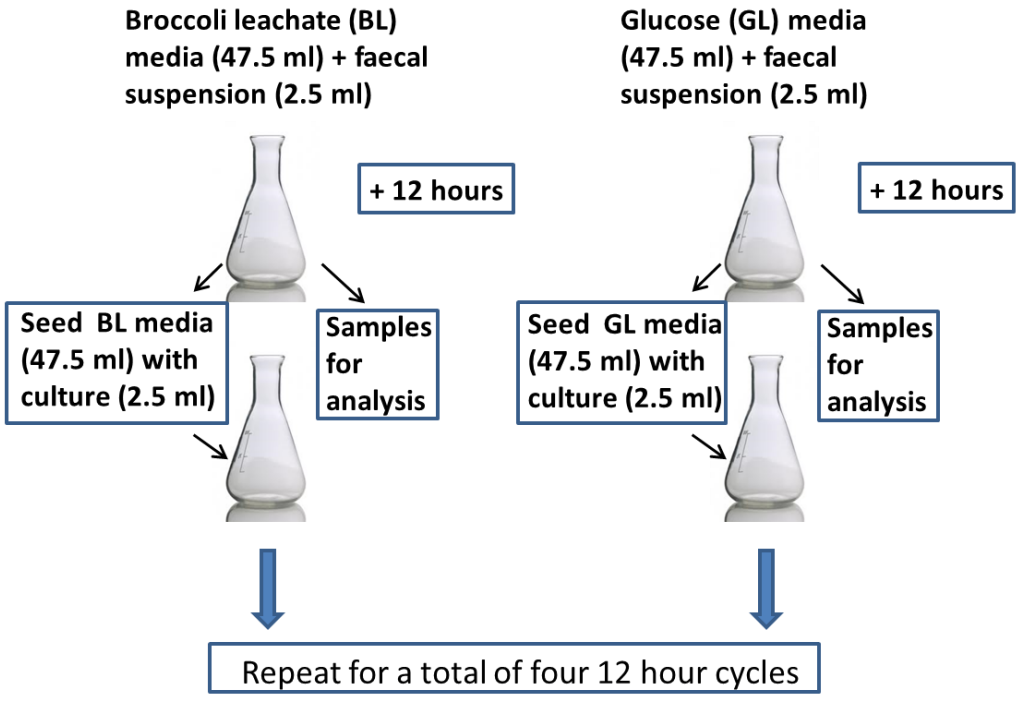


Figure 6.2: Schematic of experimental design. Broccoli leachate (BL) media (47.5 ml) and glucose (GL) media (47.5 ml) were seeded with a faecal suspension (2.5 ml) and cultured for 12 hr at 37˚C, under anaerobic conditions. After a 12 hr incubation period, samples (2 ml) were taken for ^{1}H NMR spectroscopy, and a 2.5 ml aliquot was used to seed the respective fresh media. This was repeated for four cycles of bacterial enrichment.

Table 6.1: Age, gender, Body Mass Index (BMI), and smoking status of the faecal donors.

Faecal donors	*Age (years)*	*Gender*	*BMI (kg/m^2)*	*Smoker*
Subject 1	44	Male	24.1	N
Subject 2	24	Female	23.3	N
Subject 3	52	Female	20.9	N
Subject 4	50	Female	38.4	N
Subject 5	61	Female	20.5	N

6.4 Results

6.4.1 Analysis of lactate production

Samples obtained from experiments using five human faecal microbiotas cultured in a BL media and a GL media, were examined using ^{1}H NMR spectroscopy. This analysis was performed to determine whether the production of lactic acid by LAB may have been responsible for the increased acidity of the cultured BL media.

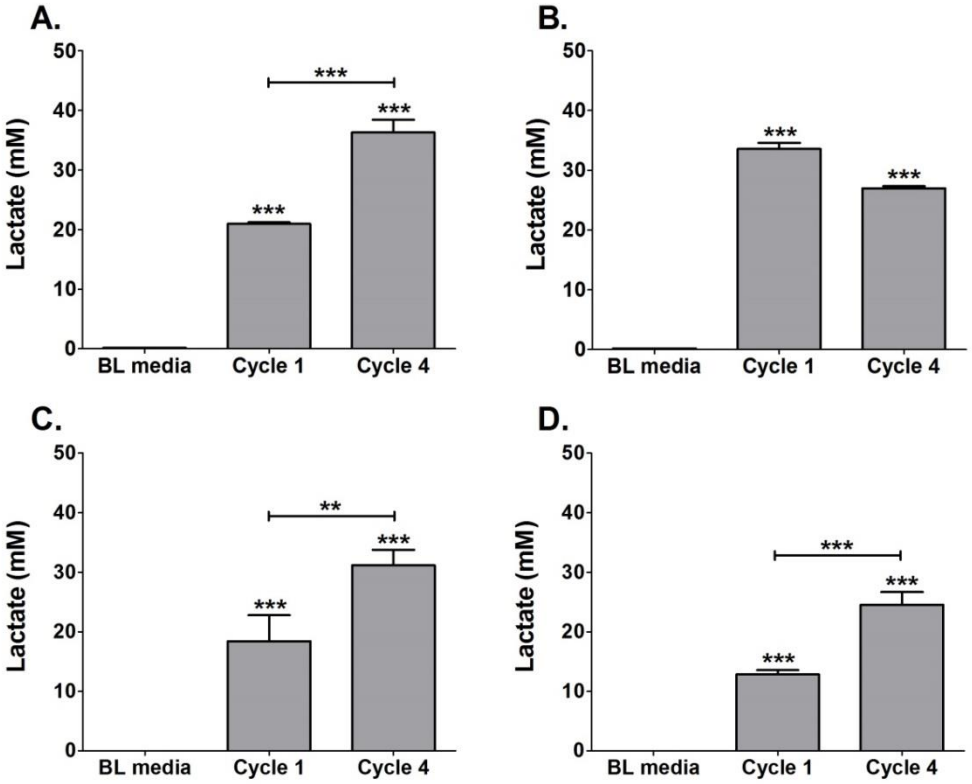


Figure 6.3: Lactate accumulated in the BL media when cultured with human faecal microbiotas. Five human faecal suspensions were cultured independently in a broccoli-leachate-containing (BL) media and a glucose (GL) media. Aliquots of the cultured media supernatant were mixed with a 0.4 mM phosphate buffer, and subjected to ^{1}H NMR spectroscopy. Sample analysis was performed using the Chenomx NMR Suite software. The figure shows the concentration of lactate in the pre-inoculated BL media, and cultured BL media after cycle 1 and cycle 4 for the microbiotas obtained from subjects 2 - 5. Data shown = mean ± SD of four technical replicates. A. = subject 2; B. = subject 3; C. = subject 4; D. = subject 5. The data was statistically analysed using one-way ANOVA followed by Bonferroni multiple comparisons tests. ** $p \leq 0.01$; *** $p \leq 0.001$.

In experiments using four of the five microbiotas (subjects 2 - 5), high levels of lactate (21.33 – 34.38 mM) were observed in the cultured BL media after cycle 4 (**Figure 6.3**). A relatively high decrease in the levels of lactate in the cultured BL media between cycles 1 and 4 was observed for the cultured microbiota of subject 1 (**Figure 6.4**).

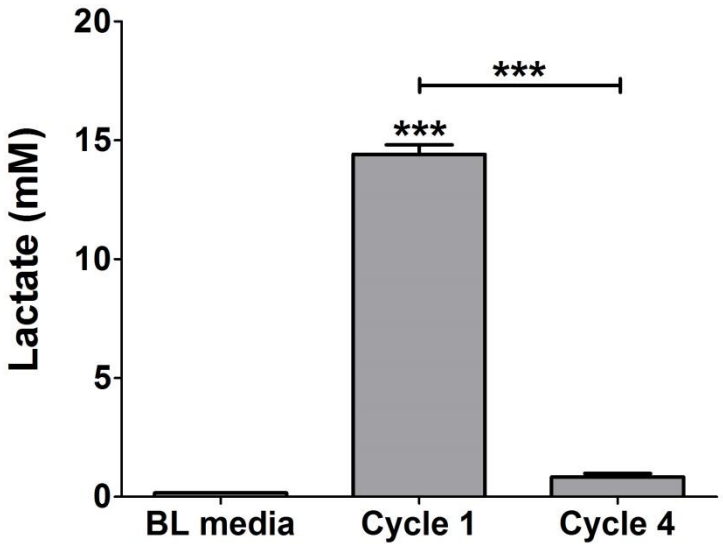


Figure 6.4: Lactate levels decreased in the BL media after cycle 4 when cultured with the faecal microbiota of subject 1. A human faecal suspension was cultured in a broccoli-leachate-containing (BL) media and a glucose (GL) media. Aliquots of the cultured media supernatant were mixed with a 0.4 mM phosphate buffer, and subjected to ^{1}H NMR spectroscopy. Sample analysis was performed using the Chenomx NMR Suite software. The bar chart displays the concentration of lactate in the pre-inoculated BL media, and cultured BL media after cycle 1 and cycle 4 for the microbiota obtained from subject 1. Data shown = mean ± SD of four technical replicates. The data was statistically analysed using one-way ANOVA followed by Bonferroni multiple comparisons tests. ***$p \leq 0.001$ cultured BL media cycle 1 vs uncultured BL media.

The decrease in lactate concentration observed between cycle 1 and cycle 4 may have been due to increased lactate utilisation by the cultured microbiota of subject 1. Although lactate was detected in the GL media after cycle 1, following inoculation with three of the faecal microbiotas (subjects 3 - 5), it was only present in low amounts (0.07 - 0.84 mM). Lactate was not present in the GL media after cycle 4 when cultured with the faecal microbiotas of subjects 1 – 4. Lactate was detected at a low concentration in the GL media after cycle 4 when cultured with the microbiota of subject 5 (**Table 6.2**).

Table 6.2: Proportions of *Lactobacillus* (Lb) and *Streptococcus* (Str) in the BL and GL media after cycle 4, and the corresponding concentrations of lactate.

Source of microbiota	***BL media***			***GL media***		
	Lb (%)	**Str (%)**	**Lactate (mM)**	**Lb (%)**	**Str (%)**	**Lactate (mM)**
Subject 1	53.4	0	0.84 (± 0.28)	0	0.1	0
Subject 2	94.1	0	36.31 (± 2.12)	0	0	0
Subject 3	35.9	0.9	23.49 (± 3.78)	0.8	1.6	0
Subject 4	4.3	77.4	31.16 (± 2.59)	0	21.9	0
Subject 5	22.5	0.3	24.53 (± 2.15)	0	0	0.17 (± 0.19)

As lactate was present at a high concentration in the BL media cultured with the microbiota of subject 4, it may be a little surprising that lactate was not detected in the corresponding GL media. LAB can be divided into two groups based on the fermentation pathways utilised for hexose metabolism: homofermenters and heterofermenters. Whilst the former produce lactate as the sole or major end-product, heterofermenters produce smaller quantities of lactate, but also produce acetate, ethanol and CO_2 [252]. Analysis of acetate and ethanol, alongside lactate, may help to explain the absence of lactate in the GL media cultured with the microbiota of subject 4 (**Table 6.3**).

Table 6.3: Products obtained from two LAB fermentation pathways.

Source of microbiota	*BL media*			*GL media*		
	Acetate (mM)	**Ethanol (mM)**	**Lactate (mM)**	**Acetate (mM)**	**Ethanol (mM)**	**Lactate (mM)**
Subject 1	21.37 (± 0.26)	1.75 (± 0.09)	0.84 (± 0.28)	10.61 (± 2.35)	1.65 (± 0.28)	0
Subject 2	20.27 (± 1.46)	18.42 (± 1.32)	36.31 (± 2.12)	8.68 (± 0.23)	1.21 (± 0.02)	0
Subject 3	24.68 (± 7.23)	7.79 (± 0.50)	23.49 (± 3.78)	7.45 (± 1.24)	1.18 (± 0.13)	0
Subject 4	3.79 (± 2.78)	0.75 (± 0.71)	31.16 (± 2.59)	7.52 (± 0.09)	0.67 (± 0.03)	0
Subject 5	16.46 (± 2.68)	3.53 (± 0.20)	24.53 (± 2.15)	6.68 (± 1.05)	0.97 (± 0.09)	0.17 (± 0.19)

Acetate and ethanol were present at relatively low concentrations in the BL media cultured with the microbiota of subject 4, which was dominated by *Streptococcus*. This may suggest that homofermentation was the primary fermentation activity. The data obtained from the corresponding cultured GL media was less suggestive, and may be indicative of a switch to heterofermentation due to the depletion of glucose from the media.

6.4.2 Short-chain fatty acid analysis of cultured media

Lactate can be used in bacterial metabolic processes to generate bacterial waste products, termed SCFAs. ^{1}H NMR spectroscopy was used to identify the concentrations of the most physiologically prevalent SCFAs (acetate, butyrate, and propionate) in both the cultured BL and GL media (**Figure 6.5**).

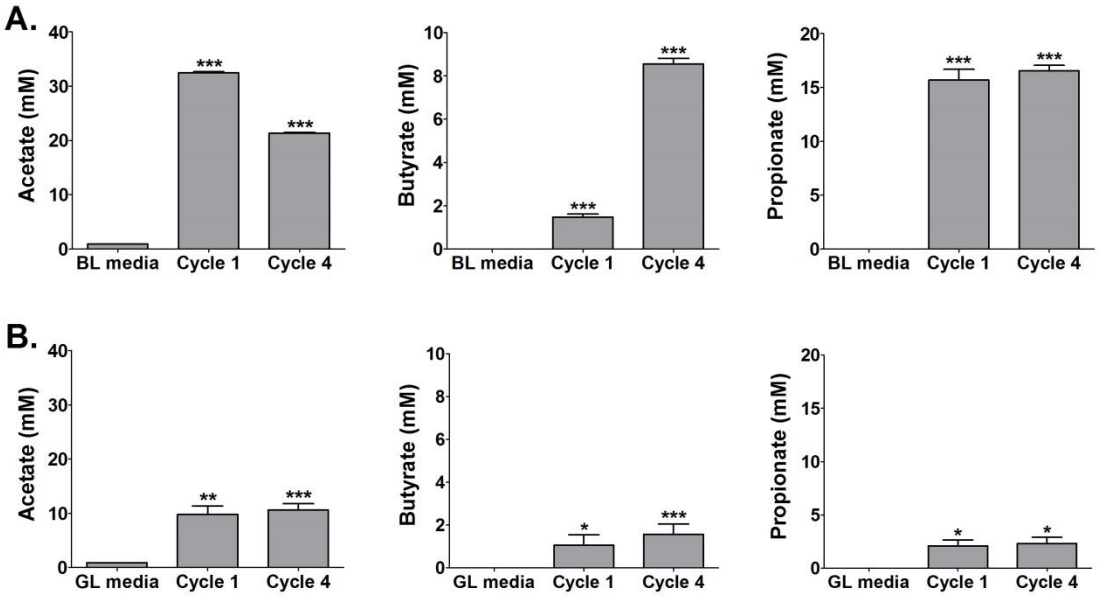


Figure 6.5: Concentrations of short-chain fatty acids differ in the BL media and GL media when cultured with human faecal microbiotas. Five human faecal suspensions were cultured independently in a broccoli-leachate-containing (BL) media and a glucose (GL) media. Aliquots of the cultured media supernatant were mixed with a 0.4 mM phosphate buffer, and subjected to ^{1}H NMR spectroscopy. Sample analysis was performed using the Chenomx NMR Suite software. The figure shows the concentration of acetate, butyrate, and propionate in the pre-inoculated media, and cultured BL and GL media after cycle 1 and cycle 4 for the microbiota obtained from subject 1. These results are representative of the differences between the media observed with all five cultured faecal microbiotas. A. Acetate, butyrate, and propionate concentrations in cultured BL media; B. Acetate, butyrate, and propionate concentrations in cultured GL media. Data shown = mean ± SD of four technical replicates. The data was statistically analysed using one-way ANOVA followed by Bonferroni multiple comparisons tests. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ cultured media vs uncultured media.

Acetate, butyrate, and propionate were present at higher concentrations in the cultured BL media, compared to the cultured GL media. On average, across all five faecal microbiotas, there was a 2.2-fold increase in total yield of SCFAs in the cultured BL media compared to the cultured GL media. The increased levels of the SCFAs found in the cultured BL media, likely reflects the increased availability of nutrients derived from the broccoli leachate, but will be directly linked to the metabolic capabilities of the bacteria within the cultured microbiotas.

6.4.3 Comparing SCFA profiles between microbiotas cultured in a BL media

It has been demonstrated that inter-individual variability exists within the metabolic potential, as well as in the composition, of the human gut microbiota [418]. Variability in SCFA production, between the five human faecal microbiotas cultured in the BL media for four 12 hr cycles, was investigated using ^{1}H NMR spectroscopy (**Table 6.4**).

Table 6.4: Mean short-chain fatty acid concentrations in the BL media after cycle 4, when cultured with human faecal microbiotas.

Source of microbiota	*Acetate*	*Butyrate*	*Formate*	*Propionate*	*Valerate*	*Total*
Subject 1	21.37 (± 0.26)	8.55 (± 0.26)	2.25 (± 0.14)	16.53 (± 1.05)	11.54 (± 0.28)	**60.24**
Subject 2	20.27 (± 1.46)	0	1.16 (± 0.06)	0	0	**21.43**
Subject 3	24.68 (± 7.23)	4.94 (± 1.34)	5.03 (± 0.68)	2.64 (± 2.85)	0.21 (± 0.41)	**37.5**
Subject 4	3.79 (± 2.78)	0	2.59 (± 2.21)	0.06 (± 0.12)	0	**6.44**
Subject 5	16.46 (± 2.68)	0	1.69 (± 0.10)	0	0	**18.15**

The cultured microbiota of subject 1 produced a variety of SCFAs, as well as the highest total yield of SCFAs (60.24 mM). Valerate was only detected in the cultured samples from subjects 1 and 3, whilst subjects 2 and 5 formed neither butyrate, nor propionate. The lowest total yield of SCFAs was observed in the samples derived from subject 4 (6.44 mM), and this largely consisted of acetate and formate. All sugars present in the starting BL media were metabolised by subjects 1 - 3, however relatively high concentrations of fructose were identified in the cultured samples from subjects 4 and 5 (6.91 mM and 2.18 mM, respectively).

6.5 Discussion

6.5.1 Lactate production by gut bacteria in the presence of a broccoli leachate

Human faecal microbiotas cultured in a BL media exhibited a proportional increase in LAB, and this was associated with a decrease in the pH of the media from 7.0 to an average of 4.87 (**Figure 5.17**). ^{1}H NMR spectroscopy was used to test the hypothesis that the decrease in pH was associated with the production of lactic acid by the LAB. Samples obtained from the cultured BL media indicated that lactate was present at high concentrations after the first 12 hours growth with all five faecal microbiotas. High levels of lactate were observed after cycle 4 in the BL media of four of the five cultured faecal microbiotas (subjects 2 - 5) (**Figure 6.3**). The cultured BL media with the microbiota obtained from subject 1, contained levels of lactate which were not significantly greater than that found in the starting media (**Figure 6.4**). It seems likely that the lactate was being utilised in metabolic processes by members of this microbiota.

6.5.2 Metabolic activity of *Streptococcus* in the glucose media

Lactate was only detected in the GL media at low levels in three of the cultured samples (subjects 3 - 5) following the first 12 hours of growth, and was only present in the cultured microbiota obtained from subject 5 after cycle 4 (**Table 6.2**). The microbiota of subject 4 exhibited an increased proportion of the potential LAB, *Streptococcus,* when cultured in both the BL and GL media (**Figure 5.12**). Lactate was absent in the corresponding cultured GL media. This may be a consequence of the different fermentation pathways between LAB, and an effect of glucose-limiting

conditions in the GL media, as higher levels of glucose were present in the BL media compared to the GL media.

Streptococcus is classified as a homofermentative LAB; lactic acid is the sole or major end-product of glucose fermentation [252]. Some lactobacilli are homofermentative, whilst others, such as *L. fermentum*, are heterofermenters, producing lactate, acetate, ethanol and CO_2 from hexose sugars [252]. These data indicate that, when cultured in the BL media, the microbiota of subject 4 produced high levels of lactate, but less acetate and ethanol than the other cultured microbiotas (**Table 6.3**). However, when the same microbiota was cultured in the GL media, the profile of acetate, ethanol, and lactate resembled that of the other microbiotas tested. This may be explained by the differences in sugar content between the two media. The BL media was richer in sugars than the GL media (**Table 3.4**). The glucose within the GL media had been fully depleted at this time-point, and this may have provided conditions in which it was favourable for *Streptococcus* to switch to a heterofermentative state. Thomas *et al* previously showed that under certain growth conditions, such as glucose-limited conditions, lactic streptococci switch to heterofermentation activity [417]. Therefore, it may be that lactate was being produced in smaller amounts in the GL media, and subsequently utilised by members of the cultured microbiota due to the depletion of the preferred carbon source, glucose.

6.5.3 Short-chain fatty acid production by cultured microbiotas

Lactate is the major metabolic end-product of carbohydrate fermentation by LAB, and can be subsequently utilised by other members of the gut microbiota to generate bacterial metabolites, such as SCFAs (**Figure 6.1**). The physiologically prevalent SCFAs (acetate, butyrate, and propionate) were detected in cultured samples from both the BL media and GL media (**Figure 6.5**). However, on average there was a 2.2-

fold increase in the total yield of these SCFAs in the cultured BL media, compared to the cultured GL media. The microbiotas cultured in the BL media may have used some of the available lactate for metabolic processes, which could lead to a greater yield of SCFAs.

Acetate was the primary SCFA produced from all cultured microbiotas, as has been observed in the human colon and faecal material [274-276]. Acetate or butyrate can be produced through the utilisation of pyruvate, which can be formed through the oxidation of lactate [419, 431]. Lactate can be used directly in the acrylate pathway to generate propionate [273]. Relatively high concentrations of propionate were only detected in the BL media cultured with the microbiota obtained from subject 1 (**Table 6.4**). This may indicate that lactate was being produced by this microbiota during cycle 4, but was subsequently utilised to produce propionate. The microbiota of subject 1 contained a relatively high proportion of the genus *Megasphaera*, which was not detected in the other microbiotas cultured (**Figure 5.11**). *Megasphaera elsdenii* is known to be present in the human intestine, and is an important rumen bacterium, which converts lactate to propionate via the acrylate pathway [432]. When glucose and lactate are both available, *M. elsdenii* produces increasing amounts of butyrate and valerate, with a concomitant decrease in the production of propionate [433]. When the glucose is depleted, the lactate is metabolised to produce propionate. The presence of *Megasphaera* in the BL media cultured with the faecal microbiota of subject 1 may be at least partly responsible for the relatively high concentration of both propionate and valerate, as well as the production of butyrate (**Table 6.4**).

6.6 Conclusions

Metabolite analysis using ^{1}H NMR spectroscopy indicated that lactate was the major bacterial metabolite produced when human faecal microbiotas were cultured in the BL media. At the end of the experimental period, lactate was only detected in the GL media cultured with the microbiota of subject 5, and was only present at relatively low concentrations. Levels of lactate, acetate, and ethanol in the cultured microbiota of subject 4 suggested that growth in the GL media may have caused *Streptococcus* species to switch from homofermentation to heterofermentation. This was likely due to the depletion of glucose from the GL media. Although SCFAs were detected in all the cultured media, the total yield of SCFA was higher in the BL media compared to the GL media. The types and concentrations of SCFAs produced in the cultured BL media differed between the microbiota. The microbiota of subject 1 produced the largest yield of SCFAs when cultured in the BL media, with relatively high concentrations of propionate and valerate. This microbiota contained a relatively high proportion of the genus *Megasphaera*, which has previously been shown to utilise lactate and glucose to produce propionate and valerate. The low pH of the cultured BL media was likely due to the increased levels of lactate and SCFAs, which were produced during bacterial carbohydrate fermentation.

CHAPTER SEVEN

7 Bacterial identification and screening assays

7.0 Summary

This chapter discusses the identification of bacteria isolated from the in vitro *fermentation experiments detailed in chapter 5, and the use of these isolates in screening assays to investigate the bacterial metabolism of broccoli compounds. 16S rDNA sequencing of eighteen bacterial isolates identified all lactobacilli tested to be* Lactobacillus fermentum*. Pure culture screening assays using glucoraphanin discovered several* Escherichia *species able to reduce glucoraphanin to a similar degree. The extent of glucoraphanin reduction was not dependent on the levels of oxygen, but differed between bacterial culture media. The* E. coli *DH5α laboratory strain was able to reduce glucoraphanin, suggesting that the bacterial reduction of glucosinolates may not be restricted to intestinal bacteria. The reduction of* S-*methylcysteine sulphoxide (SMCSO) by an isolated* E. coli *strain (1B04) suggests that the reduction reaction is not specific to glucosinolates. GC-MS analysis using* E. coli *1B04 and* E. coli *DH5α, cultured in media containing glucoraphanin or SMCSO, identified degradation products of both compounds due to the activity of each* E. coli *strain. These results suggest that although the bacterial reduction reaction was the primary metabolic activity,* E. coli *1B04 is able to degrade glucoraphanin and SMCSO.*

7.1 Introduction

Lactobacillus fermentum species are an indigenous resident of the human gut microbiota, with several strains currently used as probiotics. These acid-tolerant bacteria have been shown to strongly adhere to intestinal epithelial cells, have antimicrobial activity against pathogenic bacteria, and immunomodulatory properties [247, 250, 251]. In addition, *L. fermentum* strains have been linked to an increased production of SCFA, and cholesterol-lowering properties associated with bile salt

hydrolase activity [434-436]. Investigations into the beneficial properties of *L. fermentum* strains is an ongoing area of research.

E. coli have evolved to be highly efficient at exploiting its environment to maximise energy sources, with reductases central to many of these processes. Reductases are involved in multiple important biological processes, such as the protection of proteins from oxidative damage [314, 437, 438], altering the biological activities of proteins to maintain homeostasis [315], involvement in the synthesis of DNA [316, 317], and are central in respiratory pathways [294, 439]. The high level of flexibility built into the respiratory pathways of *E. coli* enables its growth in aerobic and anaerobic environments, as well as the utilisation of a diverse range of electron acceptors [294, 439, 440]. The *E. coli* dimethyl sulphoxide (DMSO) reductase, DmsABC has been found to be able to utilise multiple sulphoxides as electron acceptors during anaerobic growth [310, 441].

Within the plant, *S*-methylcysteine sulphoxide is thought to function as a phytoalexin, providing protection against microbial pathogens and herbivores, through its degradation by cysteine sulphoxide lyases (**Figure 7.1**) [150, 442, 443]. Bacterial cysteine β-lyase activity has been detected within gastrointestinal bacteria, and has been attributed to a diverse range of bacteria, including *E. coli* [164-166, 444-448]. The bacterial cysteine lyase has broad substrate specificity, and is able to cleave the C-S bonds of a range of *S*-alkyl-cysteine molecules, in a manner similar to the plant cysteine lyases [150]. Research into the beneficial effects of SMCSO and its metabolic products have indicated that this dietary compound may exhibit protective effects against cancer [167-174, 449, 450], diabetes [177, 179, 451], and cardiovascular disease [175, 176, 178, 452].

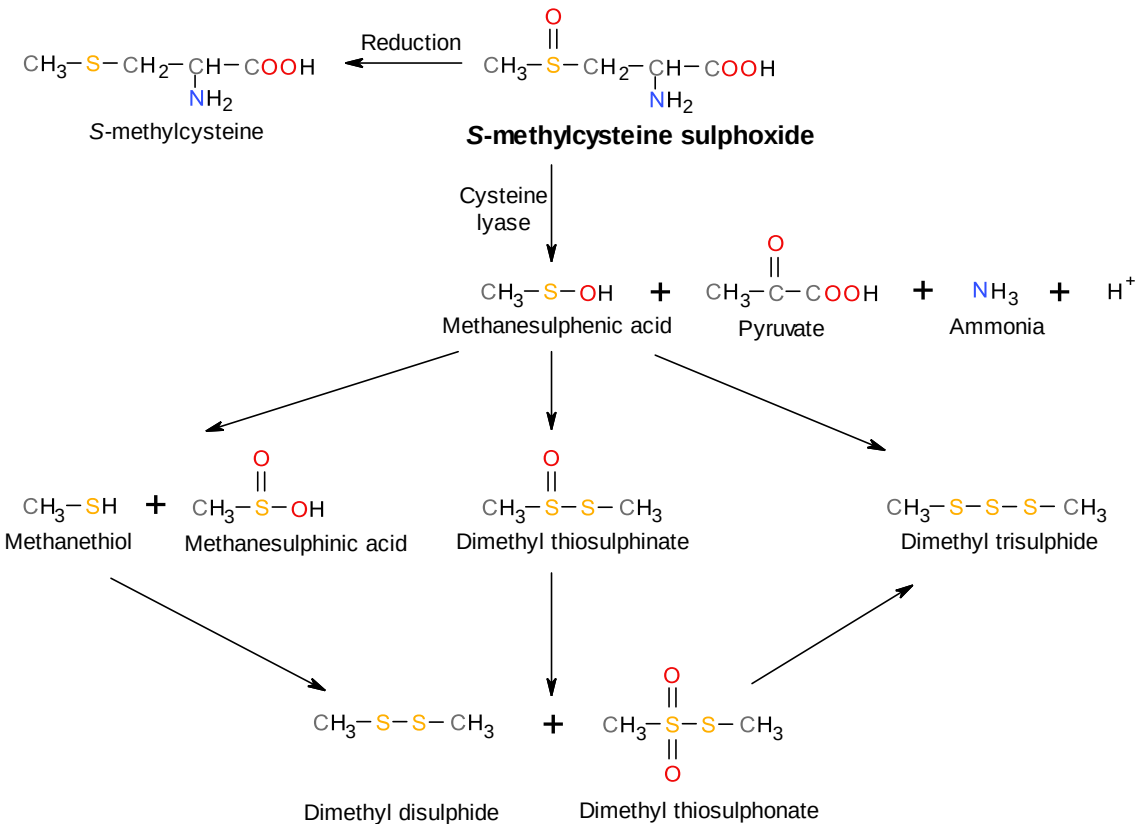


Figure 7.1: Metabolic schematic for the reduction or degradation of *S*-methylcysteine sulphoxide. Schematic displaying the metabolic products formed through the degradation of SMCSO by the action of cysteine lyase, and the reduction of SMCSO to *S*-methylcysteine.

7.2 Objectives

The hypothesis of the work contained in this chapter was that bacteria, isolated from the *in vitro* fermentation experiment (chapter 5), that were able to reduce glucoraphanin and SMCSO would be identified through pure culture experiments.

7.3 Materials and methods

Bacteria were isolated from experiments in which human faecal microbiotas were cultured in BL media, as summarised in **Figure 7.2**, and described in section 2.8.5. The 16S rDNA, of a selection of these bacterial isolates, were amplified using colony PCR (section 2.10.2), and sequenced commercially at Eurofins. The FinchTV and SeqMan software were used for DNA sequence quality checking and contig assembly, respectively, and the online Ribosomal Database Project (RDP) tool SeqMatch, was used for bacterial species identification, as detailed in section 2.10.3. Bacterial isolates were cultured in media containing ~6 mM glucoraphanin (section 2.11.1), or 0.3 mM glucoerucin (section 2.11.2). The analysis of glucosinolates within culture supernatants was performed using HPLC (section 2.5). A bacterial isolate was cultured in the presence of 0.3 mM SMCSO (section 2.11.3), and analysis of SMCSO concentrations in the culture supernatants was performed using LC-MS (section 2.4.2) and ^{1}H NMR spectroscopy (section 2.6). GC-MS was used to further investigate the products of glucoraphanin and SMCSO metabolism (section 2.12).

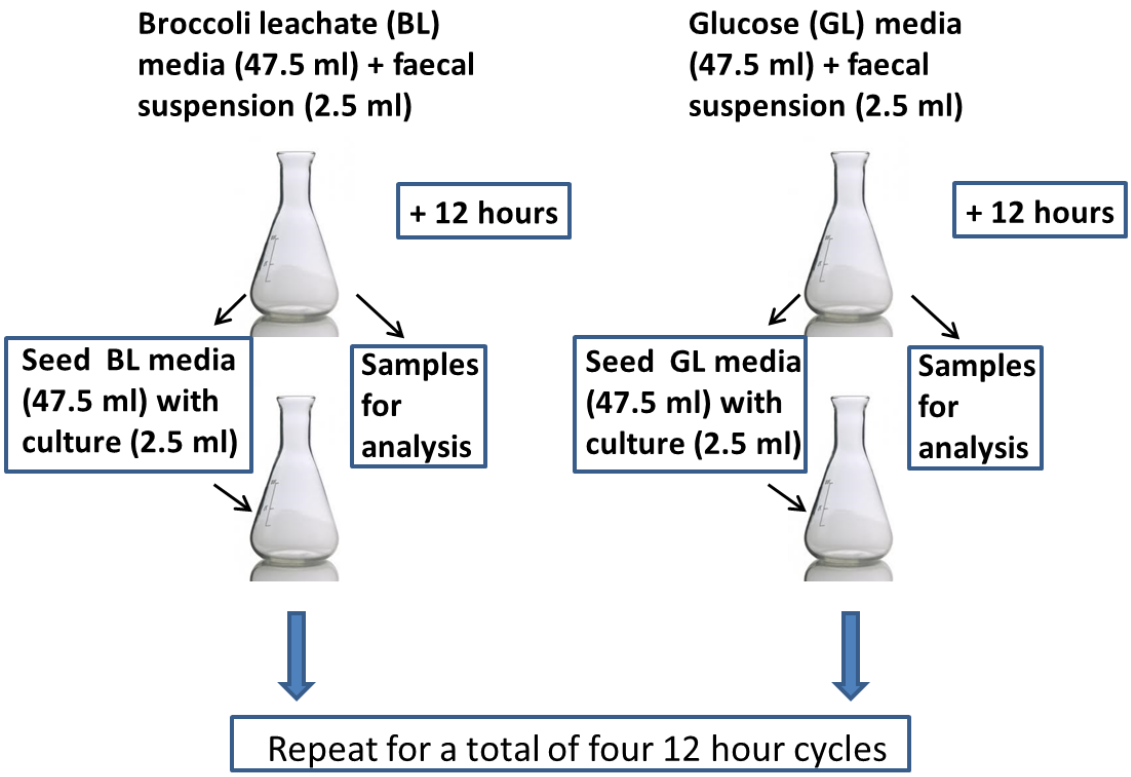


Figure 7.2: Schematic of experimental design. Broccoli leachate (BL) media (47.5 ml) and glucose (GL) media (47.5 ml) were seeded with a faecal suspension (2.5 ml) and cultured for 12 hr at 37˚C, under anaerobic conditions. Samples were taken for sequencing analysis of the variable V4 and V5 regions within 16S rDNA, and an aliquot was used to seed the respective fresh media. This was repeated for four cycles of enrichment.

7.4 Results

7.4.1 Amplification of the 16S rDNA gene from bacterial isolates

Fifty bacterial isolates, obtained from human faecal microbiotas that had been cultured in the BL media (section 2.8.5), were selected for further investigation. Following growth of colonies on MRS agar medium, 19 bacterial isolates were chosen for species identification based on growth and morphological characteristics. The 16S rDNA gene was amplified for each of these bacterial isolates, using colony PCR (section 2.10.2), and visualised using agarose gel electrophoresis (section 2.9.1.1).

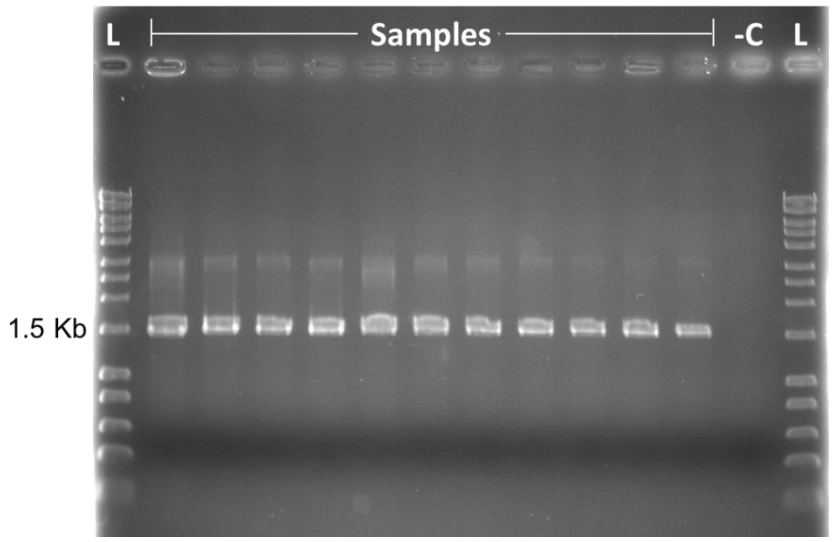


Figure 7.3: A 1% agarose gel showing the amplification of the 16S rDNA gene from bacteria isolated from the cultured BL media. Human faecal microbiotas were cultured with a broccoli leachate-containing (BL) media for four 12 hr cycles at 37˚C, under anaerobic conditions, and bacteria were isolated and stored in glycerol at -80˚C. The 16S rDNA gene (1.5 Kb) was amplified through colony PCR, and visualised using agarose gel electrophoresis and ethidium bromide staining. The figure shows an imaged gel of 11 amplicons, which is representative of all the amplicons visualised. L = Bioline Hyperladder I (10 Kb); -C = negative control (PCR reaction mixture without DNA).

Visualisation of the agarose gel suggested that PCR amplification of the 16S rDNA gene had been successful, as indicated by the presence of bands at the 1.5 Kb molecular weight marker (**Figure 7.3**).

7.4.2 Identification of bacterial isolates

Amplified PCR products of the nineteen isolates were sent to Eurofins in duplicate for sequencing of the 16S rDNA gene, each containing 2 µl of either the forward or reverse primer used in the PCR reaction. The sequences obtained were quality checked. Based on the data obtained, one of the isolates was considered as potentially contaminated and removed from further analysis. The sequence data for the remaining eighteen isolates were submitted to RDP SeqMatch for comparison to known 16S

rDNA sequences compiled in the RDP database. Taxonomic identification was based on the S_ab score (fraction of shared 7-base sequence fragments between query and reference sequences) [334].

Table 7.1: Identification of bacterial isolates based on the RDP SeqMatch S_ab score, and the number of unique oligomers within each query sequence.

Isolate code	*Taxonomic rank*	*Species name*	*S_ab score*	*Unique oligomers*
1A09	Species	*Lactobacillus fermentum*	1.000	1384
1B02	Genus	*Escherichia*	1.000	1350
1B03	Genus	*Escherichia*	0.995	1334
1B04	Species	*Escherichia coli*	1.000	1325
1C03	Species	*Enterococcus faecium*	1.000	1367
1C04	Species	*Lactobacillus fermentum*	1.000	1375
1C05	Species	*Lactobacillus fermentum*	1.000	1381
1C06	Genus	*Escherichia*	1.000	1035
My-0101	Species	*Lactobacillus fermentum*	1.000	1382
My-0103	Species	*Lactobacillus fermentum*	0.998	1398
My-0105	Species	*Enterococcus durans*	1.000	1371
My-0201	Species	*Lactobacillus fermentum*	0.997	1380
My-0204	Species	*Lactobacillus fermentum*	1.000	1384
My-0205	Species	*Lactobacillus fermentum*	1.000	1391
My-0302	Species	*Lactobacillus fermentum*	1.000	1391
My-0303	Species	*Lactobacillus fermentum*	1.000	1391
My-0401	Species	*Lactobacillus fermentum*	0.995	1385
My-0404	Species	*Lactobacillus fermentum*	0.993	1383

Of the eighteen isolates, twelve were identified as *Lactobacillus fermentum*, two 16S rDNA sequences were considered as belonging to *Enterococcus* species, and the remaining four isolates were identified as belonging to the genus *Escherichia* (**Table 7.1**).

7.4.3 Screening for glucoraphanin reduction activity

Ten identified bacterial isolates were selected and screened for their ability to convert glucoraphanin to glucoerucin in pure cultures (section 2.11.1).

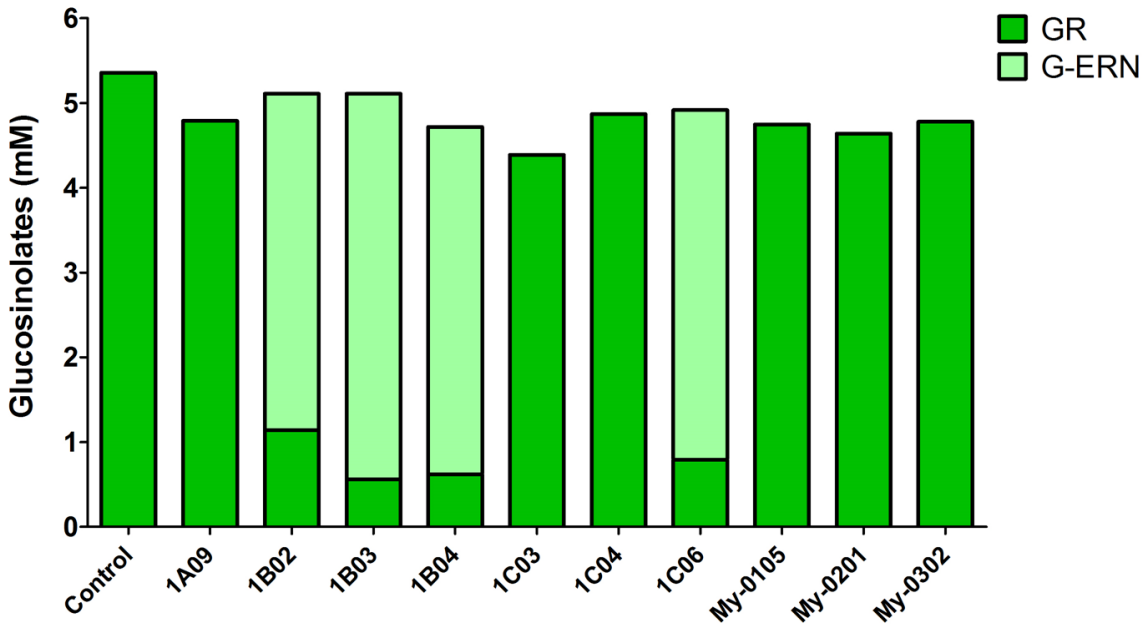


Figure 7.4: Four bacterial isolates, identified as *Escherichia*, have the ability to form glucoerucin via the reduction of glucoraphanin. Ten bacterial isolates (5 µl) were cultured in CNM containing ~6 mM purified glucoraphanin extract (total volume of 1 ml) for 24 hr at 37˚C, under anaerobic conditions. The control sample consisted of CNM containing ~6 mM purified glucoraphanin extract (995 µl) with sterile water (5 µl) added in place of the bacterial inoculum. Glucosinolates were converted to desulphoglucosinolates and measured using HPLC. GR = Glucoraphanin; G-ERN = Glucoerucin.

Of the ten bacterial isolates tested, four identified as belonging to the genus *Escherichia* were able to significantly reduce glucoraphanin to glucoerucin (74 – 85%) (**Figure 7.4**). The remaining six isolates, that did not exhibit the ability to reduce glucoraphanin, consisted of bacteria from the order *Lactobacillales*: four *L. fermentum* isolates and two *Enterococcus* species. Two *Escherichia* isolates were cultured with glucoraphanin under aerobic conditions to test whether the ability to reduce glucoraphanin was associated with the reduced environment in the anaerobic cabinet.

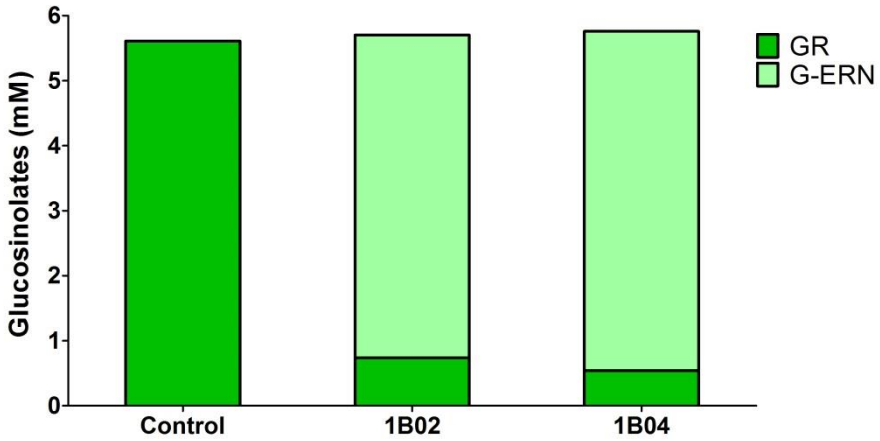


Figure 7.5: Reduction of glucoraphanin to glucoerucin by *Escherichia* isolates is not linked to a reduced environment. Two *Escherichia* isolates (5 µl) were cultured in CNM containing ~6 mM purified glucoraphanin extract media (total volume of 1 ml) for 24 hr at 37˚C, under aerobic conditions. The control sample consisted of CNM containing ~6 mM purified glucoraphanin extract (995 µl) with sterile water (5 µl) added in place of the bacterial inoculum. Glucosinolates were converted to desulphoglucosinolates and measured using HPLC. GR = Glucoraphanin; G-ERN = Glucoerucin.

The reduction of glucoraphanin to glucoerucin occurred to a similar extent when *Escherichia* isolates were cultured with purified glucoraphanin extract under aerobic conditions, compared to an anaerobic environment (**Figure 7.5**). Luria broth (LB) is a nutritionally rich medium, which is commonly used for the cultivation of *E. coli* [453]. Isolate *E. coli* 1B04 was cultured in both CNM and LB, supplemented with a purified glucoraphanin extract to a concentration of ~6 mM, to investigate whether differences

in media would affect bacterial reductase activity. No significant difference in reductase activity was observed between aerobic or anaerobic conditions within each media type, but the conversion of glucoraphanin to glucoerucin was lower in the cultured LB samples, compared to the cultured CNM samples (**Figure 7.6**).

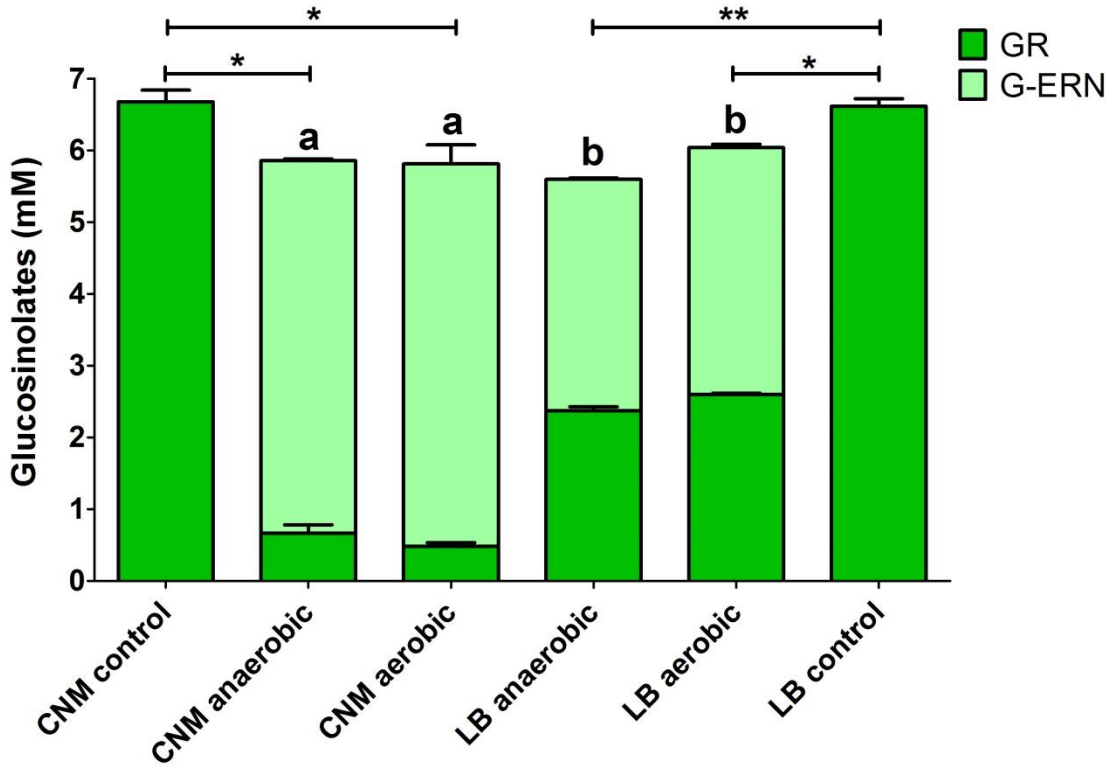


Figure 7.6: The ratio of glucoraphanin to glucoerucin differs between the LB and CNM media when cultured with *Escherichia coli* 1B04, irrespective of levels of oxygen. *E. coli* 1B04 (5 µl) was cultured in two media (CNM and LB) containing ~6 mM purified glucoraphanin extract media (total volume of 1 ml) for 24 hr at 37˚C, under both anaerobic and aerobic conditions. The control samples consisted of the equivalent media containing ~6 mM purified glucoraphanin extract (995 µl) with sterile water (5 µl) added in place of the bacterial inoculum. Glucosinolates were converted to desulphoglucosinolates and measured using HPLC. GR = Glucoraphanin; G-ERN = Glucoerucin; CNM = Chemostat nutrient media; LB = Luria broth. Data shown = mean ± SD of two technical replicates. The data for the ratios of glucoraphanin and glucoerucin between samples were statistically analysed using two-way ANOVA; a and b are statistically significantly different from one another ($p<0.001$). The data for the analysis of total glucosinolates in cultured samples vs the associated control sample were analysed using Student's t-tests (two-tailed); *$p<0.05$, **$p<0.01$.

Optical density (OD_{600}) readings indicated that *E. coli* 1B04 exhibited stronger growth in LB compared to CNM, in both anaerobic and aerobic conditions (**Table 7.2**).

Table 7.2: Mean absorbance readings of *E. coli* 1B04 when grown in either CNM or LB, containing ~6 mM purified glucoraphanin extract, over 24 hours.

Media	*Conditions*	*Time (hr)*	*Optical density (OD_{600})*
CNM	*Anaerobic*	8	0.297
		12	0.699
		24	0.806
	Aerobic	8	0.298
		12	0.799
		24	0.970
LB	*Anaerobic*	8	0.954
		12	1.279
		24	1.726
	Aerobic	8	0.943
		12	1.180
		24	1.502

Taken together, **Table 7.2** and **Figure 7.6** would seem to suggest that there is a negative correlation between the growth of *E. coli* 1B04 and the degree to which glucoraphanin is reduced. To investigate this relationship, *E. coli* 1B04 was cultured in both CNM and LB, containing ~6 mM purified glucoraphanin extract, over 72 hours in anaerobic conditions.

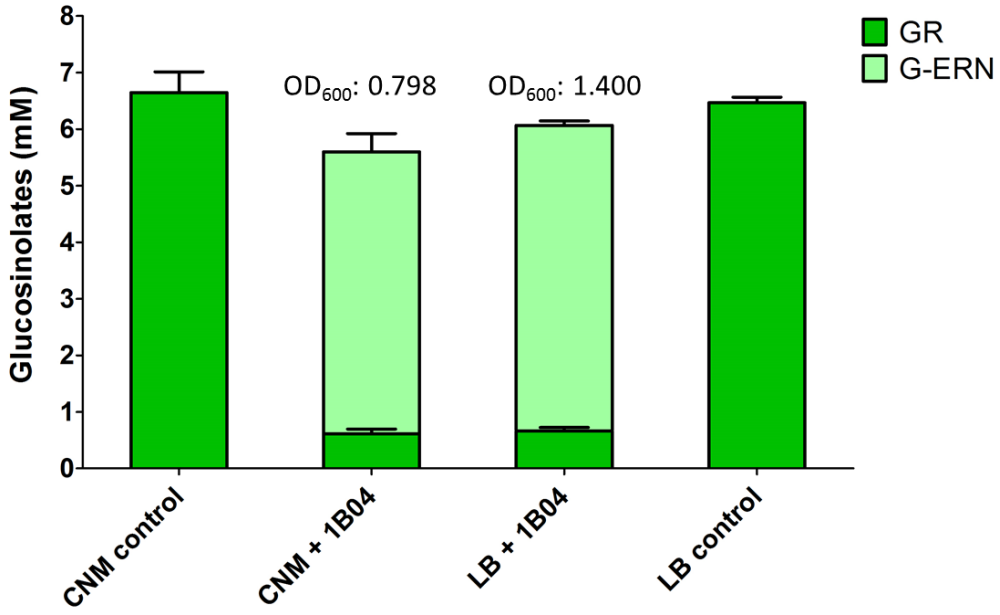


Figure 7.7: Similar levels of glucoraphanin and glucoerucin are observed within both media cultured with *E. coli* 1B04, following an extended period of culturing. *E. coli* 1B04 (5 µl) was cultured in two media (CNM and LB) containing ~6 mM purified glucoraphanin extract media (total volume of 1 ml) for 72 hr at 37˚C, under anaerobic conditions. The control samples consisted of the equivalent media containing ~6 mM purified glucoraphanin extract (total volume of 995 µl) with sterile water (5 µl) added in place of the bacterial inoculum. Glucosinolates were converted to desulphoglucosinolates and measured using HPLC. GR = Glucoraphanin; G-ERN = Glucoerucin; CNM = Chemostat nutrient media; LB = Luria broth; 1B04 = *Escherichia coli* 1B04. Data shown = mean ± SD of two technical replicates.

Extending the culturing period of *E. coli* 1B04 in both CNM and LB, containing ~6 mM purified glucoraphanin extract, from 24 hours to 72 hours resulted in similar levels of glucoraphanin reduction between the media (**Figure 7.7**). There was no statistically significant difference between the levels of glucoraphanin or glucoerucin within the two media, or the total glucosinolate content between the cultured samples and their respective control samples. The OD_{600} values indicated that increased growth of *E. coli* 1B04 was observed when cultured in LB (1.400), compared to CNM (0.798).

7.4.4 Can *Escherichia coli* 1B04 metabolise glucoerucin?

It had been determined that *E. coli* 1B04 was able to reduce glucoraphanin to form glucoerucin. Many bacterial reduction reactions are reversible; therefore a small-scale experiment was performed to investigate whether this strain was able to form glucoraphanin through the oxidisation of glucoerucin.

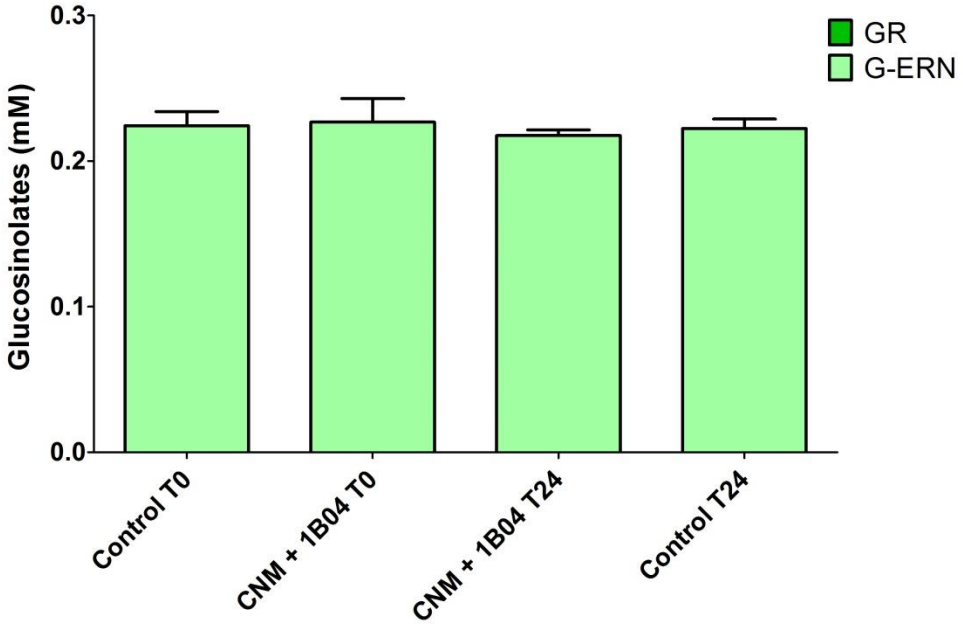


Figure 7.8: Glucoerucin is neither oxidised nor hydrolysed by *E. coli* 1B04. *E. coli* 1B04 (5 µl) was cultured in CNM containing 0.3 mM glucoerucin (total volume of 1 ml) for 24 hr at 37˚C, under anaerobic conditions. The control samples consisted of CNM containing 0.3 mM glucoerucin (995 µl) with sterile water (5 µl) added in place of the bacterial inoculum. Glucosinolates were converted to desulphoglucosinolates and measured using HPLC. GR = Glucoraphanin; G-ERN = Glucoerucin; CNM = Chemostat nutrient media; 1B04 = *Escherichia coli* 1B04; T0 = prior to inoculation; T24 = 24 hr post-inoculation. Data shown = mean ± SD of three technical replicates.

Glucoraphanin was not detected in either the control or test samples, demonstrating that, under the conditions tested, *E. coli* 1B04 did not convert glucoerucin to glucoraphanin via an oxidation reaction (**Figure 7.8**). The levels of glucoerucin in the control and test samples were not statistically significantly different, indicating that *E. coli* 1B04 did not hydrolyse glucoerucin.

7.4.5 Glucoraphanin reduction by *Escherichia coli* DH5α

The *E. coli* strain DH5α was developed as a laboratory cloning strain. *E. coli* DH5α was cultured in CNM containing glucoraphanin to assess whether a laboratory-associated strain of *E. coli* possessed the ability to reduce glucoraphanin.

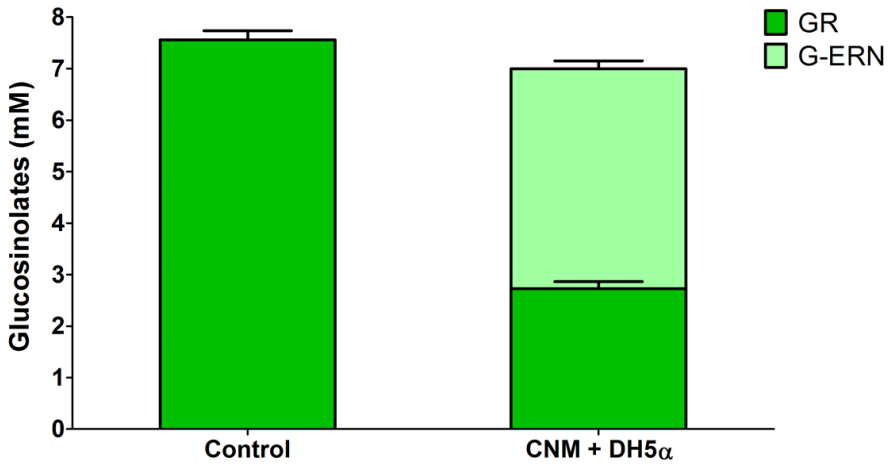


Figure 7.9: The laboratory strain, *Escherichia coli* DH5α, has the ability to convert glucoraphanin to glucoerucin. *E. coli* DH5α (5 µl) was cultured in CNM containing ~7.5 mM purified glucoraphanin extract media (total volume of 1 ml) for 24 hr at 37˚C, under anaerobic conditions. The control sample consisted of CNM containing ~7.5 mM purified glucoraphanin extract (995 µl) with sterile water (5 µl) added in place of the bacterial inoculum. Glucosinolates were converted to desulphoglucosinolates and measured using HPLC. GR = Glucoraphanin; G-ERN = Glucoerucin; CNM = Chemostat nutrient media; DH5α = *Escherichia coli* DH5α. Data shown = mean ± SD of three technical replicates.

Although *E. coli* DH5α grew relatively poorly in the media (OD_{600} = 0.286), this laboratory strain was able to convert glucoraphanin to its reduced analogue, glucoerucin (**Figure 7.9**). CNM was designed to contain the essential nutrients required for the growth of human intestinal bacteria. The weak growth of this strain may reflect the differences in nutrient requirements between gut-adapted *E. coli* and the laboratory strain DH5α. Enhanced growth of *E. coli* DH5α may have been observed if it had been grown in a nutrient rich media, such as LB media, and shaken under aerobic conditions. As *E. coli* DH5α was able to reduce glucoraphanin, this may suggest that the ability of *E. coli* to reduce sulphoxide groups could be a general metabolic process rather than a glucosinolate-specific process.

7.4.6 Is the bacterial sulphoxide reduction specific to glucosinolates?

Sulphoxide-containing compounds can be found in various foods that form part of a normal diet. To investigate whether the observed putative reductase activity was glucoraphanin-specific, *E. coli* 1B04 was cultured in a media containing SMCSO, which is also a broccoli-derived compound that contains a sulphoxide moiety.

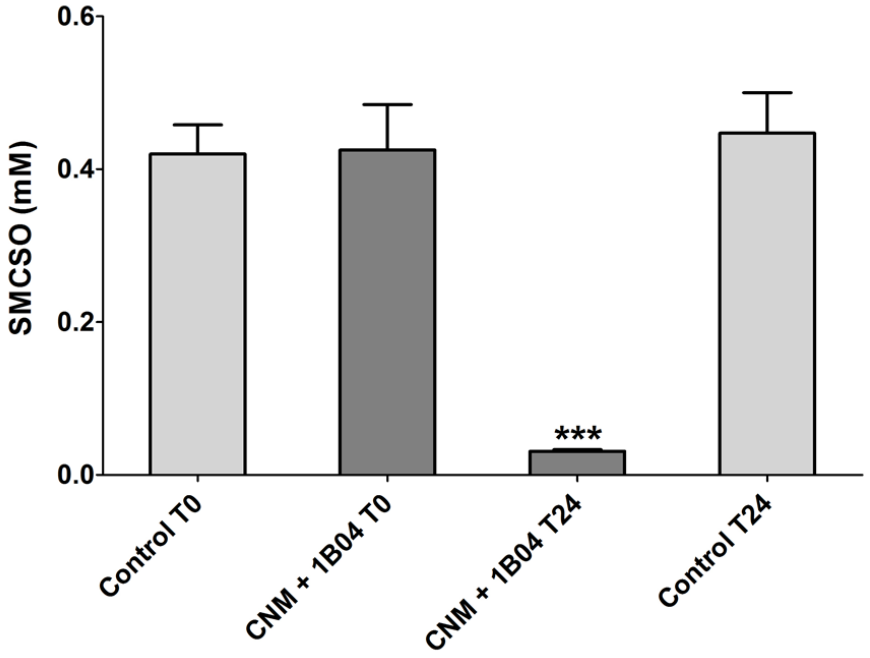


Figure 7.10: Levels of *S*-methylcysteine sulphoxide decreased when incubated with *Escherichia coli* 1B04. *E. coli* 1B04 (5 µl) was cultured in CNM containing 0.3 mM SMCSO (total volume of 1 ml) for 24 hr at 37˚C, under anaerobic conditions. The control samples consisted of CNM containing 0.3 mM SMCSO (995 µl) with sterile water (5 µl) added in place of the bacterial inoculum. Derivatisation of the samples (100 µl) was performed by mixing with a 10 mM dansyl chloride reagent (250 µl) and a 20 mM borate buffer (650 µl). SMCSO derivative analysis was performed using LC-MS. SMCSO = *S*-methylcysteine sulphoxide; CNM = Chemostat nutrient media; 1B04 = *Escherichia coli* 1B04; T0 = prior to inoculation; T24 = 24 hr post-inoculation. Data shown = mean ± SD of three technical replicates.

Levels of SMCSO decreased considerably over a period of 24 hours when exposed to *E. coli* 1B04 (**Figure 7.10**). SMCSO remained stable in the control samples devoid of bacteria, indicating that the lower concentration of SMCSO in the cultured samples was due to the presence of *E. coli* 1B04. A 200 µl aliquot was taken from the remaining supernatant of each of the control samples to form a pooled control sample with a total volume of 600 µl, and the same was performed for the cultured samples. The pooled samples were not derivatised prior to analysis using ^{1}H NMR spectroscopy, due to concerns that the derivatisation reagents may cause significant chemical shifts or severe line broadening.

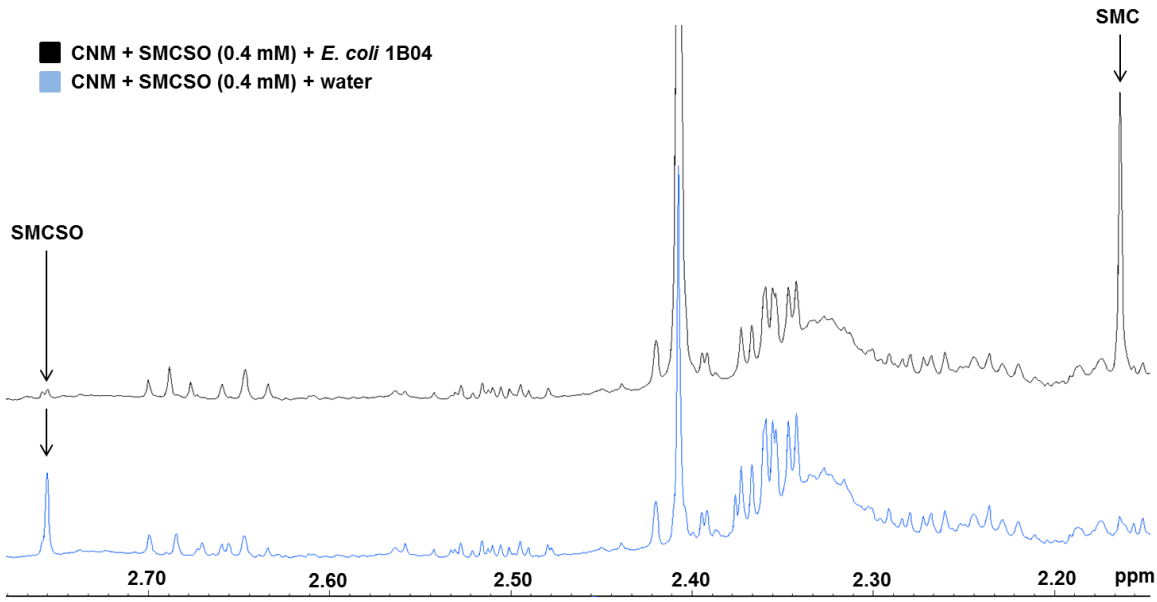


Figure 7.11: Presence of *S*-methylcysteine in the cultured sample confirms *S*-methylcysteine sulphoxide was reduced. *E. coli* 1B04 (5 µl) was cultured in CNM containing 0.3 mM SMCSO (total volume of 1 ml) for 24 hr at 37˚C, under anaerobic conditions. The control samples consisted of CNM containing 0.3 mM SMCSO (995 µl) with sterile water (5 µl) added in place of the bacterial inoculum. Aliquots (600 µl) of the samples were mixed with 70 µl of a 0.4 mM phosphate buffer, and 600 µl was analysed using ^{1}H NMR spectroscopy. SMCSO = *S*-methylcysteine sulphoxide; SMC = *S*-methylcysteine; CNM = Chemostat nutrient media; 1B04 = *Escherichia coli* 1B04.

S-methylcysteine, the reduced analogue of SMCSO, was only detected in the SMCSO-containing CNM sample incubated with *E. coli* 1B04, indicating that SMCSO was reduced by a bacterial-mediated reaction (**Figure 7.11**). In the control sample, SMCSO was present at a concentration of 0.12 mM. The SMCSO peak in the cultured sample was too small to accurately quantify, but *S*-methylcysteine was quantified at 0.11 mM. The concentration of SMCSO is lower than recorded by LC-MS (**Figure 7.10**), and this may indicate loss of these volatile compounds due to the samples not being derivatised prior to analysis.

7.4.7 Detection of metabolic products using gas chromatography mass spectrometry

E. coli 1B04 and DH5α were cultured anaerobically for 24 hours in two media: CNM containing glucoraphanin (~6 mM), and CNM containing SMCSO (0.3 mM). Gas chromatography mass spectrometry (GC-MS) was used to investigate product formation by comparing retention times, mass spectra, and fragment ion fingerprints with the National Institute of Standards and Technology (NIST) library. Compounds common to controls (CNM + water, CNM + substrate, and CNM + bacterium) and test samples (CNM + substrate + bacterium) are omitted from **Table 7.3**, as they likely originate from CNM.

Table 7.3: Metabolic products of glucoraphanin and *S*-methylcysteine sulphoxide, identified using GC-MS, following culturing with two individual strains of *Escherichia coli*. Abbreviations: *E. coli* = *Escherichia coli*; SMCSO = *S*-methylcysteine sulphoxide.

E. coli *strain*	*Substrate*	*Retention time (min)*	*NIST ID*	*Probability (%)*
1B04	Glucoraphanin	*1.9*	Carbon disulphide	94.4
		11.0	Erucin nitrile	89.9
DH5α	Glucoraphanin	*1.9*	Carbon disulphide	51.9
		11.0	Erucin nitrile	96.9
1B04	SMCSO	*1.8*	Methanethiol	96.6
		3.4	Ethanol	78.7
		4.8	Dimethyl disulphide	95.6
		5.8	2-heptanone	59.9
		5.9	3-methyl-1-butanol	55.7
		7.5	Dimethyl trisulphide	58.3
		9.2	1-nonanol	5.0
DH5α	SMCSO	*1.8*	Methanethiol	98.2
		3.4	Ethanol	90.2
		4.4	1-propanol	88.5
		4.8	Dimethyl disulphide	95.6
		5.8	2-heptanone	66.2
		5.9	3-methyl-1-butanol	48.8
		7.5	Dimethyl trisulphide	98.0
		9.2	1-nonanol	17.5

GC-MS analysis indicated that culturing either *E. coli* 1B04 or DH5α in CNM containing glucoraphanin, led to the production of carbon disulphide and, the glucosinolate hydrolysis product, erucin nitrile (**Table 7.3**). As erucin nitrile was only present in the samples containing both glucoraphanin and either *E. coli* strain, this indicates that both

E. coli strains have the ability to degrade glucosinolates. However, only relatively low amounts of glucoraphanin were unaccounted for when each strain was cultured in glucoraphanin-containing media (**Figure 7.6** and **Figure 7.9**), suggesting that glucosinolate reduction was the primary bacterial reaction. This indicates that any glucosinolate hydrolysis performed by these bacterial strains, under the conditions tested, occurred at a relatively low level. When *E. coli* 1B04 or DH5α were cultured in CNM containing SMCSO, a larger range of metabolic products were identified (**Table 7.3**). Except for the presence of a ketone (2-heptanone) these metabolic products could be classified as either alcohols or sulphur-containing compounds.

7.5 Discussion

7.5.1 Identification of *Lactobacillus fermentum*

Chapter 5 details experiments in which culturing human faecal microbiotas in a BL media led to the enrichment of *Lactobacillus* species. Bacteria isolated from these experiments were identified using 16S rDNA sequencing. Analysis of isolates derived from the BL media cultured faecal microbiotas of subjects 1 and 2 led to the identification of all twelve *Lactobacillus* isolates as *Lactobacillus fermentum* (**Table 7.1**). *L. fermentum* is an acid-tolerant, heterofermentative LAB, which has repeatedly been detected in human samples, often found in faecal samples and oral swabs [454]. Several strains of *L. fermentum* have been marketed as probiotics, and are considered to have antimicrobial and antioxidative activities [455-458].

7.5.2 Identification of *Escherichia* species able to reduce glucoraphanin

Ten bacterial isolates were tested for the ability to convert glucoraphanin to the reduced analogue, glucoerucin (**Figure 7.4**). Four of these isolates exhibited putative reductase activity, all of which were identified as members of the genus *Escherichia* (**Table 7.1**). It is interesting to note that the BL media cultured faecal microbiota of subject 2, which did not convert glucoraphanin to glucoerucin, was the only cultured microbiota that did not contain *Escherichia* bacteria at the end of cycle 4. It was determined that the reduced atmosphere of the anaerobic environment did not play a role in the bacterial reduction of glucoraphanin by *E. coli* 1B04 (**Figure 7.5**).

However, the choice of culture media was found to affect the rate at which glucoraphanin was converted to glucoerucin, with lower levels of glucoerucin seen in LB, compared to CNM (**Figure 7.6**). Higher OD_{600} readings were observed when *E. coli* 1B04 was cultured in LB, compared to CNM, indicating stronger growth in LB. This may suggest that the reduction of glucoraphanin was linked to nutrient availability, but a difference in potential co-factors between the media may be a more plausible explanation. It was recently reported that Mg^{2+} and either NAD^+ or $NADP^+$ were necessary for the reduction of sulphoxide-containing glucosinolates by an *E. coli* strain [45]. Nicotinamide and nicotinate were identified following analysis of CNM (**Figure 3.8**), and these compounds can be utilised by bacteria as precursors for the production of NAD^+ or $NADP^+$ [347].

GC-MS analysis of glucoraphanin-containing CNM cultured with *E. coli* 1B04 indicated that erucin nitrile was present in the cultured samples, but absent from the control samples (**Table 7.3**). The data presented in this chapter indicates that the reduction of glucoraphanin, rather than glucosinolate hydrolysis, was the primary metabolic activity

of *E. coli* 1B04. As the hydrolysis of glucoerucin was not observed (**Figure 7.8**), the presence of erucin nitrile indicates that this bacterial strain likely has the ability to hydrolyse glucoraphanin, and may have produced erucin nitrile through the subsequent reduction of SF or SF nitrile. The lack of glucoerucin hydrolysis may suggest that the reduction reaction itself, rather than the reduced product, was advantageous to *E. coli* 1B04. Work performed by Simala-Grant and Weiner showed that the anaerobic growth of *E. coli*, on various sulphoxides, was only supported following the reduction of these compounds by the DmsABC reductase [310]. This enabled the sulphoxides to act as electron acceptors, which can be used by *E. coli* to generate energy to drive ATP synthesis through anaerobic respiration. Therefore, the difference in the rate of glucoraphanin reduction observed in **Figure 7.6** may reflect the presence of electron acceptors in the LB media that were preferred to the sulphoxide, which were reduced by other enzymes.

E. coli can express a wide range of reductases for a variety of activities, such as terminal reductases used in anaerobic respiration [439], methionine sulphoxide reductases which counteract damage that can be caused by reactive oxygen species [314, 315], the reduction of xenobiotics [318], and ribonucleotide reductases which are involved in the synthesis of DNA [316, 317]. In a study performed by Lee and Renwick, the authors postulated that *E. coli* may possess at least five cytosolic enzyme systems capable of reducing sulphoxide-containing drugs, such as sulindac [318]. As the laboratory strain *E. coli* DH5α was able to reduce glucoraphanin (**Figure 7.9**), this suggests that the enzyme responsible for the glucosinolate reduction may be present in all *E. coli* strains. It should be considered that this reduction reaction may be possible through the activity of more than one reductase. It is not unreasonable to conclude that sulphoxide reduction in anaerobic environments may provide electron acceptors for

anaerobic respiration, whilst similar reductase activity in an aerobic environment may serve to protect *E. coli* from oxidative damage.

7.5.3 Metabolism of *S*-methylcysteine sulphoxide

To test the hypothesis that the observed glucoraphanin reduction was not glucosinolate-specific, *E. coli* 1B04 was cultured in CNM containing SMCSO. A dramatic decrease in the levels of SMCSO was observed in the presence of *E. coli* 1B04, when the culture supernatants were analysed by LC-MS (**Figure 7.10**). ^{1}H NMR analysis indicated that a large proportion of SMCSO was converted to the reduced analogue, *S*-methylcysteine, by *E. coli* 1B04 (**Figure 7.11**). GC-MS analysis of SMCSO-containing CNM incubated with *E. coli* 1B04 or *E. coli* DH5α, highlighted multiple metabolic products derived from SMCSO due to the activity of the *E. coli* strains (**Table 7.3**). Amongst these products were sulphur-containing compounds, which are indicative of SMCSO degradation (**Figure 7.1**). SMCSO is degraded in plants through the action of cysteine lyases [442, 443]. Multiple gastrointestinal bacteria, including members of the human gut microbiota, have been shown to exhibit cysteine β-lyase activity, which cleaves C-S bonds of *S*-alkyl-cysteine molecules in a similar manner to the plant cysteine lyases [164-166, 444-448]. The reduction of SMCSO to *S*-methylcysteine appears to have been the primary reaction mediated by *E. coli* 1B04, however the degradation of SMCSO also occurred, to some degree, when cultured with both *E. coli* strains. Although it is possible that glucoraphanin and SMCSO were reduced by the actions of different enzymes, it is likely that the activity of a single enzyme with broad substrate specificity is responsible for the sulphoxide reductions of both of these compounds (**Figure 7.12**).

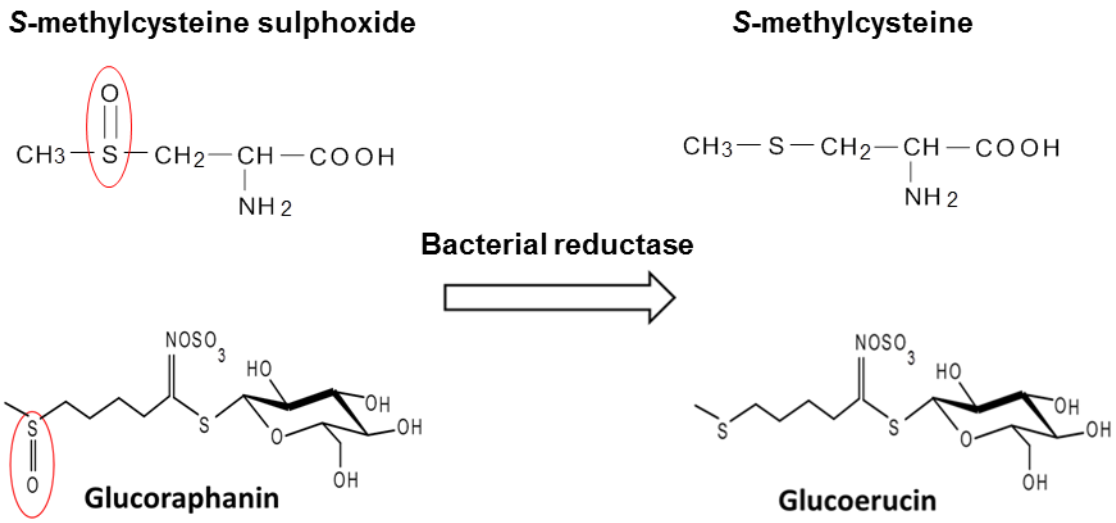


Figure 7.12: Schematic indicating the bacterial reduction of *S*-methylcysteine sulphoxide and glucoraphanin. The sulphoxide moieties (circled) on SMCSO and glucoraphanin can be reduced by human intestinal bacteria to produce the reduced analogues, *S*-methylcysteine and glucoerucin, respectively.

7.6 Conclusions

Lactobacillus isolates, obtained from culturing two human faecal microbiotas in BL media, were identified as *L. fermentum*, a known human intestinal bacterium. *L. fermentum* isolates were unable to reduce glucoraphanin, but four *Escherichia* isolates did exhibit the ability to convert glucoraphanin to glucoerucin. Further analysis was focused on *E. coli* 1B04, and it was determined that the bacterial reduction of glucoraphanin occurred in both aerobic and anaerobic environments. The rate of glucoraphanin reduction was found to be affected dependent on the media used for culturing the bacteria, and this may have been linked to nutrient, co-factor, or electron acceptor availability. There were no indications that *E. coli* 1B04 was able to further metabolise glucoerucin, and it may be that the sulphoxides are used as electron acceptors as part of anaerobic respiration. The laboratory strain, *E. coli* DH5α, was also able to reduce glucoraphanin, which may suggest that this reaction is common amongst all *E. coli* strains. *E. coli* 1B04 was found to also have the ability to convert SMCSO to its reduced analogue, *S*-methylcysteine, but it is unclear whether the reduction of both SMCSO and glucoraphanin is by the action of a single enzyme. GC-MS analysis detected breakdown products of both glucoraphanin and SMCSO, but it appears that the reduction of these compounds is the primary metabolic reaction, under the conditions tested.

CHAPTER EIGHT

8 Human intervention study investigating the effects of a low- and high-*Brassica* diet on the gut microbiota

8.0 Summary

*Although the human gut microbiota is relatively stable, short-term dietary interventions have been shown to alter the microbiota composition. This chapter presents the results of a human intervention study designed to investigate the effects of a low- and high-*Brassica *diet on the composition and metabolic activity of the human gut microbiota. Variation between microbiotas was observed to be larger than variation within microbiotas across time, as has been reported in other dietary studies. Statistical analysis identified ten bacterial taxa significantly associated (*p<*0.05) with an increase in relative proportions following the low-*Brassica *diet, and a proportional decrease after the high-*Brassica *diet, compared to proportions present prior to the respective diet phase. The association of five of these taxa to the* Brassica *diets were strongly significant (p<0.01), and these were identified as members of the* Rikenellaceae *(*Bacteroidales*),* Ruminococcaceae *(*Clostridiales*),* Mogibacteriaceae *(*Clostridiales*),* Clostridium *(*Clostridiales*), and an unclassified* Clostridiales*. No associations between the faecal metabolite profiles and the* Brassica *diets were observed, and the results of the urinary excretion of glucoraphanin hydrolysis products were inconclusive.*

8.1 Introduction

Studies indicate that although humans share a core gut microbiota, there is a greater amount of variation in community composition between individuals than within individuals across time [181, 202, 372, 459, 460]. The structure of the gut microbiota is largely formed during early life, and remains relatively stable for the majority of adult life [461]. Although stable, the structure of the microbiota exhibits a relatively large degree of plasticity, which allows for the adaptation to its environment [236, 459, 462, 463]. Small fluctuations in the abundances of different bacteria can enable the efficient

extraction of nutrients from various dietary compounds. Short-term dietary interventions have illustrated the effects of diet on the composition of the human gut microbiota [254, 401, 408, 464-467]. Diet also affects the production and utilisation of metabolites in the intestines, and this can be monitored through analysis of biological material, such as urine and stools [468-470].

A study by Li *et al* investigated the effects of cruciferous vegetables on the human gut microbiota through a controlled diet devoid of other vegetables, fruits, whole-grains, and high-fibre foods [134]. Bacteria putatively assigned as *Eubacterium hallii*, *Phascolarctobacterium faecium*, *Alistipes putredinis*, and *Eggerthella* sp. were reported to be associated with the consumption of cruciferous vegetables, however the nature of the association was unexplained. Studies investigating the urinary excretion of glucosinolate hydrolysis products, as markers of microbial glucosinolate metabolism, observed variation between individuals, with recovery ranging from approximately 1 – 45% of the initial dose of glucosinolates [111-114]. One study in which participants were retested over a period of two and a half years also reported intra-individual variation amongst urinary excretion results over time [111].

8.2 Objectives

The hypothesis for the work contained in this chapter was that a low- and/or high-*Brassica* diet would alter the gut microbiota composition. It was also hypothesised that changes to the proportions of gut lactobacilli would be observed, based on the *in vitro* work in chapter 5. It was considered that these dietary interventions would alter the microbial metabolite profile, and that the excretion of glucoraphanin hydrolysis products in the urine would differ between individuals.

8.3 Materials and methods

Ten participants were recruited onto the 'Effects of *Brassica* on human gut lactobacilli' (EBL) study, which investigated the differential effects of consuming a low- or high-*Brassica* diet on the composition of the gut microbiota, and the proportions of intestinal lactobacilli (section 2.13). The two-phase crossover study design is summarised in **Figure 8.1**, and details of the study participants are compiled in **Table 8.1**. Analysis of the *Brassica* foods used in the diets was performed using HPLC (section 2.5). ^{1}H NMR spectroscopy analysis (section 2.6.2) was performed on faecal waters to investigate changes to microbial metabolic profiles associated with the *Brassica* diets (section 2.13.3.2). Urine samples were collected to act as indicators of compliance to the dietary restrictions, and 24 hr urine collections were used to assess differences in urinary glucoraphanin hydrolysis product excretion rates between and within participants. All urine samples were prepared as described in section 2.13.3.3, prior to analysis via LC-MS/MS (section 2.4.1). The bacterial composition of faecal samples were determined using 16S rDNA paired-end sequencing (2x 250 bp) on the Illumina MiSeq platform, and bioinformatic analyses were performed using the QIIME pipeline (section 2.13.3.1). The data was modelled using multivariate analysis, prior to statistical analysis by sequential analysis of variance (section 2.14).

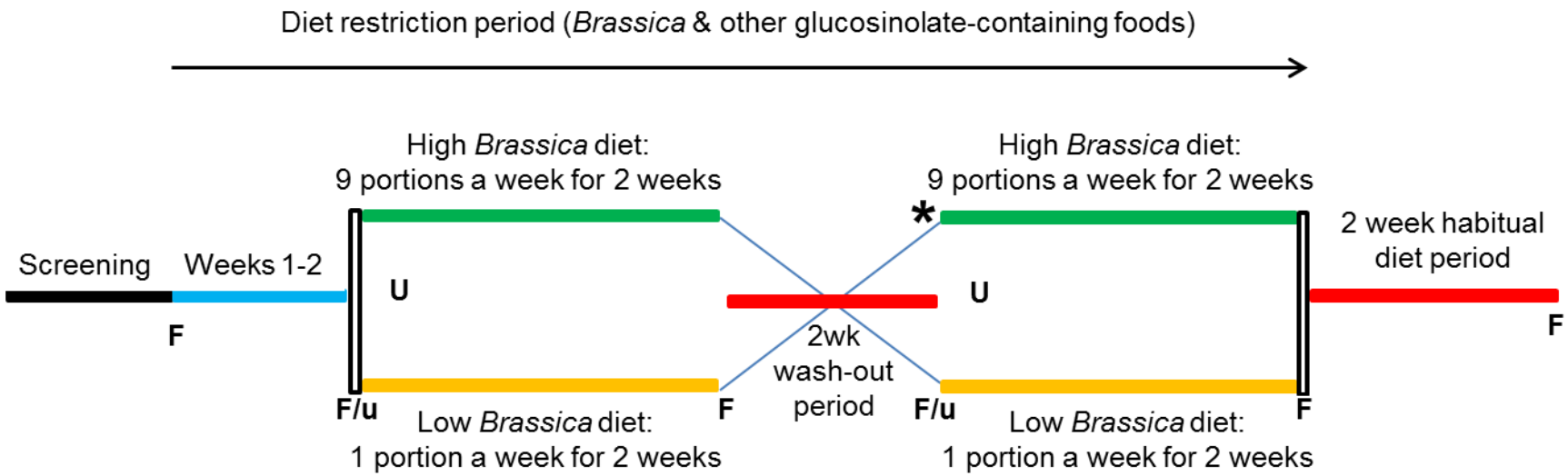


Figure 8.1: Design for the EBL human dietary intervention study. Randomised, two phase crossover, human dietary intervention study: *n* = 10. Following a successful eligibility screening appointment, participants produced a faecal sample before commencing a *Brassica* and glucosinolate-containing food diet restriction. After 2 weeks, a faecal sample (**F**) and urine (**u**) sample were collected, and the first 2 week intervention phase started (low- or high-*Brassica* diet). All urine was collected for 24 hr (**U**) directly after consuming the first portion of broccoli or broccoli-based soup. Participants were restricted from eating further portions until the urine collection was completed. Upon conclusion of the first intervention phase, a faecal sample was collected, and the 2 week washout period began. A further faecal sample and urine sample were collected on completion of the washout period, and the second 2 week intervention phase started (alternative diet to that consumed in intervention phase 1). All urine was collected for 24 hr directly after consuming the first portion of broccoli or broccoli-based soup. Participants were restricted from eating further portions until the urine collection was completed. Upon conclusion of the second intervention phase, a faecal sample was collected and the diet restriction period ceased. After 2 weeks consuming their habitual diet, participants collected a final faecal sample, completing the study. During each intervention phase, and the habitual diet phase, stool charts and food diaries were completed for a period of seven consecutive days. * At the end of the 2 week wash-out period, 8 participants were granted a short break from the study, for a period of no more than 6 weeks, to accommodate the Christmas period. Participants were required to resume the dietary restrictions for 2 weeks prior to producing an additional faecal sample, and starting the second intervention phase.

Table 8.1: Age, gender, Body Mass Index (BMI), and smoking status of the EBL study participants.

Participant code	*Age (years)*	*Gender*	*BMI (kg/m^2)*	*Smoker*
EBL16	35	Male	28.5	N
EBL28	25	Female	23.5	N
EBL51	25	Female	24.8	N
EBL56	32	Female	21.1	N
EBL74	39	Female	20.3	N
EBL84	40	Male	24.4	N
EBL88	36	Male	26.0	N
EBL89	41	Female	25.3	Y
EBL92	28	Male	27.5	N
EBL97	34	Female	23.8	N

8.4 Results

8.4.1 Analysis of the *Brassica* diet

Participants were asked to consume a total of seven 84 g portions of cauliflower, seven 84 g portions of broccoli, and six 300 g portions of broccoli and sweet potato soup (manufactured using 84 g of broccoli per portion) across the two dietary intervention phases. The soups were manufactured by Bakkavor for use in the ESCAPE study (REC ref: 13/EE/0110), performed at the IFR, and had been shown to be a rich source of glucoraphanin. Each of the portions were analysed for total glucosinolate content using HPLC (**Table 8.2** and **Table 8.3**).

Table 8.2: Mean µmoles of glucosinolates per 84 g portion of steamed cauliflower from 5 different batches. Refer to page 4 and 5 (section 1.1.1) for glucosinolate structures and semi-systematic names.

Batch code	*Cauliflower - glucosinolates (mean µmoles/84 g cooked portion)*									
	Glucoiberin	**Gluconasturtiin**	**Progoitrin**	**Glucoraphanin**	**Sinigrin**	**Glucoiberverin**	**Glucobrassicin**	**4-MIND**	**4-methoxy glucobrassicin**	**Total**
DF4217 13:58	1.00	7.09	0.79	0.80	7.68	8.24	22.56	1.30	3.93	**53.38**
DF4217 13:59	1.04	6.22	0.19	0.94	7.17	8.80	10.63	1.20	1.39	**37.59**
DF4217 07:09	1.03	7.81	2.81	0.93	11.10	3.37	32.00	1.79	4.81	**65.68**
DF4217 16:28	0.94	13.23	2.75	1.40	12.90	2.35	20.72	1.13	1.92	**57.34**
DF4217 12:56	0.92	8.25	1.97	1.40	6.55	1.78	33.29	1.64	3.71	**59.50**

Table 8.3: Mean µmoles of glucosinolates per 84 g portion of steamed broccoli from 3 different batches, and 300 g portion of a single batch of the microwaved broccoli and sweet potato soup. Refer to page 4 and 5 (section 1.1.1) for glucosinolate structures and semi-systematic names.

Batch code	*Broccoli based foods - glucosinolates (mean µmoles/cooked portion)*						
	Glucoiberin	**Glucoraphanin**	**Glucobrassicin**	**4-MIND**	**4-methoxyglucobrassicin**	**4-hydroxyglucobrassicin**	**Total**
Broccoli - DF4231 18:18	7.91	65.66	28.50	4.63	9.31	1.44	**117.46**
Broccoli - DF4231 20:37	7.71	61.64	21.13	2.31	6.55	1.45	**100.80**
Broccoli - DF4232 15:55	9.17	53.61	25.30	4.33	8.85	2.02	**103.29**
Soup – 302 (3)	93.79	158.98	13.18	2.47	20.04	0	**288.46**

The cauliflower portions were found to contain a relatively high level of glucobrassicin (**Table 8.2**), but fewer total μmoles of glucosinolates than present in the broccoli portions. The most prevalent glucosinolate in the broccoli portions and the broccoli-based soup was glucoraphanin, with the soup containing approximately 2.5-fold higher levels than the frozen broccoli (**Table 8.3**). The increased glucoraphanin levels in the broccoli-based soup is likely associated with the broccoli being cultivated in Spain under optimal growing conditions, with high quality fresh broccoli florets used to generate the soup. The frozen broccoli florets were grown in England, and purchased from a Sainsbury's superstore.

8.4.2 Urinary glucoraphanin hydrolysis product profiles

Each participant collected their urine for 24 hours following consumption of the first portion, of either the broccoli or the broccoli and sweet potato soup, for each of the *Brassica* diet phases. The urine was analysed for glucoraphanin hydrolysis products using LC-MS/MS, and the total percentage excreted was calculated (**Figure 8.2**).

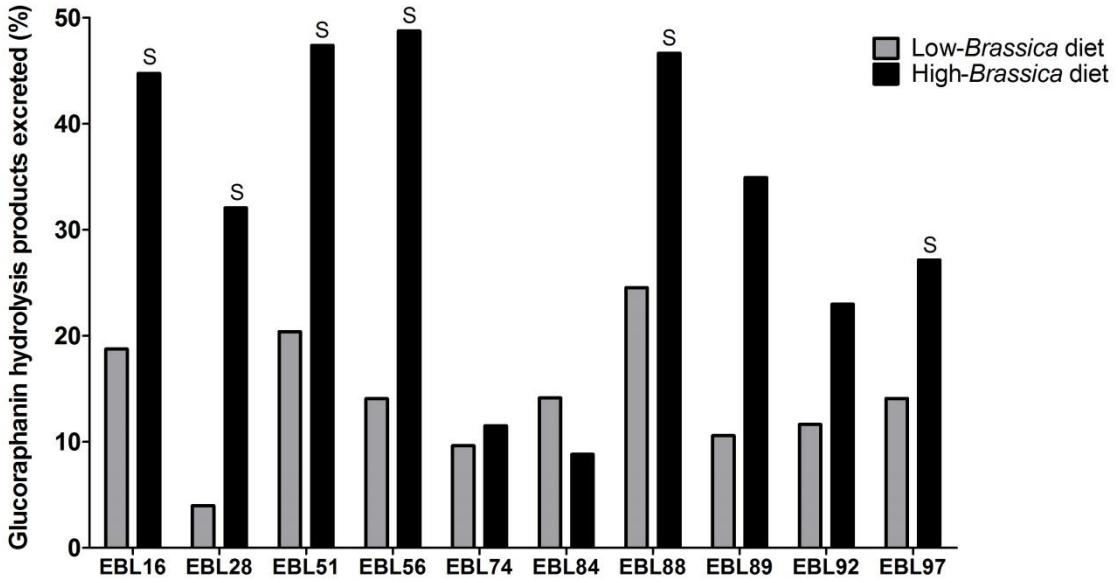


Figure 8.2: Differences in urinary glucoraphanin hydrolysis product excretion was observed between and within individuals. Ten participants collected their urine for 24 hr after eating the first portion of broccoli or the broccoli and sweet potato soup. Aliquots of urine were filtered (0.2 μm), and analysed using LC-MS/MS. Total percentage of glucoraphanin hydrolysis products were calculated based on the glucosinolate analysis of the dietary foods (**Table 8.3**). S signifies that the urine was collected following consumption of the broccoli-based soup, all other data was collected following consumption of steamed broccoli.

A general trend in 8 of 10 participants was observed for a higher percentage of glucoraphanin hydrolysis products to be excreted in the urine following consumption of the first portion of the high-*Brassica* diet (**Figure 8.2**). The urine obtained from the remaining two participants (EBL74 and EBL84) show a similar percentage of excreted glucoraphanin hydrolysis products following consumption of the first portion of either *Brassica* diet.

8.4.3 Proton nuclear magnetic resonance spectroscopy analysis of metabolite profiles in faecal waters

In total 68 faecal samples were collected from the 10 participants over the duration of the study. Each of the 68 faecal samples were homogenised in a 0.4 mM phosphate buffer to produce faecal waters (section 2.13.3.2). ^{1}H NMR spectroscopy analysis was performed on the supernatant of the centrifuged faecal waters to detect *Brassica* diet-induced changes to the metabolite profile.

8.4.3.1 Variation in metabolite profiles due to portion sampling of faecal material

It was decided to ascertain whether a single aliquot taken from a faecal sample was representative of the metabolite profile of the entire sample. Two aliquots of 54 faecal samples were taken from random positions of each sample and used to generate two separate faecal waters. The two faecal sample replicates of the 54 faecal samples were analysed using ^{1}H NMR spectroscopy to identify the level of repeatability between different areas of the faecal sample (**Figure 8.3**).

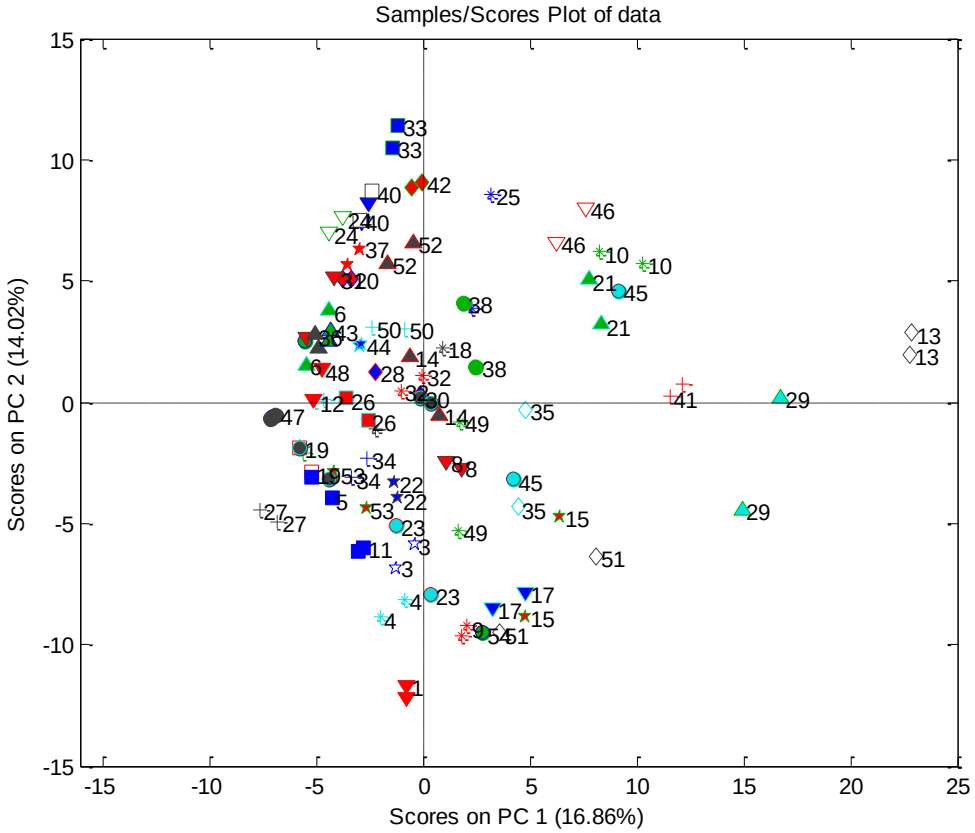


Figure 8.3: Different areas of faecal samples are representative of one another regarding the metabolite profile. Two aliquots were taken from random positions of 54 faecal samples, homogenised in a 0.4 mM phosphate buffer, and analysed using ^{1}H NMR spectroscopy. Each different number (1 – 54) represents one of the 54 faecal samples, and data-points with the same number denote replicates. The plot was generated using Matlab.

Strong repeatability was seen between the two replicates of each of the 54 faecal samples, indicating that a single aliquot of a faecal sample is likely representative of the metabolite profile of the entire sample. As such, it was not deemed necessary to analyse the remaining 14 samples for portion sampling effects.

8.4.3.2 Effects of Brassica *diets on metabolite profile*

The metabolite profiles of the 68 faecal waters showed a tendency to cluster based on the participant, indicating a low level of intra-individual variability over time (**Figure 8.4**).

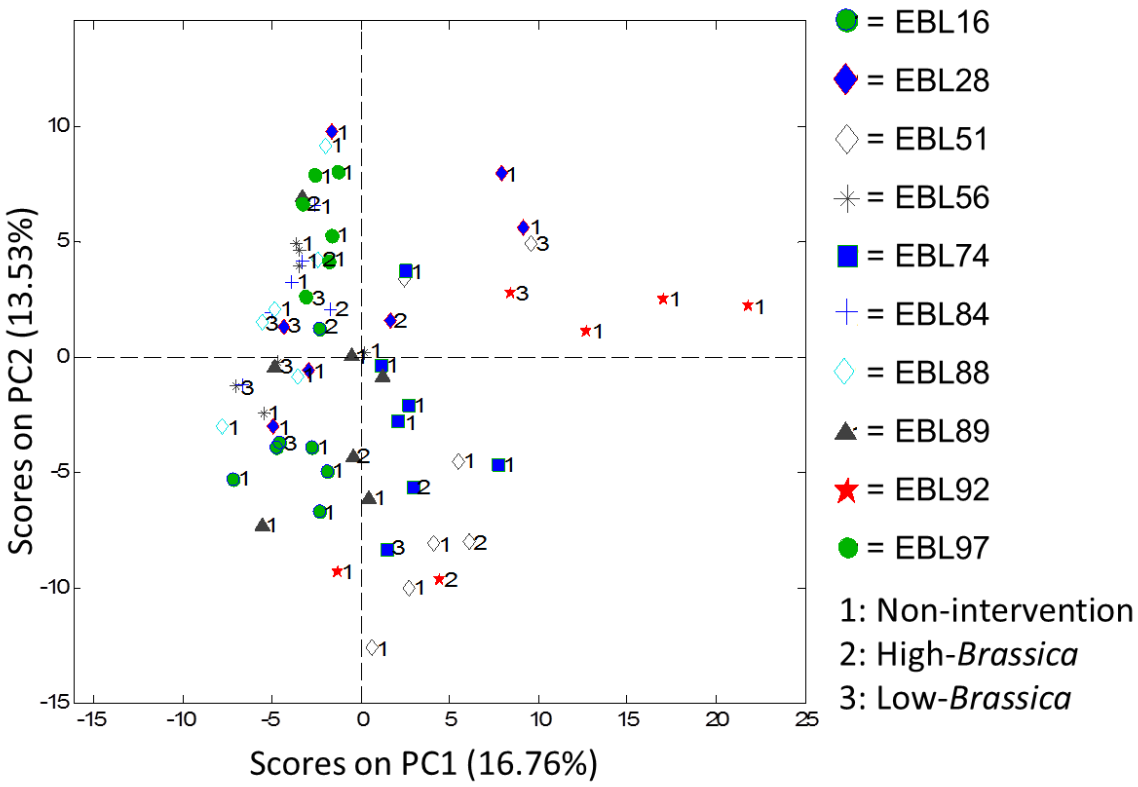


Figure 8.4: Between-individual variation of the metabolite profile is generally stronger than the within-individual variation over time. An aliquot of 68 faecal samples were each homogenised in a 0.4 mM phosphate buffer, and analysed using ^{1}H NMR spectroscopy. The ten symbols represent the ten participants (as denoted in the key), and the numbers (1 – 3) indicate the type of sample: 1 = non-intervention faecal sample, 2 = faecal sample after the high-*Brassica* diet, 3 = faecal sample after the low-*Brassica* diet. The plot was generated using Matlab.

No association between the low- or high-*Brassica* diets and the faecal metabolite profile were detected on the first 10 principal components. **Figure 8.5** shows the first two principal components.

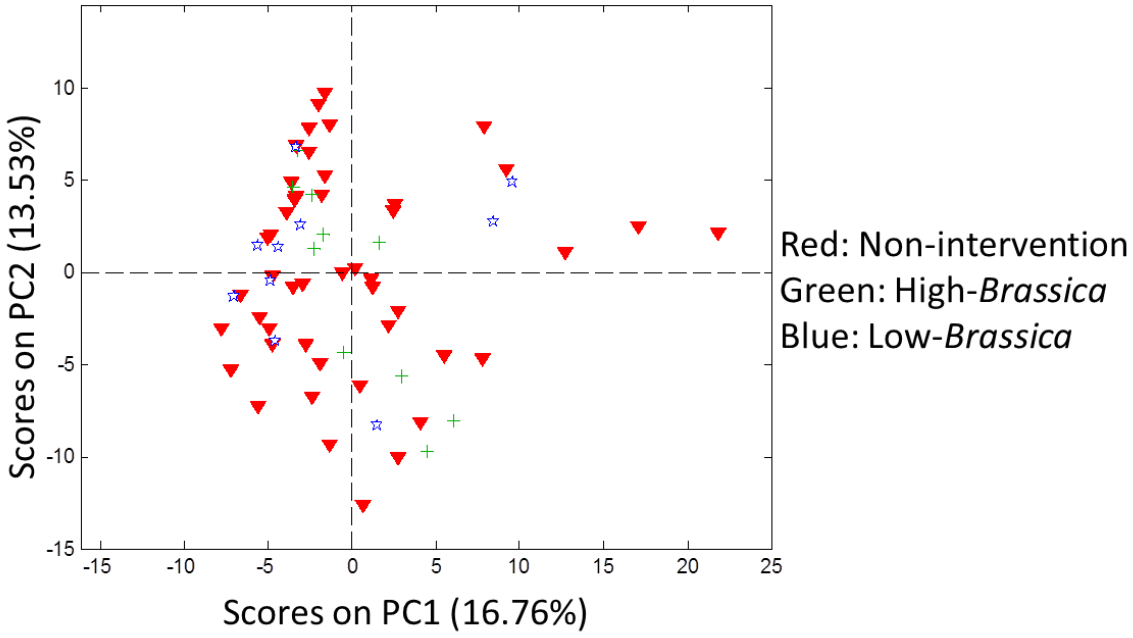


Figure 8.5: The *Brassica* diets were not associated with significant changes to the faecal metabolite profile. An aliquot of 68 faecal samples were each homogenised in a 0.4 mM phosphate buffer, and analysed using ^{1}H NMR spectroscopy. Red triangles = non-intervention faecal sample, green crosses = faecal sample after the high-*Brassica* diet, blue stars = faecal sample after the low-*Brassica* diet. The plot was generated using Matlab.

8.4.4 Community analysis of human faecal microbiotas

Faecal samples were collected during the study from 10 healthy participants, aged 25 – 41 years with a mean age of 34 years, and an average BMI of 24.5 kg/m^2. The DNA was extracted (section 2.9.1), and visualised and quantified (section 2.9.1.1), prior to normalisation to 1 ng/µl (section 2.13.3.1). The normalised DNA samples were sent to the Centre of Genomic Research (Liverpool, UK), where variable regions of the 16S rDNA gene were amplified using PCR, and sequenced using the paired-end Illumina MiSeq platform, for downstream analysis using the QIIME pipeline (section 2.13.3.1). This produced 6,503,134 high-quality reads, with an average of 95,634 ± 28,729 reads per sample, which clustered into 21,563 operational taxonomic units at 97% identity.

No associations were observed between the consumption of either of the *Brassica* diets and the relative proportions of *Lactobacillus* (≤0.2% in all samples). Unweighted (**Figure 8.6**) and weighted (**Figure 8.7**) beta-diversity analysis suggested that the consumption of a *Brassica*-rich (high) diet or a *Brassica*-poor (low) diet did not lead to a strong alteration of the faecal microbiotas towards a common composition.

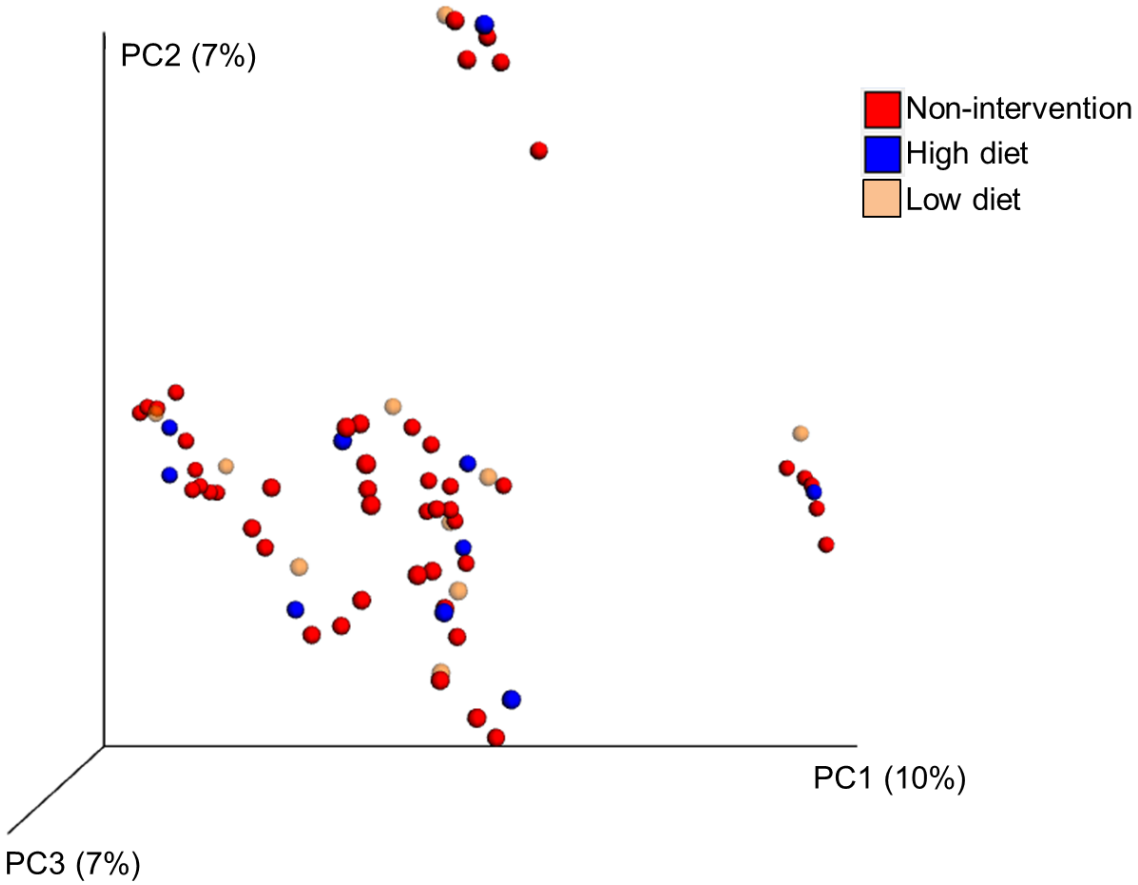


Figure 8.6: Consumption of the *Brassica* diets did not correlate with a common gut microbiota composition. Unweighted beta-diversity analysis of faecal microbiotas from ten study participants; each participant collected 4 – 5 non-intervention samples, a single sample following a 2 week low-*Brassica* diet, and a single sample following a 2 week high-*Brassica* diet. Analysis was performed using the UniFrac metric, and visualised as a 3D PCoA plot. Beta-diversity analysis was performed using QIIME 1.8.0, and plotted using Emperor.

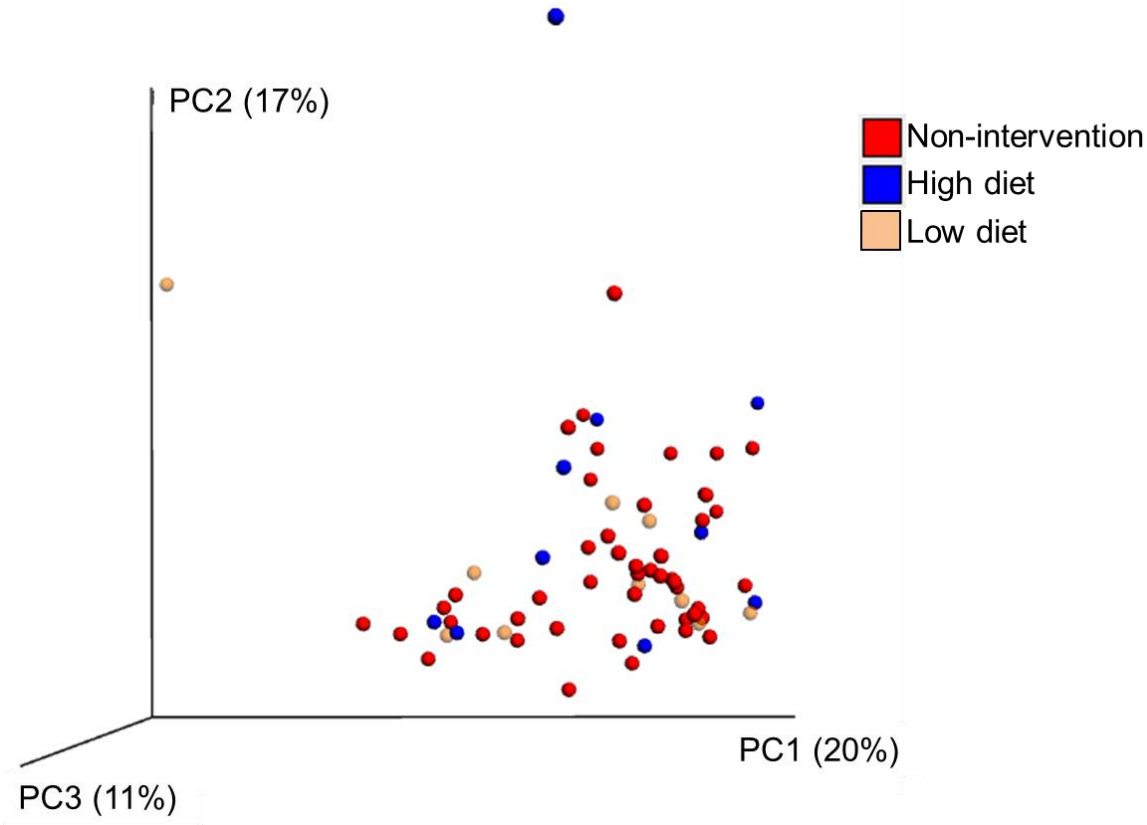


Figure 8.7: No association between gut microbiota composition and *Brassica* diets was observed when accounting for bacterial relative abundance. Weighted beta-diversity analysis of faecal microbiotas from ten study participants; each participant collected 4 – 5 non-intervention samples, a single sample following a 2 week low-*Brassica* diet, and a single sample following a 2 week high-*Brassica* diet. Analysis was performed using the UniFrac metric, and visualised as a 3D PCoA plot. Beta-diversity analysis was performed using QIIME 1.8.0, and plotted using Emperor.

Performing the unweighted (**Figure 8.8**) and weighted (**Figure 8.9**) beta-diversity analysis with samples colour-coded for each participant, indicated that the samples obtained from each individual clustered together.

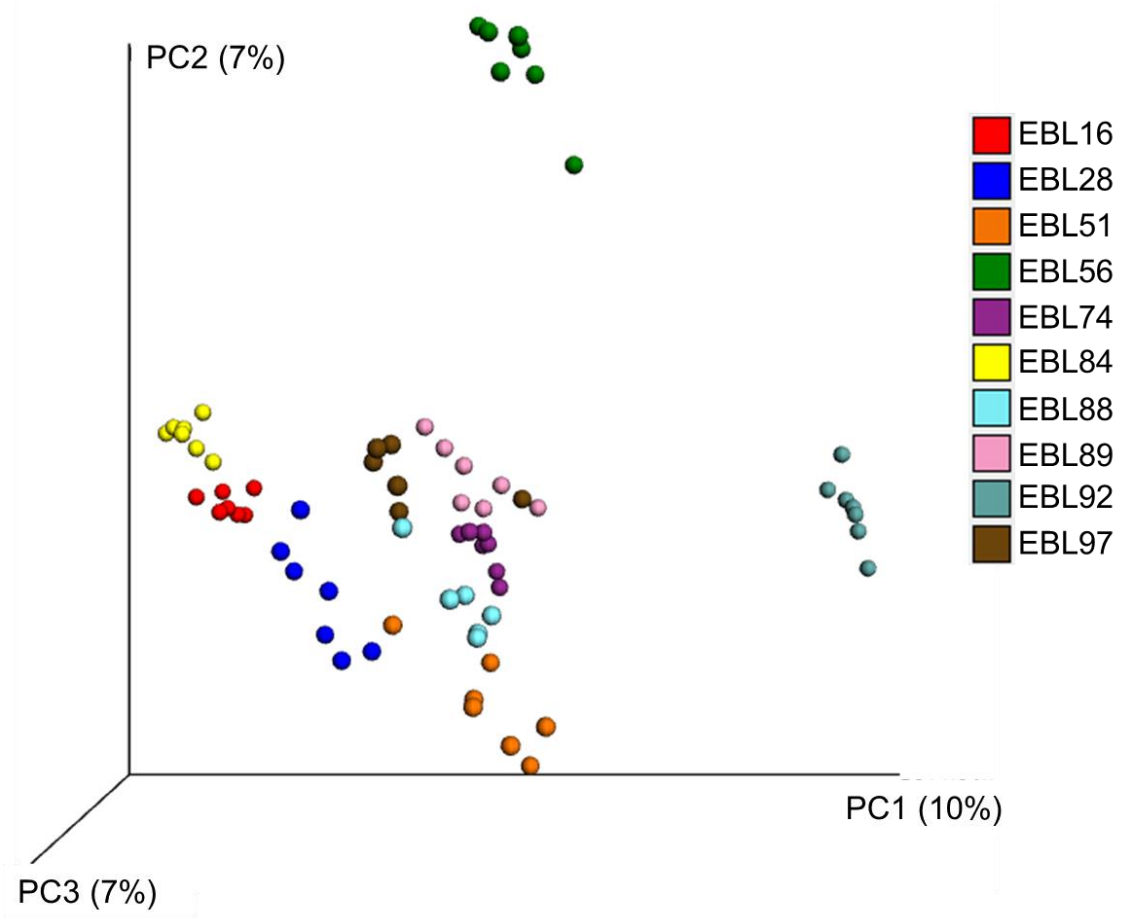


Figure 8.8: Unweighted beta-diversity analysis shows clustering of faecal microbiotas based on the individual. Unweighted beta-diversity analysis of faecal microbiotas from ten study participants; each participant collected 4 – 5 non-intervention samples, a single sample following a 2 week low-*Brassica* diet, and a single sample following a 2 week high-*Brassica* diet. Analysis was performed using the UniFrac metric, and visualised as a 3D PCoA plot. Beta-diversity analysis was performed using QIIME 1.8.0, and plotted using Emperor. The EBL code in the key refers to different participants.

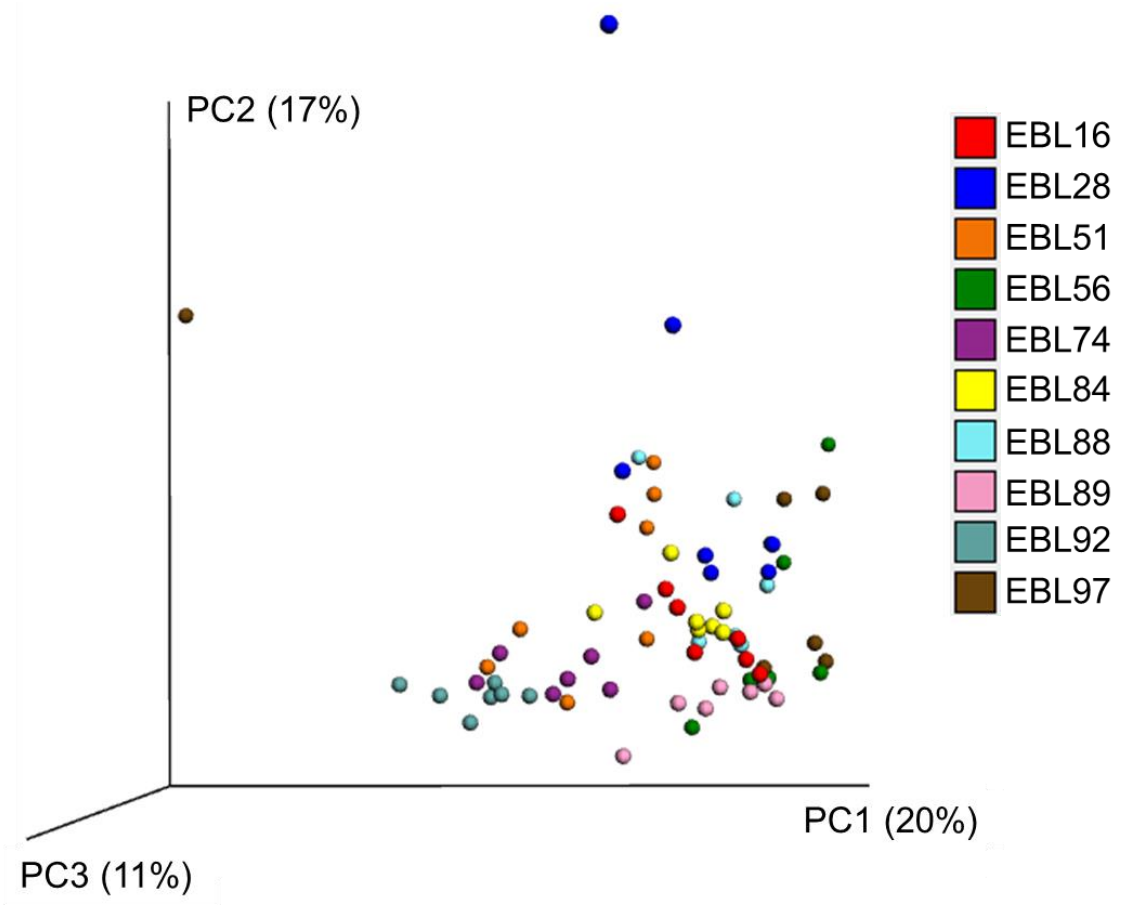


Figure 8.9: Increased variation within individuals is observed when relative abundance of bacteria within the microbiotas is considered. Weighted beta-diversity analysis of faecal microbiotas from ten study participants; each participant collected 4 – 5 non-intervention samples, a single sample following a 2 week low-*Brassica* diet, and a single sample following a 2 week high-*Brassica* diet. Analysis was performed using the UniFrac metric, and visualised as a 3D PCoA plot. Beta-diversity analysis was performed using QIIME 1.8.0, and plotted using Emperor. The EBL code in the key refers to the 10 different participants.

The unweighted beta-diversity analysis calculates the similarities between samples, based on which bacterial taxa are shared amongst the microbiotas. **Figure 8.8** suggests that the greatest degree of similarity between faecal microbiotas can be found amongst the samples that originated from each individual. The faecal microbiotas of EBL56 and EBL92 would appear to differ from one another, and are less similar to the microbiotas of the other eight participants, due to a lower number of shared bacterial taxa.

Weighted beta-diversity analysis considers the similarity between the relative abundance of the bacterial taxa, alongside which taxa are shared between microbiotas. **Figure 8.9** indicates an increased variability amongst the faecal microbiotas of individuals due to small fluctuations in the relative abundance of bacterial taxa. The effects of relative abundance are seen most strongly for the samples obtained from EBL28 and EBL97. Alpha-diversity analysis was performed to identify whether the variation observed for the samples from EBL28 and EBL97 were true indicators of natural fluctuations in the microbiota, or artifacts that reflect poor sequencing output (**Figure 8.10**).

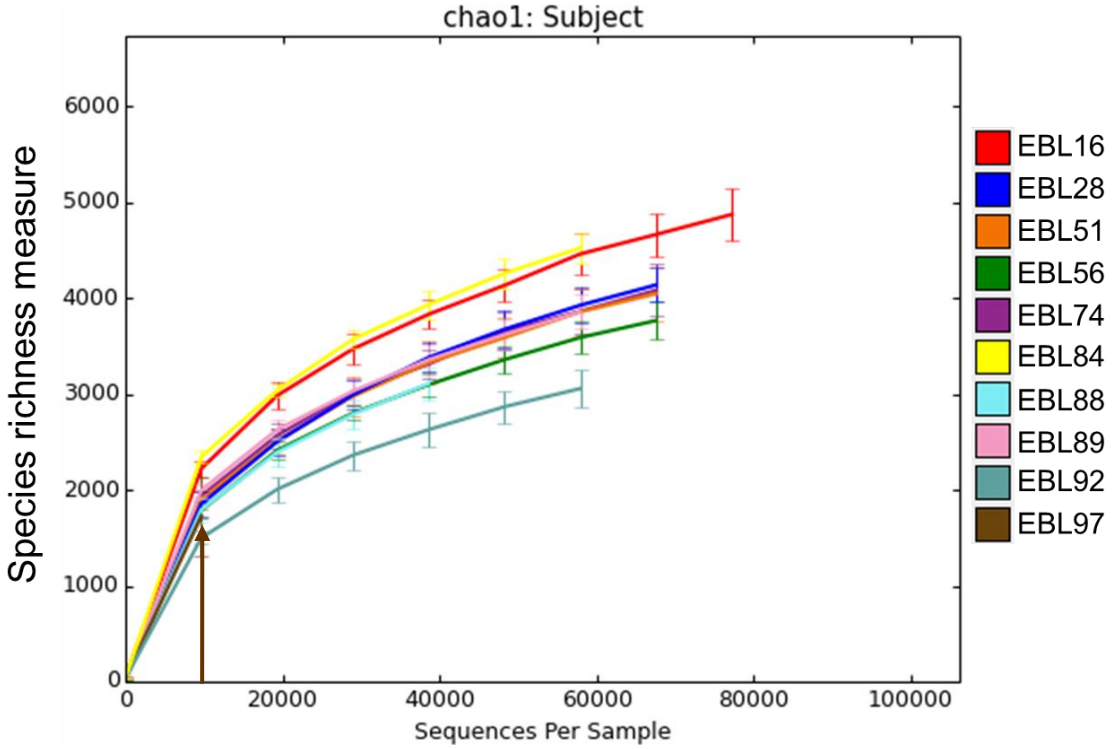


Figure 8.10: Alpha-diversity analysis indicates that at least one sample obtained from EBL97 contains poor sequencing data. Alpha-diversity rarefaction plot of species richness within the human faecal microbiota samples grouped for each of the ten individuals. The y-axis is a measure of diversity within each community, whilst the x-axis represents the number of sequences used in the diversity calculation. Rarefaction plots were generated using QIIME 1.8.0. The EBL code in the key refers to the 10 different participants. The arrow points to the diversity analysis output for participant EBL97 (brown line), which indicates that at least one sample did not contain enough sequences to accurately reflect the true microbiota.

Alpha-diversity analysis suggested that a minimum of one sequencing data file from the faecal samples of EBL97 did not produce a number of sequences large enough for use in further analysis, as indicated by the arrow (**Figure 8.10**). Interrogation of the raw sequencing data isolated a file containing only 18,731 sequencing reads, which correlated to the DNA sample obtained from the faecal sample produced by EBL97 following the two week low-*Brassica* diet intervention phase. This sample can be seen, positioned apart from the other samples, alongside the second principal component axis in **Figure 8.7** and **Figure 8.9**, indicating that the number of sequencing reads was insufficient for this sample to be used in further analysis. Each of the remaining files, including the file corresponding to the isolated sample of EBL28 (**Figure 8.7** and **Figure 8.9**), contained sufficient sequencing reads to reflect the true composition of the microbiotas. Therefore, the subsequent analysis was performed on the data obtained from the 67 samples that were considered to be reliable, and the data corresponding to the sample collected from EBL97 following the low-*Brassica* diet was excluded.

8.4.5 *Brassica* diet-associated changes to bacterial taxa within the gut microbiota

Although the consumption of a low- or high-*Brassica* diet were not sufficient to show large changes to the gut microbiota community composition, smaller diet-associated alterations to members of individual bacterial taxa may still have occurred. Multivariate analysis was performed on the bacterial taxa that were present at a proportion >0 in a minimum of 45 of the 67 faecal microbiotas (excluding the poorly sequenced sample of EBL97). This criterion designated 66 of the total 385 bacterial taxa as candidates for further analysis, with this subset accounting for >90% of the total population in 64 of 67 faecal microbiotas. After the data was log-transformed to allow for a clearer visualisation of the data-points, and variance scaled to account for the uneven

distribution, 14 principal components were retained based on the Kaiser criterion (factors with an eigenvalue >1). A varimax rotation (orthogonal rotation of the factor axes to simplify interpretation) was performed on the 14 principal components to produce principal component analysis (PCA) factor scores that correlated with the 66 bacterial taxa within each participant's faecal microbiotas. The variance explained by the retained 14 principal components and the varimax-rotated factors are displayed in **Table 8.4**.

Table 8.4: Variance and cumulative percentage variance explained by the first 14 principal components, and the corresponding values for the varimax-rotated factors.

PCA factor	*Variance*	*Cumulative (%)*	*Rotated axis variance*	*Cumulative (%)*
1	15.3	23.2	13.9	21.1
2	7.7	34.9	5.3	29.2
3	6.7	45.0	5.4	37.4
4	5.1	52.8	3.7	43.0
5	3.5	58	3.2	47.9
6	3.1	62.7	3.2	52.8
7	2.6	66.7	2.5	56.6
8	2.6	70.5	3.4	61.8
9	2.2	73.9	2.3	65.4
10	2.0	76.9	2.3	68.9
11	1.5	79.1	4.5	75.7
12	1.2	81.0	2.1	78.8
13	1.1	82.7	2.0	81.8
14	1.0	84.2	1.6	84.2

The 66 bacterial taxa were plotted as a single variate for each of the 67 faecal samples, against the first two varimax-rotated factors in the form of a score-plot. This highlighted a trend, in which the 66 bacterial taxa were associated with lower first factor scores after participants consumed the high-*Brassica* diet, compared to the

corresponding taxa following consumption of the low-*Brassica* diet (**Figure 8.11**). In addition, samples from the same participants were observed to form clusters, although these often overlapped.

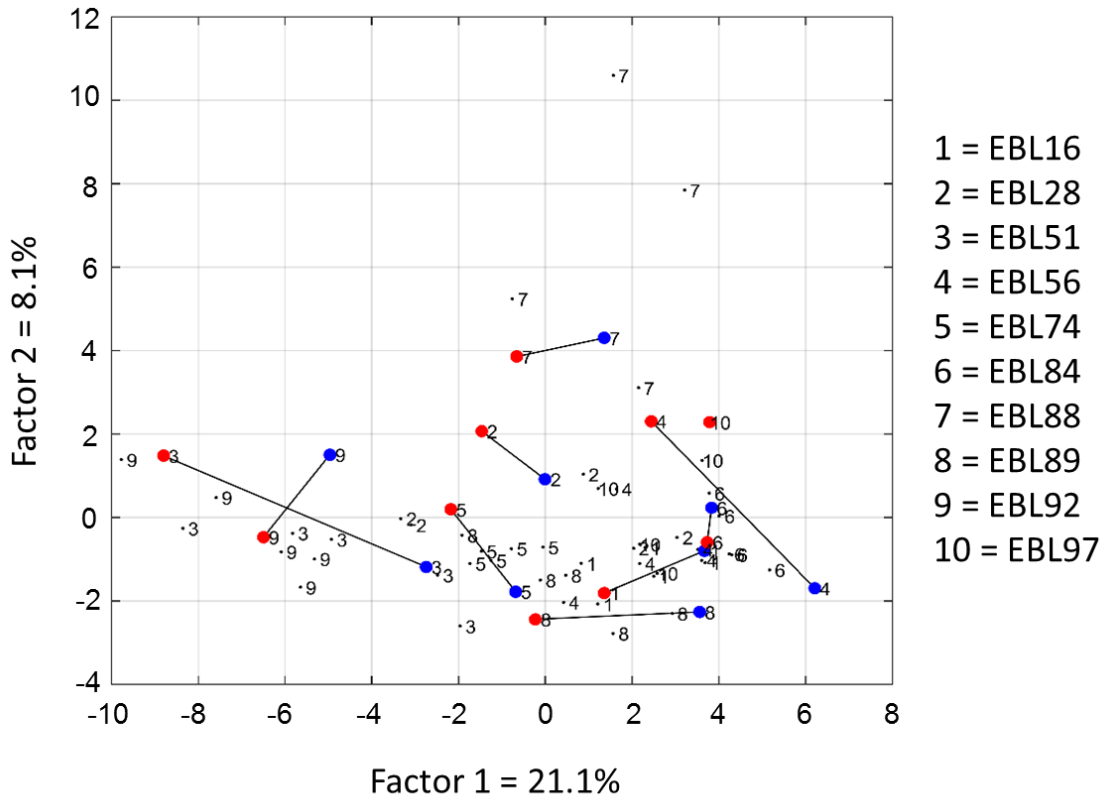


Figure 8.11: Variates corresponding to 66 bacterial taxa within faecal samples collected after the high-*Brassica* diet are associated with a lower first factor score than those collected after the low-*Brassica* diet. Score-plot of the first two varimax-rotated factors: black dots signify scores of 66 bacterial taxa within non-intervention faecal samples, filled red circles represent scores faecal samples collected after the high-*Brassica* diet, filled blue circles denote scores of 66 bacterial taxa within faecal samples collected after the low-*Brassica* diet. Scores labelled with the same number (1 – 10) correspond to a single individual, and scores obtained for the low- and high-*Brassica* diet for each individual are connected by a black line. As the data from the sample collected after the low-*Brassica* diet for EBL97 (10) was excluded, no connecting line is present for the samples of this participant.

As a consequence of this perceived trend, correlations between the first and second varimax-rotated factor scores and each of the log-transformed variates were examined to identify which log-transformed variates were most strongly associated with the first factor score. Those variates in which the greater amount of variance was explained by the first factor score were selected as the most likely candidates to have had a significant association with the *Brassica* diets. This allowed the identification of 19 bacterial taxa with a strong association with the first factor score (listed in **Table 8.5**): 18 bacterial taxa were positively associated, whilst a member of the genus *Veillonella* was negatively associated.

Sequential analysis of variance was performed using Matlab to identify whether there were any statistically significant changes to the log-transformed proportions of the 19 bacterial taxa, following the consumption of the *Brassica* diets (**Table 8.6**). No significant difference was detected for the order in which the participants consumed the diets.

Table 8.5: Bacterial taxa associated with the first factor score (*Veillonella* (19) was the only taxa negatively associated), and the maximum (Max.) proportion of each in the total population, obtained from 67 faecal samples. p = phylum, c = class, o = order, f = family, g = genus.

Identifier	***Bacterial taxa***	***Max. proportion***
1	Unclassified bacteria	1.64%
2	p_Bacteroidetes; c_*Bacteroidia*; o_*Bacteroidales*; f_*Rikenellaceae*	1.23%
3	p_Bacteroidetes; c_*Bacteroidia*; o_*Bacteroidales*; f_*Rikenellaceae*	2.53%
4	p_Bacteroidetes; c_*Bacteroidia*; o_*Bacteroidales*; f_*Rikenellaceae*; g_*Alistipes*	0.37%
5	p_Bacteroidetes; c_*Bacteroidia*; o_*Bacteroidales*; f_*Barnesiellaceae*	1.94%
6	p_Firmicutes	1.26%
7	p_Firmicutes; c_*Clostridia*	0.12%
8	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*	5.53%
9	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Christensenellaceae*	4.41%
10	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Dehalobacteriaceae*; g_*Dehalobacterium*	0.23%
11	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Ruminococcaceae*	11.88%
12	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Ruminococcaceae*	4.0%
13	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Ruminococcaceae*; g_*Anaerotruncus*	0.22%
14	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Ruminococcaceae*; g_*Clostridium*	0.07%
15	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Ruminococcaceae*; g_*Oscillospira*	4.45%
16	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Ruminococcaceae*; g_*Ruminococcus*	18.15%
17	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Mogibacteriaceae*	0.38%
18	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Mogibacteriaceae*	3.11%
19	p_Firmicutes; c_*Clostridia*; o_*Clostridiales*; f_*Veillonellaceae*; g_*Veillonella*	0.88%

Table 8.6: Summary statistics and modelling results for 19 bacterial taxa associated with the first factor score. Identifier = numerical code assigned to bacterial taxa in Table 8.5; Day 0 and Day 15 = values obtained before and after each dietary intervention phase; Diff = paired differences between values obtained before and after each dietary intervention phase; Day 15 high – Day 15 low = paired differences between values obtained after the high- and low-*Brassica* diet intervention phases; *P* = significance of *Brassica* diet in analysis of variance model, with bold values indicating $p<0.05$. Bacterial proportions expressed as the natural logarithm of the fractional proportion of population after the addition of a small offset (0.00001) to counter zero-proportions.

Identifier	***Low*-Brassica *diet***						***High*-Brassica *diet***								
	Day 0		**Day 15**		**Diff**		**Day 0**		**Day 15**		**Diff**		**Day 15 high – Day 15 low**		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	***P***
1	-8.12	1.56	-8.39	1.37	-0.27	0.84	-8.90	1.45	-8.37	2.14	0.53	1.16	0.02	1.71	0.1879
2	-6.17	1.00	-5.76	0.72	0.42	0.79	-6.24	1.18	-6.58	1.39	-0.33	1.40	-0.82	1.03	**0.0295**
3	-5.06	0.87	-4.69	0.59	0.38	0.83	-5.09	1.14	-5.35	1.03	-0.26	1.02	-0.66	0.77	**0.0078**
4	-7.85	1.45	-7.28	1.31	0.57	0.76	-8.29	1.44	-8.38	1.60	-0.09	1.51	-1.10	0.88	**0.0254**
5	-7.08	1.84	-6.84	1.82	0.24	0.83	-6.97	2.03	-7.07	2.12	-0.11	1.18	-0.23	1.66	0.4902
6	-7.15	2.17	-6.88	1.71	0.26	0.88	-7.41	1.41	-7.80	1.74	-0.38	1.11	-0.91	1.04	**0.0206**
7	-9.05	1.35	-9.11	0.82	-0.06	0.72	-9.51	1.07	-10.03	1.42	-0.51	0.91	-0.92	1.31	0.1230
8	-5.02	2.12	-4.93	2.05	0.09	0.68	-5.10	1.56	-5.75	1.89	-0.64	1.29	-0.82	0.93	**0.0037**
9	-6.92	2.60	-6.94	3.01	-0.02	1.22	-7.48	2.53	-8.32	2.68	-0.85	0.79	-1.39	1.73	0.2320
10	-8.84	1.76	-8.72	1.90	0.12	0.52	-9.46	1.79	-9.42	1.79	0.04	1.18	-0.69	0.86	**0.0206**
11	-3.55	1.19	-3.43	1.06	0.12	0.37	-3.64	1.05	-3.67	0.84	-0.03	0.32	-0.24	0.35	0.0917
12	-6.35	2.34	-5.86	2.29	0.49	0.80	-6.95	2.34	-7.23	2.38	-0.28	0.79	-1.37	1.26	**0.0001**
13	-9.17	1.65	-8.97	1.63	0.20	1.27	-9.67	1.42	-9.38	1.29	0.29	0.53	-0.41	0.66	0.1336
14	-8.98	0.83	-9.17	0.96	-0.20	1.07	-9.42	0.87	-9.94	1.19	-0.53	0.70	-0.77	0.45	**0.0059**
15	-4.32	0.82	-4.17	0.74	0.15	0.43	-4.30	0.72	-4.66	1.15	-0.36	0.63	-0.50	1.10	0.1677
16	-2.75	0.55	-2.77	0.51	-0.02	0.39	-2.82	0.58	-3.26	1.16	-0.44	0.97	-0.49	0.97	0.1384
17	-7.83	1.65	-7.90	1.81	-0.07	0.84	-8.31	1.61	-8.60	1.76	-0.29	0.82	-0.70	0.77	**0.0360**
18	-5.86	1.31	-5.57	1.15	0.29	0.49	-5.99	1.15	-6.31	1.30	-0.31	0.31	-0.74	0.36	**0.0024**
19	-8.31	1.84	-9.10	0.91	-0.79	1.62	-8.02	1.64	-8.43	2.15	-0.41	1.76	0.68	1.44	0.2591

The *p*-values obtained though the statistical analysis indicated that 10 members of the selected 19 bacterial taxa were present at significantly different relative proportions ($p<0.05$), when comparing the endpoint of each *Brassica* diet intervention phase. **Table 8.6** indicates that the relative proportions of members of these taxa were lower after the high-*Brassica* diet, compared to after the low-*Brassica* diet. In addition, the proportions of the members of these taxa were often found to be lower at the end of the high-*Brassica* diet compared to the beginning, whilst the reverse was observed for the low-*Brassica* diet. The log-transformed proportions of the members of the 5 bacterial taxa with a *p*-value<0.01 after each of the *Brassica* dietary interventions are shown for the 9 participants analysed, in **Figure 8.12**.

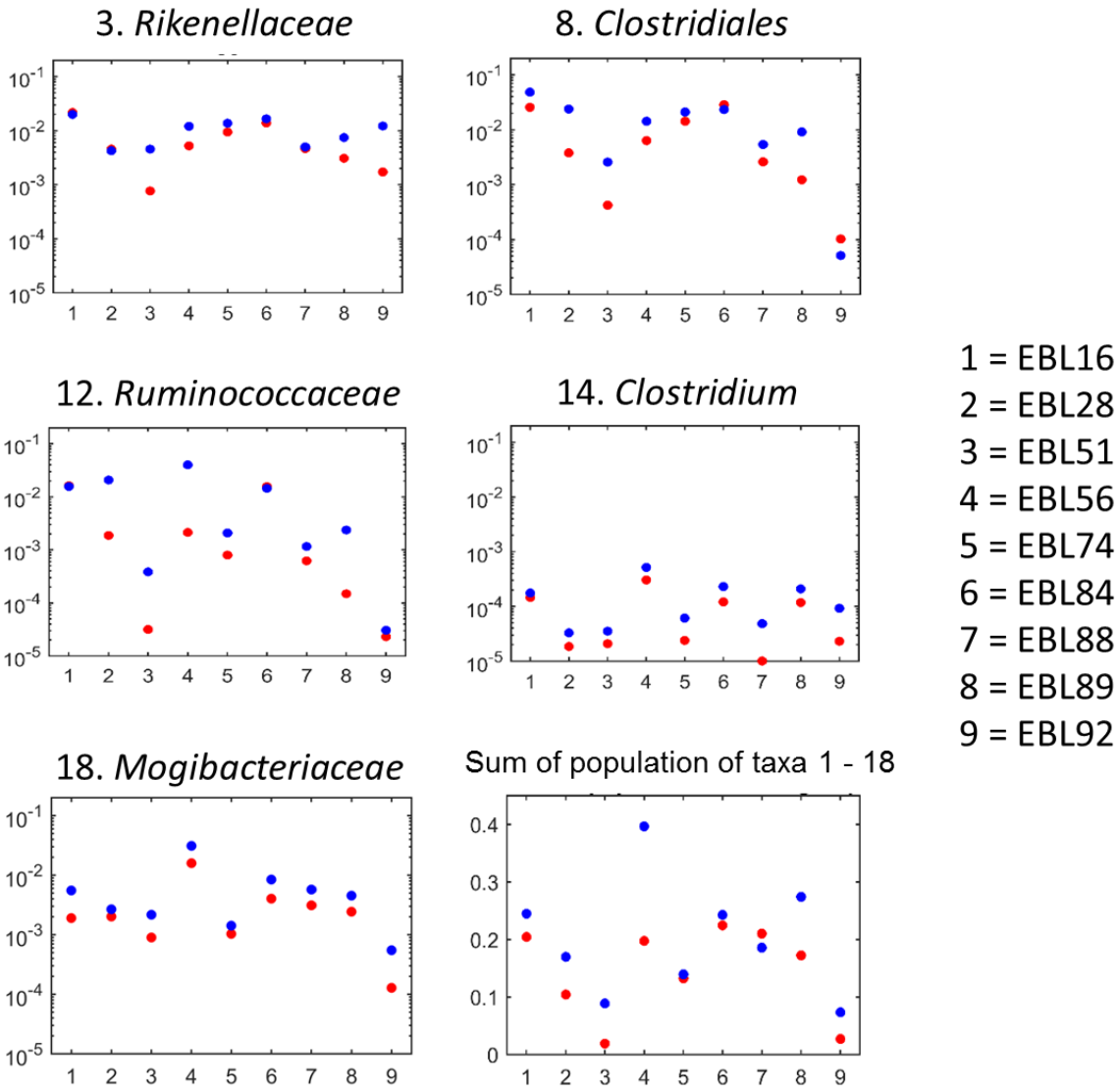


Figure 8.12: Decreased proportions of the strongly significant members of the bacterial taxa associated with the *Brassica* diets ($p<0.01$) are observed in most of the 9 participants tested after consuming the high-*Brassica* diet, compared to the low-*Brassica* diet. Population proportions of members of the 5 bacterial taxa significantly associated with consumption of the *Brassica* diets ($p<0.01$) in 9 participants after the low-*Brassica* (blue) and high-*Brassica* (red) diets. The lower right sub-plot shows the summed proportions of the 18 bacterial taxa positively associated with the first varimax-rotated factor in 9 participants after each *Brassica* diet. The proportions are presented on a log scale and have had a small offset (0.00001) added. The plots were produced using Matlab.

8.5 Discussion

8.5.1 Differences in urinary glucoraphanin hydrolysis product excretion profiles between and within participants

Participants were asked to collect their urine for 24 hours following the consumption of the first portion of *Brassica* for each of the two diets, and the percentage of glucoraphanin hydrolysis products excreted in the urine were calculated (**Figure 8.2**). Differences in the proportions of glucoraphanin hydrolysis products excreted in the urine were observed between and within individuals. Variation in urinary excretion profiles between individuals has been observed in several other studies, ranging from 1 – 45% of the initial glucosinolate dose [111-114]. Fahey *et al* observed intra-individual differences when participants were re-tested between a period of a few days and two and a half years [111]. As the urinary excretion rate of glucosinolate hydrolysis products is likely to directly reflect the microbial conversion of glucosinolates, this could be linked to minor fluctuations in the relative abundances of certain gut bacteria able to metabolise glucosinolates.

A limitation of the study that forms the basis of this chapter was that participants were free to consume either a portion of steamed broccoli or a broccoli and sweet potato soup, prior to the 24 hour urine collection. Interpretation of the urinary excretion data would be more reliable if all participants had consumed the same type of food prior to the urine collection period. The soup contained approximately 2.5-fold higher levels of glucoraphanin, and differences in the food matrix between the steamed broccoli and the soup may affect absorption, gut transit time, and microbial accessibility to glucosinolates.

8.5.2 Changes to faecal metabolite profiles associated with *Brassica* diets

Metabolite analysis, using two replicates from 54 faecal samples, suggested that two distinct areas of the faecal samples tested contained a similar metabolite profile, and indicated good reproducibility of the technique (**Figure 8.3**). This mirrors the results from a study performed by Mai *et al*, which concluded that a pea sized aliquot of faecal material could be taken from any part of a faecal sample and that this would give an accurate representation of the entire stool with regards to both the composition of the microbiota and the microbial diversity [471].

When the metabolite profiles of all 68 faecal samples collected throughout the study were analysed, samples tended to cluster based on the participant (**Figure 8.4**). Statistical analysis indicated no significant association between the *Brassica* diets and the metabolite profile of the participants (**Figure 8.5**). However, this is not entirely unexpected as the components of the *Brassica* diet will have only represented a fraction of the daily food intake of the participants, and any effects may be transient. In addition, the metabolite profiles of faecal samples may not always be fully representative of activity in the colon. An increase in the production of metabolites may not be reflected in the faecal metabolite profile, if there is a corresponding increase in the utilisation of these metabolites by the gut microbiota or human host.

8.5.3 Effects of the *Brassica* diets on the relative proportions of intestinal lactobacilli

The relative proportions of *Lactobacillus* were observed to range between <0.1% and 0.2% of the total bacterial populations, and were not found to be associated with the consumption of either of the *Brassica* diets. This suggests that the results obtained from the *in vitro* batch fermentation model, discussed in chapter 5, do not correlate with

the *in vivo* effects of consuming broccoli. This is likely due to the continuous flow of contents through the intestines, which does not allow for extreme changes to pH occurring in localised areas for extended periods of time.

8.5.4 Bacterial community diversity analysis

Phylogenetic analysis of the faecal microbiotas of 10 participants was performed to determine whether the consumption of a low- or high-*Brassica* diet was associated with an alteration in the gut bacterial community composition. It was observed that the faecal samples clustered by participant (**Figure 8.8**), indicating the unique nature and inherent stability of an individual's gut microbiota. This appears to reflect the low intra-individual variability across time that was also observed with the faecal metabolite profiles (**Figure 8.4**). Inter-individual differences in the composition of gut bacterial communities have been observed in other studies [134, 201, 227]. Weighted beta diversity analysis suggested that the microbiotas clustered, but that there was variation in the abundances of bacteria within the communities over time (**Figure 8.9**). This likely reflects the dynamic nature of the gut microbiota, which is able to adapt to environmental alterations, such as a change of dietary habits, while retaining its core structure.

In a previous study by Li *et al*, changes to the composition of human gut bacterial communities were reported following a 'double-cruciferous diet' (basal diet + 14 g cruciferous vegetables/kg body weight per day), versus the basal diet [134]. As the basal diet was devoid of other vegetables, fruits, whole-grains and high-fibre foods, this would mean that the addition of a large portion of cruciferous vegetables would provide a range of nutrients, which the bacterial community may not have been able to obtain from other dietary sources. The study described in this chapter only required the participants to restrict their diet of *Brassica* vegetables, thereby allowing the

consumption of other foods that may contain similar vitamins, minerals, and fibres. Consumption of the *Brassica* diets did not appear to alter the composition of the gut microbiotas in a uniform direction (**Figure 8.6** and **Figure 8.7**). This is not surprising considering that any effects of the *Brassica* diet would need to be strong enough to be detectable over the background of other foods containing similar nutrients, and the natural fluctuations of the microbiota.

8.5.5 Statistical analysis of *Brassica* diet-associated changes to gut bacteria

Data modelling and statistical analysis identified a tendency for the relative proportions of members of ten bacterial taxa to be higher following the consumption of the low-*Brassica* diet, compared to the high-*Brassica* diet ($p<0.05$) (**Table 8.6**). Additionally, the relative proportions of the members of these ten bacterial taxa were often found to increase following the consumption of the low-*Brassica* diet, compared to the proportions measured prior to the low-*Brassica* diet phase, with the opposite trend observed for the high-*Brassica* diet. Three members of these bacterial taxa belonged to the *Bacteroidales* order, whilst six taxa were members of the *Clostridiales* order, and the remaining taxon could not be classified at a greater level than the phylum Firmicutes. The association of members of five of these taxa to the *Brassica* diets were found to be strongly significant ($p<0.01$): *Rikenellaceae* (*Bacteroidales*), *Ruminococcaceae* (*Clostridiales*), *Mogibacteriaceae* (*Clostridiales*), *Clostridium* (*Clostridiales*), and an unclassified *Clostridiales*.

The cruciferous vegetable study performed by Li *et al* (discussed in section 8.5.4) found four terminal restriction fragment length polymorphism (tRFLP) fragments that were significantly associated with the double cruciferous diet intervention [134].

However, it was not reported whether the identified fragments were positively or negatively associated with the cruciferous diet. These fragments were putatively assigned to *Eubacterium hallii*, *Phascolarctobacterium faecium*, *Alistipes putredinis*, and an *Eggerthella* species. Both *E. hallii* and *P. faecium* belong to the *Clostridiales* order, whereas *A. putredinis* is a member of the *Rikenellaceae* family. This may indicate that the *Brassica* diets described in this chapter affected similar bacterial taxa to those reported in the study of Li *et al*. Additionally, in this study the diet–bacteria association was observed whilst participants were able to consume other vegetables, fruits, whole-grains, and high-fibre foods, suggesting that the effects on the members of these bacterial taxa were associated with the *Brassica* diets, rather than nutritious foods in general.

It is unclear why the high-*Brassica* diet was associated with a decrease in the relative proportions of the members of the bacterial taxa observed, but one explanation may be an inhibitory effect caused by relatively high concentrations of sulphur-containing compounds. *Brassica* and *Allium*, such as broccoli and garlic, respectively, are known to contain relatively large amounts of sulphur-containing compounds, compared to plants from other genera [472]. A study performed by Filocamo *et al* investigated the effects of garlic powder on pure cultures of human gut bacteria, and found a strong inhibitory effect on *Clostridium nexile* [473]. However, further research would be necessary to determine whether members of the order *Clostridiales* are sensitive to sulphur-containing compounds when present at relatively high concentrations.

8.6 Conclusions

A human pilot dietary intervention study was performed to investigate the effects of a low- and high-*Brassica* diet on the human gut microbiota. It was shown that each participant had a unique bacterial community with small fluctuations in the relative abundances of bacterial members. Data modelling and statistical analyses identified members of ten bacterial taxa whose relative proportions were significantly altered following consumption of the *Brassica* diets. Members of these taxa tended to be present at higher proportions following the consumption of the low-*Brassica* diet, compared to before the low-*Brassica* diet intervention, and after consuming the high-*Brassica* diet. Of these taxa, five members were strongly associated with the *Brassica* diet: *Rikenellaceae* (*Bacteroidales*), *Ruminococcaceae* (*Clostridiales*), *Mogibacteriaceae* (*Clostridiales*), *Clostridium* (*Clostridiales*), and an unclassified *Clostridiales*. Faecal metabolite profiles were similar within individuals, but no changes were detected following the consumption of the *Brassica* diets. Inter- and intra-individual variation was observed in the urinary excretion of glucoraphanin hydrolysis products amongst participants over time, but an accurate interpretation of these results are complicated due to the different sources of glucoraphanin.

CHAPTER NINE

9 General discussion

9.1 Summary of findings

The overall aim of this research was to investigate the effects of *Brassica* on the human gut microbiota composition, through the use of an *in vitro* batch fermentation model and a human intervention study. It has been established that short-term dietary interventions, using dietary components, whole foods, or different types of diet are able to alter the composition of the human gut microbiota [373]. The work presented in this thesis aimed to determine what effects broccoli may have on the microbiota community composition and metabolic output of human faecal bacteria when cultured in an *in vitro* batch fermentation model. These results formed the basis of a human study investigating what effects dietary *Brassica* may produce *in vivo*, with a focus on changes to the composition of the gut microbiota.

9.1.1 Bacterial reduction of glucosinolates

The bacterial metabolism of glucosinolates has been reported when human faecal bacteria have been cultured in media containing single glucosinolates [45, 124, 142], or a purified glucosinolate extract [144]. However, to the best of my knowledge the use of a broccoli leachate, containing components of broccoli in addition to glucosinolates, has not been previously explored. The data presented in chapter 4 indicated that the aliphatic glucosinolates, glucoraphanin and glucoiberin, were converted to their reduced analogues, glucoerucin and glucoiberverin, respectively, by human faecal microbiotas cultured in a media containing a broccoli leachate (**page 104** and **107**). Chapter 7 discusses the isolation of bacteria from these *in vitro* microbiota experiments, and presents data indicating that *Escherichia coli* 1B04, and the laboratory strain *E. coli* DH5α, were able to convert glucoraphanin to glucoerucin through the reduction of the sulphoxide moiety (**page 177** and **183**, respectively). Previous studies observed the reduction of glucoraphanin to glucoerucin by human

faecal microbiotas, but did not identify the bacteria responsible [124, 142]. Research in two independent groups identified three bacteria of human origin that were able to convert glucoraphanin and glucoiberin, to glucoerucin and glucoiberverin, respectively [45, 144]. All three bacterial species were members of the *Enterobacteriaceae*: *E. coli* Nissle 1917, *E. coli* VL8, and *Enterobacter cloacae* ATC13047.

9.1.2 Is the bacterial reduction of glucosinolates substrate-specific?

E. coli DH5α is a bacterial strain that was developed specifically for laboratory work. The ability of this strain to reduce glucoraphanin raises the question of whether the reduction of sulphoxide-containing glucosinolates is an outcome of a general bacterial reductase reaction, rather than a reaction specific to glucosinolates. The query regarding the specificity of the glucosinolate reduction reaction led to investigations into the metabolism of the plant secondary metabolite SMCSO (chapter 7). It was found that SMCSO was reduced by *E. coli* 1B04 to produce *S*-methylcysteine (**page 186**), and that both *E. coli* 1B04 and *E. coli* DH5α were able to degrade SMCSO (**page 188**). Multiple studies have indicated that bacteria, many of them residents of the gastrointestinal tract of animals, have the ability to degrade SMCSO via cysteine β-lyase activity [444-448], but no evidence could be found for previous reports of SMCSO reduction by human gut bacteria.

A previous study showed that *E. coli* is able to use sulphoxides as electron acceptors to generate energy for ATP synthesis, through their reduction by the DMSO reductase, DmsABC [310]. In another study, which focused on the reduction of sulphoxide-containing drugs by an *E. coli* strain, the thioredoxin system was implicated in the reduction of sulindac. The authors concluded that there were likely to be a minimum of five different cytosolic enzyme systems present in *E. coli* that were able to reduce the sulphoxide-containing drugs tested [318]. As *E. coli* 1B04 did not metabolise

glucoerucin when cultured in a glucoerucin-containing medium (**page 182**), it may indicate that this bacterium benefits from the reaction rather than the product of the reaction. Although further characterisation of the bacterial reductase activity against sulphoxide-containing glucosinolates and SMCSO would be necessary, it seems unlikely that the reduction of glucosinolates is a substrate-specific bacterial reaction.

9.1.3 *In vitro* effect of the broccoli leachate on the microbiota composition

In chapter 5 it was identified that the repeated exposure of human faecal microbiotas to broccoli leachates, led to multiple observations of an increase in the relative proportions of lactobacilli (**page 137**), and in one experiment the potential LAB *Streptococcus* (**page 138**). Sequencing of bacterial isolates obtained from the cultured BL media indicated that all of the lactobacilli tested were *L. fermentum* (**page 176**). The increased relative proportions of the genus *Lactobacillus* observed in the cultured BL media was associated with a decrease in the pH of the media (**page 146**). The major end-product of *Lactobacillus* fermentation is known to be lactate [474], and analysis indicated relatively high levels of lactate accumulation in the BL media, with only a small amount observed in the GL media (**page 161**).

Although LAB are acid-tolerant, it is known that some intestinal bacteria are sensitive to changes in pH [270, 271]. The decrease in pH will have likely inhibited the growth of some bacteria in the BL media, and killed those most sensitive to acidic conditions, while providing *Lactobacillus* and *Streptococcus* species with a growth advantage. However, lactobacilli generally account for ≤1% of the human gut microbiota [246], which was reflected in the pre-cultured faecal samples, so it is likely that the broccoli leachate contained nutrients that allowed the increased growth of

Lactobacillus/Streptococcus, prior to the effects of a decrease in pH. There would likely have been strong competition amongst the bacteria to metabolise the sugars contained within the media, but the broccoli leachate may have contained compounds less desirable to bacteria other than *Lactobacillus*. A study was recently published investigating the metabolism of phenolic compounds in a broccoli puree by various species of *Lactobacillus* [399]. A strain of *L. fermentum* was found to be an effective metaboliser of all of the measured phenolic compounds in the broccoli puree, with the exception of caffeic acid. It was hypothesised that the catabolism of phenolic compounds would provide these bacteria an energetic advantage through NAD^+ regeneration [399].

9.1.4 Influence of broccoli leachate on SCFA production

In chapter 6, metabolite analysis indicated a variation in the range and concentrations of SCFAs produced by the cultured microbiotas (**page 164**). Higher concentrations of SCFAs were observed in the cultured BL media, compared to the cultured GL media (**page 163**), and there is some evidence that the production of SCFAs may have been enhanced by lactate metabolism. Lower levels of lactate and a higher total concentration of SCFAs were detected in the BL media at the end of the experiment with the faecal microbiota of subject 1, compared to the other cultured microbiotas (**page 159** and **164**, respectively). This microbiota contained relatively high proportions of the genus *Megasphaera* (**page 137**), which includes the known ruminal lactate-utilising species *Megasphaera elsdenii* [475, 476]. *Megasphaera* was only observed in the microbiota of subject 1, which fits in with the current literature suggesting that levels of *Megasphaera* are variable amongst human intestinal populations [237, 477-481]. Relatively high concentrations of propionate and valerate, in addition to acetate and butyrate, were only detected in samples from the *Megasphaera*-containing microbiota

cultured in the BL media (**page 164**). This genus is known to contain species able to utilise lactate and generate butyrate, propionate, and valerate, with the final product dependent on environmental conditions, such as pH and the availability of lactate and glucose [432, 475]. SCFAs in general are associated with positive effects on human health, with individual SCFAs linked to different beneficial properties [284-287, 421-430].

9.1.5 *In vivo* effects of *Brassica* on the human gut microbiota

Human intervention studies allow *in vitro* driven hypotheses to be tested in an *in vivo* environment. Chapter 8 presented a randomised, 2-phase crossover, human dietary intervention study, in which the effects of a low- and high-*Brassica* diet on the gut microbiota of 10 participants was investigated (**page 199**). Changes to the relative proportions of members of ten bacterial taxa were significantly associated with the *Brassica* diets, with five members showing a strongly significant association (p<0.01): *Rikenellaceae* (*Bacteroidales*), *Ruminococcaceae* (*Clostridiales*), *Mogibacteriaceae* (*Clostridiales*), *Clostridium* (*Clostridiales*), and an unclassified *Clostridiales* (**page 218**). The relative proportions of the members of these taxa were found to generally increase after consumption of the low-*Brassica* diet, and decrease following consumption of the high-*Brassica* diet, compared to the proportions measured prior to each diet (**page 220**).

Similar results were obtained from a study performed by Li *et al*, in which participants were asked to consume a basal diet (devoid of vegetables, fruits, whole-grain foods, and high-fibre foods) and a double-cruciferous diet (basal diet supplemented with 14 g cruciferous vegetables/kg body weight per day) [134]. Significant associations were observed between the double-cruciferous diet and bacteria putatively assigned as *Eubacterium hallii*, *Phascolarctobacterium faecium*, *Alistipes putredinis*, and an

Eggerthella species, compared to the basal diet. However, it was not reported whether these bacterial species increased or decreased in proportion following consumption of the double-cruciferous diet [134]. The *Rikenellaceae* family includes *A. putredinis,* whilst *E. hallii* and *P. faecium* are members of the *Clostridiales* order, thus this represents similar observations of diet-bacteria associations in both studies.

The greatest differences between the study of Li *et al* and the human study presented in this thesis are found in the diets and the analysis method used. During the double-cruciferous dietary phase, participants on the study performed by Li *et al*, were not consuming other vegetables, fruits, or high-fibre and whole-grain foods [134]. Throughout the duration of the study presented in this thesis, participants were asked to restrict their diet of all glucosinolate-containing foods, but could otherwise continue to eat their normal diet. Therefore any changes in the gut microbiota would be against a background of effects caused by dietary fibres, lignans, vitamins, minerals, polyphenols and other compounds obtained from vegetables and fruit in general. This strengthens the hypothesis that the observed effects on the gut microbiota were due to the *Brassica* vegetables specifically, rather than components of vegetables in general. The structure of the human gut microbiota is known to be unique between individuals, as was observed in chapter 8 of this thesis, and has been shown in numerous other studies [134, 201, 227]. In conjunction with this, analysis performed in 2010 indicated that faecal samples obtained from a cohort of 124 Europeans contained between 1000 – 1150 different bacterial species, with each individual harbouring a minimum of 160 bacterial species in their gut microbiota [181]. The Li *et al* study identified bacterial species corresponding to tRFLP fragments, based on a library of 96 16S rDNA genes obtained from a single individual [134]. Therefore, accurate identification of bacterial 16S rDNA sequences may have been limited to the number of shared species amongst the participants, and may have overlooked bacterial species present at low

abundances. A strength of the dietary study presented in this thesis, was that the analysis of the bacterial populations was carried out using the Illumina sequencing platform in conjunction with the RDP classifier. This provided a high level of sequencing depth that maximised the ability to include the less abundant members of the bacterial communities, and allowed the comparison of bacterial sequences to a database currently containing 3,224,600 high-quality 16S rDNA sequences [331].

9.1.6 *Brassica*, the gut microbiota, and health

The diet, gut microbiota, and human health are inextricably linked. Research indicates that the administration of probiotic bacteria can have beneficial effects on human health, such as helping to alleviate the symptoms of IBS [482-484], preventing antibiotic-associated diarrhoea [485, 486], reducing the duration of infectious diarrhoea [487-489], reducing hypercholesterolemia [490], and preventing necrotising enterocolitis in pre-term infants [491-493]. However, the gut microbiota of a healthy individual is stable, and therefore more resistant to changes in composition compared to that of a diseased individual. The 'healthy' gut microbiota likely contains bacteria that have probiotic effects on the host, and their growth can be encouraged through the use of prebiotics. Prebiotics were recently defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" [494]. *Brassica* vegetables contain components, such as fibres, that are resistant to digestion and enter the large intestine, thereby potentially having a prebiotic effect on the indigenous microflora. Coupled with the potential beneficial effects on human health that have been attributed to the hydrolysis products of glucosinolates, dietary *Brassica* may positively impact on human health in different ways, through the bacterial metabolism of fibres and glucosinolates.

9.1.7 Completion of thesis aims

The aims of this thesis can be separated into two parts:

1. To culture human faecal microbiotas in media containing a broccoli leachate using an *in vitro* batch fermentation system to investigate the bacterial metabolism of glucosinolates, and the effects of the broccoli leachate on the microbiota composition and bacterial metabolite profiles. In addition, to use bacteria isolated from these experiments to test for the ability to perform the reduction of the sulphoxide moieties on glucoraphanin and SMCSO.

Under *in vitro* conditions, glucoraphanin and glucoiberin were converted to their reduced forms by a bacterial-mediated reaction in four of the five experiments. The exposure of human faecal microbiotas to a broccoli leachate resulted in an increase in the relative proportions of LAB, particularly the genus *Lactobacillus*, and an associated increase in lactate, which led to an enhanced production of SCFAs. An *E. coli* strain, designated *E. coli* 1B04, was isolated from the *in vitro* experiments, and was observed to exhibit the ability to reduce glucoraphanin and SMCSO.

2. To perform a human intervention study to investigate the effects of two *Brassica* diets on the human gut microbiota.

The results from the human intervention study indicated a trend for the low-*Brassica* diet to be associated with an increase in the relative proportions of certain gut bacteria, with the inverse relationship observed when the high-*Brassica* diet was consumed. Four of the five bacterial taxa most strongly associated with these effects were members of the order *Clostridiales*, with the family *Rikenellaceae* also represented.

9.2 Limitations of the research

The characterisation of the broccoli leachates, discussed in chapter 3, could have been enhanced by the measurement of sulphate through the use of techniques, such as anion-exchange chromatography. This would have allowed a greater understanding of the partitioning of the sulphur measured within the BL media. In addition, it may have been informative to investigate how the processes used to generate the broccoli leachates may have affected constituents, particularly sulphur-containing compounds, within the leachates. Cysteine, methionine, and glutathione are known constituents of broccoli, however they were not detected in the final broccoli leachates when analysed. The most plausible explanation is that these compounds were degraded during the steps necessary to process the broccoli leachates, most likely through thermal degradation when the broccoli was steamed, or when the broccoli leachates were concentrated at a temperature of 60˚C using a rotary evaporator. ^{1}H NMR analysis of samples collected before and after steaming the broccoli and evaporating the broccoli leachate, may have provided information regarding the fate of these sulphur-containing compounds during the process of generating the broccoli leachates.

The design of the glucose control (GL) media was based on the hypothesis that a large degree of glucoraphanin hydrolysis by human faecal bacteria would be observed. The hydrolysis of one mole of glucoraphanin releases one mole of an unstable aglycone, and one mole of glucose. Therefore, supplementing the basal media with 30 μmoles of glucose would control for the presence of glucose in the BL media, following the full conversion of 30 μmoles of glucoraphanin. However, the presence of sugars in the BL media, derived from broccoli, was not taken into consideration. The GL media would have been a more robust control media if it had matched the content of glucose, sucrose, and fructose found in the BL media.

A limitation of chapter 4 was that the potential production of erucin and erucin nitrile was not investigated. Within each of the five independent experiments, a portion of glucoraphanin, or glucoraphanin hydrolysis products, present in the starting BL media was unaccounted for at the end of the experimental period. Although glucoraphanin hydrolysis products, such as SF and SF nitrile, were analysed, the glucoerucin hydrolysis products were not investigated. The reduction of glucoraphanin produced glucoerucin, which may have been further metabolised by the faecal microbiotas to generate products, such as erucin or erucin nitrile. In addition, it has been reported that SF or SF nitrile can be reduced by human gut bacteria to form erucin or erucin nitrile, respectively [45]. Therefore, erucin or erucin nitrile may have been present in the cultured BL media, and analysis of the samples for these products using GC-MS may have allowed a greater understanding of the fate of the missing glucoraphanin.

In chapter 5, the main limitation was that the pH was not controlled during the culturing of the faecal microbiotas in the BL and GL media. Batch fermenters are often used due to their simplicity, affordability, and ease of which several can be run in parallel [481]. However, with the continuous flow of nutrients, and ability to control the pH, continuous culture fermenters produce a more accurate simulation of the luminal conditions of the human colon. The decision to use an *in vitro* batch model system meant that the acidic products produced during the culturing experiments accumulated in the media, causing the pH to become lower than is physiologically relevant in the colon. However, although a continuous culture system would be a more accurate model, it would have necessitated the production of a greater amount of the broccoli leachate, which was a time-consuming process and may have ultimately limited the number of faecal microbiotas that could have been tested.

During the human dietary intervention study (chapter 8), participants were asked to collect their urine across a 24 hour period in each of the diet phases, after consuming a single portion of the *Brassica* diets. The participants were asked to consume either a portion of the steamed broccoli, or a portion of the broccoli and sweet potato soup, prior to the urine collection. The study design would have been strengthened if all of the participants had been requested to collect their urine after consumption of the steamed broccoli. As the soup was not a component of the low-*Brassica* diet, all participants collected their urine after consumption of steamed broccoli in this diet phase, but many of the participants consumed the broccoli-based soup prior to the urine collection in the high-*Brassica* diet phase. The results of the urine analysis were difficult to interpret, and this may be partly due to the different food matrixes and the increased amount of glucoraphanin in the broccoli-based soup. Standardisation of the diet would have allowed for a greater interpretation of the urinary excretion results.

9.3 Future research

The data presented in this thesis identified an *E. coli* strain (1B04) isolated from human faeces, and an *E. coli* strain (DH5α) developed for laboratory cloning techniques, that were able to form glucoerucin via the reduction of glucoraphanin. However, it is unclear whether this is a metabolic reaction that is specific to glucosinolates, or a general mechanism of *Escherichia* species. Pure culture screening assays of a large range of, both pathogenic and commensal, *E. coli* strains (isolated from humans, animals, soil, and food sources) in a glucoraphanin media could help to identify whether this reduction reaction is a common metabolic process amongst *E. coli*. Should isolates be identified that are unable to convert glucoraphanin to glucoerucin, it may be possible to group reducers and non-reducers by environment, suggesting an environmental adaptation. Whereas, should all *E. coli* tested exhibit reductase activity on

glucoraphanin, it may indicate enzymic activity common to *E. coli* regardless of environment.

Further pure culture screening assays using glucoraphanin could be performed using a range of phylogenetically diverse human gut isolates, associated with the colonic lumen or the mucosa. This may help to identify whether the reduction of glucoraphanin is widespread amongst the human gut microbiota, or restricted to a single phylogenetic group, as presently, all bacteria that have been shown to have the ability to reduce glucosinolates belong to the *Enterobacteriaceae* [45, 144].

E. coli 1B04 was also shown to have the ability to reduce SMCSO to form *S*-methylcysteine, but it was not identified whether the reduction of the sulphoxide group of glucoraphanin and SMCSO were the result of the action of a single enzyme, or whether the activity is restricted to sulphoxides. The sulphoxide specificity could be tested using compounds that contain other oxide groups, such as the glucosinolate 7-methylsulphinyl-3-oxoheptyl, which contains a sulphoxide moiety and a carbon oxide. If the reduction only occurs at the sulphoxide moiety, this may indicate that the reductase is sulphoxide-specific. To identify whether the reduction of glucoraphanin and SMCSO is performed by a single enzyme, gene expression analysis followed by gene knockout experiments could be performed. Growth of *E. coli* 1B04 in a i) glucoraphanin media, ii) glucoerucin media, iii) SMCSO media, and iv) *S*-methylcysteine media, followed by RNA-Seq analysis, may enable the identification of common reductases expressed during glucoraphanin and SMCSO reduction. This could be followed by *E. coli* 1B04 gene knockout experiments to identify which gene(s) may be responsible for the reduction of glucoraphanin and/or SMCSO. If appropriate, growth of *E. coli* 1B04 and the associated knockout strain with a range of sulphoxide-containing dietary

compounds may help to elucidate the potential role of commensal *E. coli* in the formation of dietary metabolites within the human colon.

The three-stage continuous culture system could be used to culture identified probiotic strains, such as lactobacilli and *Bifidobacterium* strains, in a glucoraphanin-containing media to identify whether glucoraphanin can be metabolised by bacteria beneficial to human health. Should probiotic bacteria with the ability to hydrolyse glucoraphanin be identified, this could form the basis of a synbiotic, whose efficacy could be tested in human intervention studies with the aim of commercialisation to improve human health.

The results of the phylogenetic and statistical analyses of faecal microbiotas obtained from the human dietary study, indicated an association between the *Brassica* diets and five bacterial taxa: four members of the *Clostridiales*, and a member of the *Rikenellaceae* family. The nature of the association between the members of these taxa and the *Brassica* diets is unclear, but may be indicative of an inhibitory effect caused by the exposure to relatively high levels of sulphur-containing compounds. This hypothesis could be tested by culturing a range of human intestinal *Clostridiales*, reflecting the bacterial groups identified by the statistical analysis, and *Rikenellaceae* in media containing increasing amounts of a broccoli extract, in which concentrations of sulphur-containing compounds are measured. If an inhibitory effect was observed, the affected strains could be cultured in media containing each individual compound (e.g. sulphate, SMCSO, glucoraphanin, etc) to identify the minimum inhibitory concentration, and whether any inhibitory effects were due to a single compound or total sulphur.

9.4 Conclusion

In conclusion, the research presented in this thesis provides evidence that the bacterial reduction of sulphoxide-containing glucosinolates may not be a glucosinolate-specific reaction, or limited to bacteria that have adapted to an intestinal environment. The use of an *in vitro* model, combining phylogenetic and metabolite analysis, provided an interesting view of the importance of bacterial cross-feeding during the production of bacterial metabolites beneficial to human health. Results from a human intervention study highlighted the importance of testing *in vitro* result-driven hypotheses in an *in vivo* model, and identified gut bacterial taxa whose members are potentially associated with the consumption of *Brassica* vegetables. This urges further exploration into the effects of dietary *Brassica* on the human gut microbiota and human health.

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Appendix

IFR Institute of Food Research

A human intervention trial investigating the effects of Brassica on gut lactobacilli

Short title: Effects of Brassica on human gut Lactobacilli (EBL study)

Protocol
Version 3
26/09/2014

Chief Investigator:
Professor Richard Mithen

Investigators:
Study Manager: **Mr Lee Kellingray**
Study Scientist: **Dr Joanne F Doleman**

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Background

Numerous studies indicate that the health-promoting effects that can be obtained through eating a diet rich in Brassica vegetables are likely due to the biological activities of isothiocyanates (ITCs), which are the breakdown products of glucosinolates [1, 2]. Glucosinolates are sulphur-rich plant secondary metabolites that can be found in Brassica food crops, such as broccoli (*Brassica oleracea* L. *var. italica*), cabbage (*Brassica oleracea var. capitata*) and oilseed rape (*Brassica napus*) [3, 4]. Glucosinolate-containing plants also contain β-thioglucosidase enzymes (myrosinases) which are spatially separated from glucosinolates within the plant cells, however tissue damage, such as can be caused by chewing, results in cellular breakdown and exposes the glucosinolates to the enzyme. Myrosinase is then able to hydrolyse the thioglucose bond of the glucosinolate, causing the formation of a hydrolysis product, the most common of which are ITCs, and the release of glucose and sulphate [5, 6].

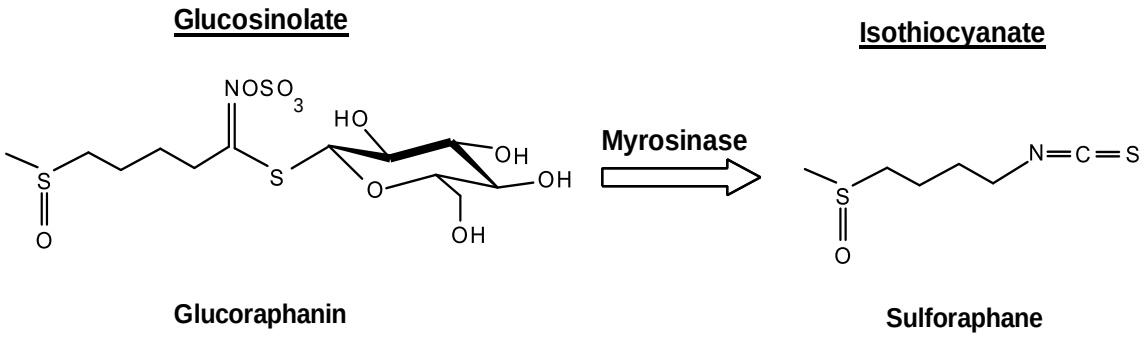


Figure 1. The isothiocyanate sulforaphane is generated via the hydrolysis of the aliphatic glucosinolate glucoraphanin, by the myrosinase enzyme.

There are >120 known glucosinolates, all of which contain the same core structure but vary by their side-chain which is derived from different amino acids. It is thought that this side-chain variation may be responsible for the different biological activity of ITCs. Brassica plants have different glucosinolate profiles, therefore affecting which ITCs are formed, for instance the prevalent glucosinolate found in broccoli is glucoraphanin and this is hydrolysed to form the ITC sulforaphane (SF) (Figure 1). Proposed beneficial effects of SF include anti-inflammatory and anti-oxidant effects, reduction of cholesterol

levels and systolic blood pressure, and a possible role in modulating the mitochondrial function [7-10]. However, when Brassica are cooked the myrosinase enzyme is denatured due to the heat, and some of the glucosinolates are able to pass intact through the gastrointestinal tract (GIT) to the colon.

In recent years it has become clear that the GIT plays a larger role in the metabolism of dietary compounds than previously thought. The main reason behind this is the realisation that the complex microbial ecosystem housed within the human intestine play a large role in human health, largely due to its metabolic potential. The human colon alone contains an estimated 200g of living cells, at a concentration of approximately 10^{11}-10^{12} cells/ml [11]. The large number of bacteria present in the colon are not reflected in the microbial diversity, with over 90% of the intestinal microbiota belonging to the Firmicutes and Bacteroidetes [12]. Despite this, it is estimated that the microbial gene catalogue residing in the gut consists of approximately 3 million genes, which is in the region of 100 times more than their host [13].

In contrast to the diversity of organisms, the bacterial genes present are highly diverse, in particular the genes that code for metabolic enzymes [14]. This is largely due to the range of non- or partly-digested food components that reach the colon, such as carbohydrates, proteins and phytochemicals. The carbohydrates that reach the colon undigested undergo bacterial fermentation, generating bacterial waste products (short-chain fatty acids) that are beneficial to the host. In contrast to this, colonic protein fermentation can lead to the generation of toxic products, such as phenols and amines [15]. Phytochemicals are exposed to a range of possible metabolic processes, which may affect their final biological activity. Upon entering the small intestine, a portion of the phytochemicals may be absorbed through the cell wall into the liver. These may undergo conjugation with glucuronic acid or sulphate before entering the bloodstream, or being recycled back to the intestine through the bile as glucuronides and/or sulphates [16, 17]. Therefore, both the parent phytochemicals and those that have been bioconverted can enter the colon where they may be absorbed or act as a substrate for the colonic bacterial community with their extensive metabolic potential [14]. Previous studies have shown that some colonic bacteria can produce a myrosinase-like enzyme which can metabolise the glucosinolate to produce the corresponding ITC [18], whilst other bacteria have been found to exhibit reductase activity, converting the glucosinolate to its reduced form [19-21]. Although it has been

shown that gut bacteria from a range of phyla have the ability to metabolise glucosinolates to their corresponding ITCs, the conversion of glucosinolates to their reduced analogue has so far only been seen in bacteria from the Enterobacteriaceae family [22-25].

As well as playing a large role in human nutrition, our gut bacteria also have a profound impact on human physiology and immunology. It is thought that alterations in the composition of our gut bacteria, known as gut dysbiosis, may be associated with some diseases, such as colon cancer and inflammatory bowel disease, as well as metabolic disorders such as obesity [26, 27]. It is currently thought that preserving the appropriate compositional balance of the gut microbiota, may help to maintain the health of the host. With this in mind, there has been an increase in research relating to probiotic bacteria. The World Health Organization defines probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”, the most common of which are *Lactobacillus* and *Bifidobacterium* strains [28]. Although the precise molecular mechanisms by which probiotic bacteria confer beneficial effects on the host are unclear, there is a body of evidence that indicates that it is generally via modulation of the host’s immune response, strengthening of the intestinal barrier, or the antagonism of pathogens by either the competition of mucosal binding sites, or the production of antimicrobial compounds [29].

The surge in commercially available probiotic supplements was preceded by increased research into these beneficial bacteria. A recent study using rats found that there was a higher abundance of both *Lactobacillus* and *Bifidobacterium* in diabetes-resistant rats compared to diabetes-prone rats, suggesting that an altered gut microbiota may be associated with the progression of type 1 diabetes [30]. Other studies, using *Lactobacillus casei* Shirota in experimental mouse models of immune disorders, have shown that this particular strain may be able to suppress the progression of arthritis, type 1 diabetes [31], chronic inflammatory bowel disease [32], and murine lupus [33]. A study using 135 human volunteers found that the daily consumption of a probiotic drink containing three lactic acid bacteria (2 of which were *Lactobacillus*) during a course of antibiotics led to a significant decrease in both antibiotic-associated diarrhoea and *Clostridium difficile* associated diarrhoea compared to the placebo group [34]. It is known that *Lactobacillus* spp. dominate the infant gut [35] and that enteric lactobacilli have been detected in adult human faecal samples, but they have also been found at

high levels in the stomach and small intestine [36-38]. Comparative genomics has been used to identify differences in milk-adapted and intestinal lactobacilli, which seem to reveal specific intestinal adaptation. Whilst, the lactobacilli from milk environments contained various genes used for growth on lactose, intestinal lactobacilli were found to have genes necessary for sugar-uptake systems and mucus-binding proteins, and certain lactobacilli are predicted to encode typical intestinal enzymes such as bile salt hydrolase [39-42]. This would seem to suggest that lactobacilli have adapted to survive in various niches throughout the human GIT, and modulation of their numbers may offer health benefits to the host.

An alternative method of altering the human gut microbiota is through the use of prebiotics, which promote the selective growth of beneficial indigenous gut bacteria. Prebiotics are defined as “selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host wellbeing and health” [43]. A prebiotic effect requires three criteria: a resistance to gastric acidity and hydrolysis by mammalian enzymes and gastrointestinal absorption; the ability to be fermented by intestinal microflora; and that it selectively stimulates the growth and/or activity of intestinal bacteria associated with health and wellbeing [43]. Studies using prebiotics suggest that they have the potential to reduce the symptoms and severity of Crohn’s disease and ulcerative colitis [44, 45], help treat diarrhoea [46, 47], and enhance calcium absorption and bone health [48, 49]. Alongside this, there is interest in whether prebiotics may have beneficial effects on immunomodulation of the gut immune system [50], lipid metabolism, gut hormones, glycaemic control, weight loss and satiety [51], as well as behavioural effects and obesity [52]. Numerous studies focusing on prebiotic effects use purified oligosaccharides, for example fructans and galactans, but many foods, whole-grains, fruits, and vegetables in particular, are naturally high in prebiotics such as fibre. As such, in the last decade there has been an increase in human dietary studies focused on changes in the gut microbiota caused by diet.

There have been several studies that have looked at the differences in ITC excretion between individuals, but as yet there have not been any links found with an individual’s gut bacteria [18, 24, 53-57]. A recent study attempted to correlate *in vivo* glucosinolate metabolism with *ex vivo* glucosinolate degradation from a small number of volunteers,

but was unable to uncover any direct links with specific bacterial species, although they did not examine whether any particular genes may have been responsible [24].

This study is primarily focused on whether a diet rich in Brassica vegetables can modulate the human gut microbiota by stimulating the growth of the beneficial lactobacilli. Experiments carried out during my PhD have indicated that components found within Brassica vegetables may specifically stimulate the growth of lactobacilli (unpublished data). In addition, there is evidence from a previous human intervention study that a diet rich in Brassica vegetables can cause a change in the composition of the gut microbiota compared to a fruit and vegetable free diet, however the study did have several limitations [58]. The amount of Brassica vegetables that the participants were asked to consume daily was based on their body weight and ranged from 660 to 1080g/d for the high Brassica arm, therefore the intake of glucosinolates and fibre varied between individuals. Terminal restriction fragment length polymorphism (tRFLP) was used to analyse the differences in the participants' gut microbiota following the consumption of the two different diets. A limitation of this method is that relies on comparing fragments of the 16S rRNA genes present in the samples, to a generated bacterial 16S rRNA gene clone library. For this study, the clone library was established from one faecal sample from one individual and therefore does not allow for the variability of gut bacteria between individuals. Also, as the library only consisted of 96 rRNA gene sequences, it is possible that only the dominant members of the community were selected for phylogenetic analysis. I plan to overcome these limitations by giving each participant a set number of portions of Brassica vegetables, irrespective of body weight, and use Illumina sequencing techniques to analyse the bacterial communities. Illumina is far more powerful than tRFLP as it doesn't rely on a clone library, instead it compares the sequences obtained from the samples analysed, to a database containing thousands of known sequences from different origins.
Alongside this, I will also investigate any changes in short chain fatty acids, bile acids, and bacterial gene expression due to the intervention, and attempt to associate different urinary ITC excretion rates with the composition of the faecal microbiota.

Hypothesis

A diet rich in Brassica vegetables will cause a change in the composition of the gut microbiota, and specifically an increase in the proportion and number of lactobacilli

commensals, compared to both the baseline faecal sample and a diet low in Brassica vegetables.

Objectives:

Primary

To investigate whether eating a diet rich in Brassica vegetables for two weeks will cause an increase in human gut lactobacilli, compared to the number of lactobacilli present in the participants' normal gut microbiota.

Secondary

To ascertain whether eating a diet rich in Brassica vegetables modulates the gut microbial community as a whole, as compared to the consumption of a low Brassica diet, and the participants' normal gut microbiota.

To determine whether the consumption of a high and/or low Brassica vegetable diet causes a change in short chain fatty acids and bile acids in the faeces.

To determine whether the consumption of a high and/or low Brassica vegetable diet causes a change in bacterial gene expression in the faeces.

To determine the extent of glucosinolate conversion to ITCs from Brassica vegetables, as measured by ITC excretion in the urine.

To determine the gut microbiota of participants and relate these data to the amount of urinary ITCs following the consumption of high and low Brassica diets.

Study design

A randomised, two-phase crossover dietary intervention study (Figure 2) will be undertaken to assess whether Brassica vegetables have the potential to cause an increase in the proportion and/or numbers of the beneficial lactobacilli in the human gut

microbiota. In this study, male and female participants aged between 18 and 50 years will be recruited until 10 complete the study, as this was the number deemed sufficient to see an increase in lactobacilli within the gut microbiota based upon power calculations (detailed on page 33 of this protocol).

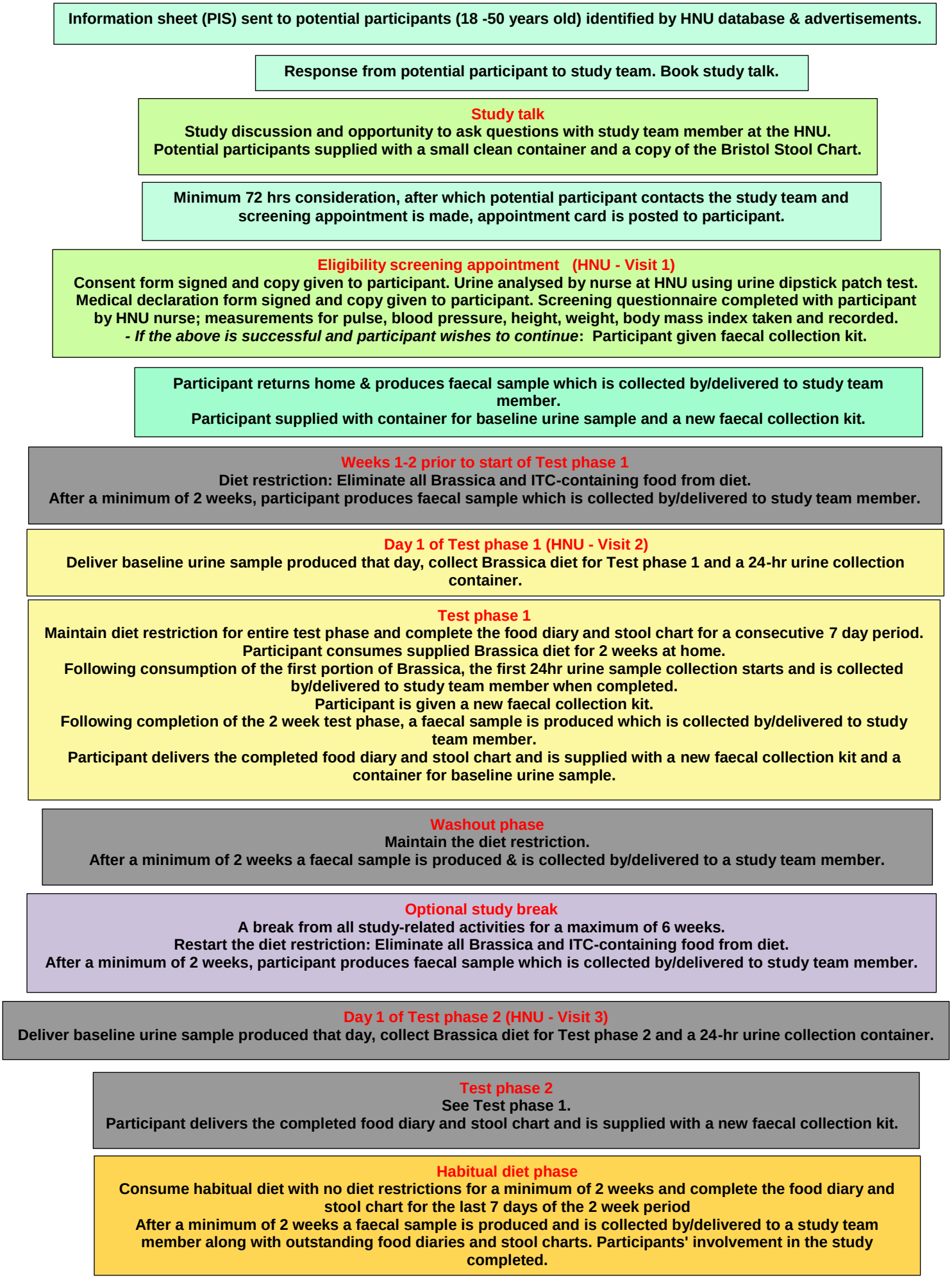
In one phase, the participants will consume a low Brassica diet for 2 weeks, consisting of one 84g portion of broccoli (or cauliflower) in week one, followed by one 84g portion of cauliflower (or broccoli) in week two, with a minimum period of five days between each portion. In the other phase, the participants will consume a high Brassica diet for 2 weeks, consisting of three 84g portions of broccoli, three 84g portions of cauliflower, and three broccoli and sweet potato soups (300g with a broccoli content of 84g), in each of the 2 weeks, eating a minimum of one portion per day. The broccoli and sweet potato soups were made by Bakkavor for another human intervention study (ESCAPE; REC Ref: 13/EE/0110), which is currently being run at the IFR. These two dietary phases will be separated by a minimum two week washout phase, with the option of a break period before commencing the second diet phase.

The participants will be asked to provide six faecal samples**,** this will increase to seven should they require the optional break period, during their involvement in the study: after the screening appointment, prior to starting each test phase, the first bowel movement after completing each 2 week test phase, and at the end of the study. This will allow for comparisons between the participants baseline gut microbiota to the composition present following both the low and the high Brassica diet, as well as determining any differences in the gut microbiota between the two Brassica diets. The faecal sample obtained at the end of the first two week diet restriction will determine any effects on the gut microbiota caused by the diet restriction, the faecal sample between the two test phases will be used to ascertain whether any effects on the gut microbial community seen at the end of test phase 1 have disappeared, and the faecal sample obtained at the end of the study will be compared with the baseline faecal sample to ascertain whether the gut microbiota has reverted back to its original composition. If participants require the optional break period (described earlier) it will be necessary for the participant to provide an additional faecal sample once the break period has finished and participants have undertaken an additional two week dietary restriction period. This extra faecal sample will act as the baseline sample to determine whether the second dietary phase has caused a change in the numbers of lactobacilli, or modulated the gut community composition as a whole.

The participants will be asked to provide a urine sample, prior to each test phase, which will be used to test compliance to the dietary restriction, and two 24 hour urine collections, which will start after eating the first portion of Brassica in each of the two test phases. The 24 hour urine collection samples will be used to analyse the amount of ITCs excreted in the participant's urine after consuming a single portion of Brassica. The participants will be asked to eliminate all Brassica vegetables and ITC-containing food (except those provided) from their diet for a minimum of 8 weeks: two weeks prior to test phases 1 and 2, as well as during the full 2 weeks of each test phase. There will be an additional minimum 2 weeks dietary restriction, should the study break be required. In order to assess the compliance of the participants to the dietary intervention and monitor any dietary changes during the study, they will be asked to keep food diaries for a consecutive 7-day period in each 2 week test phase, and for the last seven days in the 2 week period following the second test phase, in which the participants will be asked to eat their habitual diet with no dietary restrictions. The purpose of the dietary restriction is to ensure that set amounts of Brassica are being consumed during the test phases, therefore all of the ITCs recovered from the urine during the test phases should originate from the Brassica vegetables provided.

The study will be carried out at the Institute of Food Research (IFR) in Norwich and will be led by Professor Richard Mithen and run by Mr Lee Kellingray with assistance from Dr Joanne F Doleman where necessary. Dr Shikha Saha will be responsible for some of the sample analysis carried out at IFR. Students on placements may also help with supervised administrative and simple laboratory duties associated with the study at the IFR.

Figure 2: Flow diagram of EBL study



Approach

The study is a randomised human dietary intervention cross-over study and will require the participants to consume both a low and a high Brassica vegetable diet. For the low Brassica diet test phase, the participants will be provided with one 84g portion of frozen broccoli and one 84g portion of frozen cauliflower, to be consumed across a period of two weeks. The participants will be asked to consume one of these portions of Brassica (either broccoli or cauliflower) in week one, and then consume the remaining portion of Brassica in week two, with a minimum of 5 days between consuming the two portions. When on the high Brassica diet test phase, the participants will be provided with six 84g portions of frozen broccoli, six 84g portions of frozen cauliflower, and six 300g portions of frozen broccoli and sweet potato soups (each with a broccoli content of 84g), to be consumed across a period of two weeks. The participants will be asked to consume three portions of broccoli, three portions of cauliflower, and three portions of the broccoli and sweet potato soups each week for two weeks, consuming a minimum of one portion per day. The Brassica diet test phases will be separated by a washout period, which will be a minimum of 2 weeks.

The participants will be asked to restrict their diet of Brassica vegetables and ITC-containing foods for a period which includes 2 weeks prior to test phases 1 and 2, as well as during the full 2 weeks of each test phase. The participants will be asked to provide faecal samples, urine samples, complete food diaries and stool charts at various stages throughout the intervention. This is described in more detail below. Participants will be asked to complete a food diary, recording the consumption of all fruits and vegetables, for a consecutive seven day period, during each of the two test phases (test phases 1 and 2), and for the entire seven days of the last week of the study when consuming their habitual diet. Participants will also need to be willing to complete stool charts noting the frequency and consistency of their bowel movements. Participants who do not have a BMI between 19.5 and 30 kg/m^2 will be excluded on the grounds that their gut microbiota will be negatively affected, as studies have shown that the gut microbiota of individuals with a BMI between 18.5 and 30 kg/m^2 are significantly different to obese or underweight individuals [26, 59].

Recruitment strategy

The study population will consist of men and women between the ages of 18 and 50 who meet the study inclusion criteria, and recruitment will continue until 10 participants complete the study. If a potential participant registers an interest in taking part in the study, and we are near to ten participants completing the study, the potential participant may be asked if they would be happy to be placed on a standby list. Those placed on the standby list will neither be consented nor screened until they are recruited onto the study, and will be notified if their participation is not required. Those on the standby list may ask to be removed from the list at any time. The HNU volunteer database will be accessed by the HNU Senior Research nurse and it is anticipated that this will be the prime source of participant recruitment for the study. Apparently healthy potential participants from the HNU volunteer database, who meet the basic inclusion criteria, will be sent a letter of invitation (Annex 1) to participate in the study. This will be supported by the Participant Information Sheet (PIS, Annex 2) and a response slip and pre-paid envelope will be included for returning the slip if they are interested and wish for further information. The HNU volunteer database contains names and contact details of approximately 1600 people above the age of 18 years who have registered an interest for participating in human studies at IFR. Initial contact with potential participants from the database, including the posting of the invitation letter and PIS, is carried out solely by the HNU Senior Research nurse. The available numbers for recruitment on the database are subject to variation as a result of the recruitment of participants by other studies.

Along with using the database as a source of recruitment, advertisements (Annex 3a & 3b) will be placed around the Norwich Research Park (University of East Anglia (UEA), John Innes Centre (JIC), The Genome Analysis Centre (TGAC) and IFR), in the local press and other appropriate locations for example, supermarkets, social clubs, church newsletters, golf and other sporting clubs, gymnasiums and leisure facilities within the local area (a 40 mile radius of IFR). The posters advertising this study may have tear off contact slips attached to the poster in order to facilitate participant recruitment. If required, we will also obtain radio (and television) coverage, and place advertisements in the local press to raise local awareness of the project. Social networking sites like Facebook and Twitter may also be used to either display the ethically approved advert for the study, or to direct potential participants to an IFR website containing the ethically approved advertisement and contact details of members of the study team. A

member of the study team will send interested responders a letter of invitation (Annex 4) and a copy of the PIS (Annex 2) with accompanying response slip and pre-paid envelope in which to return the completed response slip to the study manager, if interested in taking part in the study.

Following an expression of interest, potential participants will be contacted by telephone or e-mail by a member of the study team (study manager or study scientist) to arrange an appointment for a pre-study talk. This talk, in which all aspects of the study will be discussed, will take place in the HNU by a member of the study team, and will involve visual aids to increase understanding of what will be required from the participant should they decide to take part in the study. The potential participants will be encouraged to ask questions at this point, prior to making any commitment. At the end of the talk all potential participants will be given a minimum of 72 hours to consider whether they wish to participate in the study, and during this consideration period they will not be contacted. If, following this period of consideration, the participant still wishes to take part they will be asked to contact the investigator named on the PIS. If however, potential participants have decided after the talk that they are keen to take part in the study and request to book their eligibility screening appointment, the screening appointment will be made for a minimum of 72 hours after the study talk. Before leaving the HNU, all potential participants will be supplied with a small clean container and a copy of the Bristol Stool Chart. Should the potential participant wish to take part in the study, the container will be used for a midstream urine sample from within 2 hours of their eligibility screening appointment. The potential participant will be told that should they decide to take part in the study, the HNU nurse will go through the Bristol Stool Chart with them at their eligibility screening appointment and ask them what their typical stool type is using the Bristol Stool Chart as a guide. Therefore, the potential participants will be advised that they will need to assess their typical stool type, referring to the Bristol Stool Chart, prior to their eligibility screening appointment. The potential participant will be informed that should they decide against taking part in the study, they may dispose of these as they see fit. On booking an eligibility screening appointment, a member of the study team will post an appointment card (Annex 5) to the potential participant.

All those responding positively following this period of consideration will be invited to attend the HNU for an eligibility screening. Participants will be reminded to bring a midstream sample of urine in the container supplied to them following their pre-study talk. Participants will need to collect their urine sample from within 2 hours prior to the

screening appointment as this is a required specification for the validity of the urine dipstick patch test (this will not be tested until after the consent form has been signed). Participants will also be reminded to bring with them details of any prescribed medication, herbal remedies or dietary supplements (i.e. name of medication, dose taken). The HNU is situated on the main site of the IFR, however it is independent of the main building. The HNU facilities include a clinical room; diet kitchen; a lounge/dining area; a laboratory, interview rooms/rest rooms; anthropometry/PWV room, temperature monitored food fridges/freezers/walk in food freezer and dedicated parking area. The unit is permanently staffed by a registered general nurse, however on screening days, members of the study team will also be present.

Eligibility screening (HNU - Visit 1)

On arrival at the HNU the participant will be taken into a confidential room where a member of the study team (study manager or study scientist) or the HNU Senior Research Nurse will go through the consent form (Annex 6) with the participant and encourage any questions they may have at this stage. Participants will then be asked to sign the consent form agreeing to participate in the study and to all their eligibility screening results being sent to their GP. The participants will also be asked to sign a medical declaration form (Annex 16) agreeing to inform the study team of any medication they may have to take, illnesses suffered, or if they become pregnant during the study. A signed copy of both the consent form and medical declaration form will be given to the participant to keep, and each participant will be assigned a unique eligibility screening code number.

Following consent, the HNU nurse will test the urine sample using Multistix ® SG urine dipstick patch test strips (Siemens Healthcare Diagnostics). The nurse will then complete a screening questionnaire with the participant (Annex 7), measure and record blood pressure (BP), pulse, height (cm) and weight (kg) and calculate Body Mass Index (BMI, kg/m^2). The screening questionnaire contains specific questions pertaining to stools, the HNU nurse will explain the Bristol Stool Chart and should the participant indicate that their typical stool type is abnormal (type 1, 2, or 7), they may be referred to their GP and will be excluded from the study. The urine dipstick results will be known immediately. If any of the results for the urine dipstick patch test are flagged, the nurses will refer to the HNU Protocol ‘For the referral of abnormal urinalysis results at

screening' which provides advice on exclusion or re-screening of the participant. This protocol has been authorised by the HNU medical advisor. In the event of a flagged urinalysis indicating a re-screen is appropriate, the HNU nurse will speak to the participant regarding their results and they will be advised to visit their GP/practice nurse prior to returning for a re-screen. If the urinalysis results are flagged on the second occasion the participant may be excluded depending on the tests flagged. If blood is flagged in the urine sample of female participants they will be asked if they are menstruating or have just finished menstruating, if they answer yes to either they will be asked to provide a second urine sample for testing 5 days after finishing menstruation. In the event of a flagged urinalysis on the second occasion, which indicates they may be re-screened, the HNU nurse will speak to the participant and they will be advised to speak to their GP regarding their results prior to coming back to the HNU for a re-screen.

If the flagged urine results indicate exclusion from the study is appropriate, the HNU nurse will speak to the participant, they will be excluded from the study and the participant will be advised to speak to their GP regarding their results. Although participants will be given a copy of their flagged urine results to take with them to their GP, a member of the study team will send a copy of all the eligibility screening results (blood pressure, pulse, weight, BMI, and urinalysis results) to the GP (Annex 10), and this will be supported with a letter to their GP detailing their clinical results (urine analysis, blood pressure, pulse, weight and BMI) (Annex 11).

The GPs of those successfully recruited onto the study will be informed of their patient's participation in the study by letter (Annex 8) along with a study description (Annex 9). It is expected that all participants who successfully pass the screening, and who wish to continue, will commence on the study within six weeks of their screening appointment otherwise a re-screen will be necessary.

Once recruited onto the study, the participant's code number, which they were assigned at screening, will be used on all of their samples (faecal and urine). Only the members of the study team named in the approved documentation will be able to link the codes to participants' names, however the code may be broken in the event of a medical emergency as deemed appropriate and necessary by the HNU Senior Research Nurse, the HNU medical advisor, or a UEA doctor who provides emergency medical cover. All personal information will be kept confidential and known only to the

chief investigator, members of the study team, HNU nurses, HNU medical advisor and the participant's GP.

Inclusion criteria

- Men and women aged between 18 and 50.
- Smokers and non-smokers.
- Those with a body mass index (BMI) between 19.5 and 30 kg/m^2.
- Those that live within a 40 mile radius of Norwich.

Exclusion criteria

- Women who are or have been pregnant within the last 12 months, or are lactating and/or breast feeding.
- Those currently suffering from, or have ever suffered from, any diagnosed gastrointestinal disease, gastrointestinal disorders including regular diarrhoea and constipation (excluding hiatus hernia unless symptomatic) and/or have undergone gastrointestinal surgery, or the study intervention/procedure is contraindicated.
- Have been diagnosed with any long-term medical condition that may affect the study outcome (e.g. diabetes, haemophilia, cardiovascular disease, glaucoma, anaemia). These will be assessed on an individual basis.
- Those diagnosed with a long-term medical condition requiring medication that may affect the study outcome.
- Those regularly taking self-prescribed over the counter medications for digestive/gastrointestinal conditions.
- Those on long-term antibiotic therapy. Those who have been on a course of antibiotics are able to participate in/continue on the study once 4 weeks has elapsed from the end of the course of antibiotics. This will be assessed on an individual basis.
- Those regularly taking laxatives (once a month or more).
- Those intermittently using pre &/or probiotics unless willing to abstain for 1 month prior to and during study period. (If used regularly (3+ times a week, and

for more than one month) and will continue throughout study period then do not exclude).

- Those on a diet programme or those who plan to start a diet programme during the study that may affect the study outcome (e.g. the 5:2 fasting diet) unless willing to abstain for 1 month prior to and during study period. These will be assessed on an individual basis.
- Those taking dietary supplements or herbal remedies (including those derived from Brassica plants) which may affect the study outcome – unless the participant is willing to discontinue taking them for 1 month prior to and during study period. Please note that some supplements may not affect the study and this will be assessed on an individual basis.
- Regular/recent (within 3 months) use of colonic irrigation or other bowel cleansing techniques.
- Recently returned to the UK following a period abroad, and who have suffered gastric symptoms during the period abroad or on return to the UK. These will be assessed on an individual basis.
- Parallel participation in another research project which involves dietary intervention and/or sampling of biological fluids/materials. Sampling of certain biological samples, such as saliva, may not affect the study and this will be assessed on an individual basis.
- Those who record blood in their stools or have two or more episodes of type 1, 2, or 7 stools during the study.
- Any person related to or living with any member of the study team.
- Those who are unwilling to provide GPs contact details.
- Those who are unable to provide written informed consent.
- Those who are not suitable to take part in this study because of their eligibility screening results.
- Those who do not have access to a freezer.
- Those who regularly consume more than 15 units of alcohol (women) or 22 units of alcohol (men) a week.
- Those who are allergic to any of the foods/ingredients within the foods supplied.

Study procedures:

Stool chart

During the study the participants will be asked to keep a record of the frequency and consistency of their bowel movements using the Bristol Stool Chart as a guide (Annex 12). The participants will be asked to keep this record for a period of seven consecutive days during each of the two test phases (test phases 1 and 2), as well as the seven days that constitute the final week of the study. These scores will be used as an estimate of gut function. If the participants stool chart indicates an abnormal stool pattern, they may be advised by the HNU Senior Research Nurse to speak to their GP, who will receive a copy of the participants stool chart. Any participant who records two or more episodes of type 7 stools, or the presence of blood in their stools, on their stool chart, will be excluded and a copy of their stool chart will be sent to their GP.

Food diary

During the study the participants will be asked to record the types and amounts of fruits, and vegetables they have consumed in a food diary (Annex 13). The participants will be asked to keep this record for a period of seven consecutive days during each of the two test phases (test phases 1 and 2), as well as for the seven days that constitute the final week of the study. The participant will be asked to complete this diary on the same days as the stool chart, but if for any reason this is not possible, then the participant will be free to complete the diary on days within the set periods that are suitable for them. This will be used to discern whether the participants' customary food intake may influence the effects seen during test phases 1 and 2, and to help confirm compliance to the dietary restriction. The food diaries will be analysed within the IFR using software such as Dietplan 6, which has been used successfully in previous human intervention studies at the IFR.

Faecal collection

Participants who successfully pass the eligibility screening will be provided with a faecal collection kit. The faecal collection kit will consist of an IFR carrier bag

containing a faecal collection pot, freezer blocks or ice cube bags, an autoclavable sample bag for collecting the faeces in, a plastic clip (for sealing the autoclavable bag), nappy sack and an insulated container for transporting the sample securely back to IFR. The participant will also be provided with study specific instructions (Annex 14) as to how to collect and store the faecal sample and how to contact members of the study team or HNU nurse regarding arrangements for collection or delivery of the faecal sample to the HNU, for processing at the IFR.

Prior to collecting the faecal sample each participant will be required to either a) place the provided freezer blocks in their own freezer overnight, or b) fill the provided ice cube bag with water and place in their own freezer overnight, ready to place in the insulated container with their faecal sample. Detailed instructions will be provided as to how to collect their faecal sample directly into the labelled plastic autoclavable sample bag in the collection pot. Once the sample has been collected the autoclavable sample bag should then be sealed closed with the plastic clip, removed from the collecting pot and placed into the nappy sack which should be closed by tying the handles in a double knot. The tied nappy sack, containing the sample bag and the previously prepared freezer blocks/ice cube bags should be placed into the insulated container which should then be sealed shut. Participants will be advised to wash their hands after collecting the samples. Participants will be asked to write the date and time of sample collection on a label on the lid of the insulated container and contact a member of the study team to arrange delivery or collection of the sample.

All containers used for faecal collections will be sterilised prior to being given to the participant, to prevent contamination and minimise infection risk. The importance of hand washing after sample collection by participants will be reiterated in the faecal collection instruction sheet.

It is hoped that the participants will be able to bring their faecal sample to a member of the study team at the HNU, however it may be that a participant requires a member of the study team to collect their faecal sample, and this will be arranged on an individual basis.

When receiving a faecal sample, a member of the study team will collect the entirety of the faecal collection kit from the participant, except for the sample collection pot which will have been disposed of. Once the first faecal sample has been received by a member of the study team, an appointment will be booked with each participant for the

start of test phase 1. The appointment will be booked for a minimum of two weeks after the faecal sample delivery in order to allow the participant to commence the Brassica dietary restriction, two weeks of which must be complete before the start of test phase 1 (optional appointment card Annex 5). In total there will be an eight-week dietary restriction: 2 weeks prior to, and during, each 2 week test phase (1 and 2). However, should participants require the optional study break this will increase to a 10 week diet restriction in total. Each participant will be supplied with a new faecal collection kit and a small clean container for the urine sample. The participants will be reminded that the urine sample they provide must be from the first day of test phase 1, i.e. after the first two weeks of the dietary restriction period.

Identical faecal collection kits and instructions will be provided to the participants prior to the collection point of all further faecal samples, and participants will be asked to collect the faecal samples in an identical manner to the first faecal sample. Should any participant suffer from diarrhoea during the study, they will be required to contact a member of the study team and will be asked to refrain from collecting a faecal sample for a minimum of 48 hours after the last episode of diarrhoea. Should the diarrhoea persist for more than 72hrs, the participant may be advised by the HNU Senior Research Nurse to speak to their GP and will be excluded from the study.

2 weeks prior to test phase 1 – Weeks 1-2 of diet restriction

Participants will be asked to eliminate all Brassica vegetables and other ITC-containing foods from their diet for two weeks prior to test phase 1. Following a minimum period of two weeks, the participant will collect a faecal sample in the collection kit provided. This faecal sample will be delivered to the HNU by the participant, or collected by a study team member if necessary. This will help to interpret any changes in the participants gut microbiota that are caused by the dietary restriction. A separate sheet detailing foods to be avoided will be provided to each participant (Annex 15), as well as the information being found in the PIS (Annex 2).

Test phase 1 (HNU - Visit 2)

Participants will be asked to visit the HNU and bring both their faecal sample, if they have not already done so, and a urine sample from that day in a container that will have been provided with their faecal collection kit. The scientist will check with the participant that the urine was produced that day and that they are happy to continue on the study. Should the participant forget to bring the sample with them, they will be given the opportunity to produce one at the HNU. The participant will be asked to complete both the food diary and stool chart for seven consecutive days during the two week intervention period, and a container for the 24hr urine collection will be provided. The participant will receive either two or eighteen portions of frozen Brassica vegetables/soups, dependent on whether they have been randomly assigned to the low or high Brassica diet in test phase 1, and will be asked to store them in their home freezer. In addition, the participant will be supplied with a steamer and will be asked to use this to steam the provided Brassica vegetables following detailed instructions which will be provided (Annex 18). The participant will be asked to refrain from steaming other items in the steamer at the same time as the supplied diet. If the participant does not have enough room in their freezer for the two week supply of Brassica, they may take one week's supply and collect the remainder towards the end of the first week. Those randomly assigned to the low Brassica diet will receive one portion of frozen broccoli (84g) and one portion of frozen cauliflower (84g). Those randomly assigned to the high Brassica diet will receive six portions of frozen broccoli (84g), six portions of frozen cauliflower (84g), and six portions of broccoli and sweet potato soups (300g with a broccoli content of 84g). The participant will be asked to consume either 1 (low Brassica diet) whole portion, or 9 (high Brassica diet) whole portions of these vegetables/soups a week, for a two week period. Participants on the low Brassica diet will be asked to leave a minimum of five days between consuming each portion, whilst those on the high Brassica diet will be asked to consume a minimum of one portion a day, across the 2 week period. The participants will be provided with instructions for cooking the vegetables & soups (Annex 18).

Following the consumption of the first portion of Brassica, the participant will be asked to collect all the urine they produce over the next 24 hours in the urine container provided. The urine containers for the 24hr urine collections will contain approximately 1g of powdered ascorbic acid (vitamin C) as a stabilising agent to prevent rapid deterioration of the urine. The participants will be advised to keep the containers away

from children and pets, and should they get the powder on their skin they should wash it off as soon as possible. The participants will be reminded to maintain their Brassica vegetable and ITC-containing food dietary restriction. The participants will be asked to refrain from consuming any further Brassica until the 24hr urine collection is complete. The participants will return to the HNU with their 24hr urine collection once completed, however it may be that a participant requires a member of the study team to collect their 24hr urine collection, and this will be arranged on an individual basis. Upon return of the 24hr urine collection, the participant will be given a faecal collection kit (as described in Study procedures: Faecal collection). Following the completion of test phase 1, the participant will be asked to collect their first bowel movement as described in Study procedures: Faecal collection. Once collected, the faecal sample, along with the food diary and stool chart, will be delivered to the HNU by the participant, or collected by a study team member if necessary.

Washout phase

Upon return of the faecal sample from the end of test phase 1, the participant will be reminded to continue the Brassica vegetable and ITC-containing food dietary restriction. The participant will be provided with a faecal collection kit and a small clean container for the urine sample required on the first day of test phase 2. The participant will be asked to provide a faecal sample (as described in Study procedures: Faecal collection) after a minimum of two weeks following the end of test phase 1. If, shortly after this two week period, the participant has not contacted the study team, a member of the study team will contact the participant to ascertain whether the participant would like to continue with the study. Upon delivery of the faecal sample to the HNU, the participant will be reminded that the urine sample they provide must be from the first day of test phase 2. Following the delivery of the faecal sample, there is an opportunity for a break in the study, should this have been previously agreed between the participant and the study team. This would allow the participant to cease the dietary restrictions for a maximum of six weeks, providing they are willing to restrict their diet for a minimum of 2 weeks and produce a faecal sample prior to continuing the study.

Test phase 2 (HNU - Visit 3)

See test phase 1 for description.

Habitual diet phase

Once the faecal sample, food diaries, and stool charts from test phase 2 have been delivered to the HNU or collected by a study team member, the participant will be reminded that the Brassica vegetable and ITC-containing food dietary restriction has ended and to consume their normal diet. The participant will be provided with a faecal collection kit and will be asked to provide a faecal sample (as described in Study procedures: Faecal collection) after a minimum of two weeks on their normal diet following the end of test phase 2. During the last week of this two week period, the participant will be asked to complete both the food diary and stool chart for the entire 7 days. If, shortly after this period, the participant has not contacted the study team, a member of the study team will contact the participant to ascertain whether the participant would like to complete the study. Upon delivery of the faecal sample, food diaries and stool charts to the HNU, the participant will have completed the study.

Completion of the study

Upon completion of the study, the general findings of the study will be reported back to the participants in the form of a basic brief summary.

Adverse event (AE) and Serious adverse events (SAE)

Any Adverse Events and Serious Adverse Events/Reactions will be reported in accordance with The National Research Ethics Service (NRES) and International Conference on Harmonisation – Good Clinical Practice (ICH-GCP) rules and the appropriate NRES form will be used to report the occurrence of any SAEs/Serious Adverse Reactions (SARs). All adverse events/reaction and SAEs/SARs will be

documented in the participant-specific case report form (Annex 17), signed by the HNU Medical Advisor and Chief Investigator, and held on file at the IFR. The report will include, the nature of the event, severity (mild, moderate or severe), and relationship to the study (definitely, probably, possibly, not related). Advice will be given by the HNU Medical Advisor as to the cause, relationship and any required further action (depending on the type of reaction); duration (dates and times), interventions and outcomes.

A serious adverse event (SAE) is defined by ICH GCP as an untoward occurrence that:

- Results in death
- Is life threatening
- Requires hospitalisation or prolongation of existing hospitalisation
- Results in persistent or significant disability or incapability
- OR, for IFR purposes, is otherwise considered medically significant by the HNU medical advisor

The participant will only be deemed to have suffered an adverse event/reaction or a serious adverse event/reaction if the participant has taken part in any of the intervention. The Chief Investigator will report all SAEs/SARs to the research ethics committee (REC) and human research governance committee (HRGC) as soon as they become aware, using the standard NRES form. Reports of SAEs/SARs will be provided to the REC within 15 days of the event being reported to a member of the study team. Once the incident has been resolved, both REC and HRGC will be informed of the outcome. However, as participants are asked questions relating to allergy at screening, no adverse events or reactions are expected.

Methods:

Study sample urine analysis

The total volume of urine collected will be recorded, mixed thoroughly and sub samples totalling no more than 40ml will be stored at –80°C until analysis. The remaining urine will be discarded.

ITC analysis

ITCs will be analysed in 2 ways:

1. A cyclocondensation assay will be used to quantify the total ITCs (PEITC, BITC, SF & ER and their conjugates; glutathione, cysteine-glycine, cysteine and N-acetyl cysteine) in the urine samples according to the method stated in Ye *et al* [60]

2. A validated liquid chromatography-mass spectrometry method will be used to measure the profile of the ITCs in the urine samples via an adapted method from Gasper *et al* [61]. The following ITCs and their conjugates will be measured: phenyl ethyl isothiocyanate (PEITC); benzyl isothiocyanate (BITC); sulforaphane (SF) and erucin (ER) and their conjugates; nitrile, glutathione, cysteine-glycine, cysteine and N-acetyl cysteine. Authentic standards of the conjugates are already available at the IFR laboratory.

Assessment of dietary restriction compliance

The urine samples collected prior to test phases 1 and 2 will be analysed for ITCs in the same way as the 24hr urine collection. It is hoped that this will provide baseline 'ITC free' urine samples for each participant. The presence of urinary ITCs in the baseline samples would indicate the participant's non-compliance with the Brassica vegetable and ITC-containing food dietary restriction period, prior to the test phases. Should non-compliance be evident, any data generated from that participant will be removed from the study analysis. From our previous experience with human dietary intervention studies we expect that the vast majority of participants will comply with the dietary restriction and do not foresee that it will be necessary to recruit further participants to compensate for the lack of data from any non-compliant participants.

Faecal sample analysis:

Phylogenetic analysis

The faecal samples produced by the participant will either be delivered to a member of the study team by the participant, or a member of the study team will arrange to go and collect the samples. Upon receipt of a faecal sample, part of it will be divided up into aliquots totalling no more than 10g, and stored at –80°C until analysis. The DNA will be extracted from faecal aliquots using the FastDNA spin kit for soil (MP Biomedicals) according to the method of Maukonen *et al* [62] and stored at –20°C. Part of the extracted DNA from the faecal samples will have the 16S rDNA genes amplified by PCR, prior to being commercially sequenced using a Next Generation platform such as Illumina. Faecal bacterial phylogenetic analysis will give both the bacterial genera present and the relative proportions of each genus within the sample. This will make it possible to observe shifts in the proportions of lactobacilli within the faecal microbiota of each participant, due to a) the consumption of the different Brassica diets compared to one another, and b) the consumption of the different Brassica diets compared to the baseline sample obtained at the start of the study. This information can then be compared with the ITC excretion data and used to try and determine whether the faecal microbiota composition correlates to the rates of ITC excretion.

Bacterial enumeration

Quantitative polymerase chain reaction (qPCR) is a validated laboratory technique which can be used to identify the numbers of specific bacteria present in a sample. This technique will enable an examination of how the consumption of the two Brassica diets may have caused a change in the actual numbers of lactobacilli, rather than proportions of lactobacilli, within the faecal bacterial communities, compared to one another, and compared to the baseline faecal sample. qPCR will be used to amplify the 16S rDNA genes using lactobacilli-specific primers to give the number of lactobacilli present in the samples. This will be performed using DNA extracted from faecal aliquots, which will be precipitated and concentrated before being washed and the resultant pellet re-suspended in 20µl buffer TE for use as the qPCR template.

Faecal metabolite analysis

Short chain fatty acids are bacterial waste products that are beneficial to the host, and as such can be considered a biomarker of good gut health. Nuclear magnetic resonance spectroscopy (NMR) will be used to determine the profile of metabolites such as short chain fatty acids and bile acids from faecal aliquots. Faecal aliquots will be diluted 10-fold using PBS, centrifuged at 10000rpm and the supernatant passed through a 0.2um filter. 70ul of a buffer solution will be added to 630ul of the filtered supernatant. This will be vortexed and 600ul will be added to an NMR tube for spectral acquisition. Data analysis will be performed at the IFR.

Metatranscriptomic analysis

If differences are seen in the phylogenetic, metabolomic, or ITC excretion rate between participants which warrant further investigation, metatranscriptomic analysis will be performed to monitor changes in bacterial gene expression. Metatranscriptomics is an approach which allows an examination of which genes are expressed at a given time, and to what extent. Metatranscriptomic analysis will use faecal aliquots which have had 20% v/v phenol/ethanol buffer added, before being mixed and incubated on ice for 30 minutes. The samples will then be centrifuged and the pellet will be re-suspended using an aliquot of the supernatant, prior to being stored at –80°C until analysis. The samples will be homogenised using a FastPrep instrument (MP Biomedicals), and centrifuged to obtain the supernatant from which the total RNA will be extracted. mRNA enrichment, cDNA synthesis and, if necessary, cDNA amplification may be performed at the IFR, prior to the samples being commercially sequenced.

Statistics:

Power calculations

The average expected population of lactobacilli in human faecal samples, and the expected change due to a dietary intervention has been estimated using data contained in the paper by Tannock *et al* [63]. These data suggest that the mean lactobacilli present in human faecal samples are 4.57 (log10 bacteria/g) with a standard deviation of 2.8 (log10 bacteria/g).

The proposed study design will use participants as their own controls by measuring the lactobacilli present in their faecal sample prior to the test phases (control) and comparing this to the lactobacilli present in their faecal samples produced after completing each two week test phase (intervention).

For the sample size calculation, we set the power to be 80% and alpha (α) to be 0.05 (2-tailed). Using the information from Tannock *et al* [63], the study would require 10 participants (in total) to detect a significant difference in the number of lactobacilli (2.79 log10 bacteria/g) present in faecal samples, between the normal diet (baseline faecal sample) and the high Brassica diet intervention.

Analysis of data

The data obtained from analysis of the urine and faecal samples, except the metatranscriptomic data, will be analysed using a paired t-test or non-parametric equivalent. The correlation analysis of ITC excretion rates and the gut microbiota composition will be performed using a Pearson product-moment correlation with a test for significance versus zero correlation (r=0). All analysis will be carried out in the R statistical package [64]. The metatranscriptomic analysis will be carried out using the R statistical package with the use of add-on packages, such as Bioconductor, which are specifically designed for RNA analysis. The data analysis will be undertaken by IFR's own statisticians, including Dr Jack Dainty who performed the power calculations for this study.

Ethical considerations:

Informed Consent

Before participation in the intervention study, all participants will be asked to give written informed consent. Prior to consent being given, the participant will be provided with the Participant Information Sheet, which provides all the information about their involvement in the study and we will ensure that all their questions are answered. The ability of participants to give informed consent will depend on them receiving enough information about the study, the participant exercising their right to choose, the

participants ability to understand the information and the ability of the participant to make a decision.

Food safety

The HNU follows local Environmental Health Guidelines for the preparation and storage of food for study participants. All staff handling, preparing or delivering food for the participants will hold Level 2 Food Safety Certificates or will be supervised by a member of staff who holds a Level 2 Food Safety Certificate.

Toxicity

There is no evidence from animal or human studies that a diet rich in Brassica is harmful. In the Li *et al* study [58], 17 participants were asked to consume between 660 and 1080g per day for 14 days when on the high Brassica diet, and they did not report any tolerability issues. For the high Brassica diet in this study, we will be asking participants to consume 504g of Brassica vegetables and 900g of soup, containing 84g of broccoli per soup, per week. That is a total of 756g of Brassica vegetables per week for a period of two weeks, which is lower than the daily amount some of the participants in the Li *et al* study were asked to consume. We therefore do not expect any tolerability issues with the amount of Brassica consumed in this study.

Participant's expenses/inconvenience payments

Eligibility screening urine sample (x1)
£2
Urine sample on first day of test phases (x2) ∴ 2 x £2 £4
24hr urine collection (x2) ∴ 2 x £10 £20
Individual stool collection (x6) ∴ 6 x £5
£30
8 week diet restriction ∴ 8 weeks at £14 per week £112
21 day 'Household measures' food diary ∴ 21 days at £5 per day completed £105
21 day Stool chart ∴ 21 days at £2 per day completed £42
20x Brassica consumption ∴ 20 portions at £1 per portion consumed £20

TOTAL = **£335**

Participants will receive £335 as an inconvenience payment if the study is completed; if the participant withdraws or is excluded from the study, *payment will be pro-rata*. However, if the participant undergoes rescreening the inconvenience payment could total £339. Furthermore, if it is necessary for there to be a study break (as described previously), an additional £33 will be paid in recognition of the added inconvenience, due to the extra 2 weeks dietary restriction and faecal sample. Participants travelling by car will be reimbursed travel expenses to and from the HNU (at IFR). This will be reimbursed at the IFR's current mileage rate. Those participants travelling by public transport will be reimbursed costs on production of a ticket or receipt. If participants require transport, the study can provide a taxi to and from HNU. This is paid for by the study.

Study partners

The study will be sponsored by the IFR and funded through the NAT4LIFE project by the Danish Council for Strategic Research.

The study will be led by Professor Richard Mithen and run by Mr Lee Kellingray with assistance from Dr Joanne F Doleman.

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Date

Dear_____,

Thank you for your interest in studies at the Institute of Food Research.

I have sent you the details of,

Effects of Brassica on human gut Lactobacilli (EBL study)

which is one of the studies in progress at present, as your details currently held on the database indicate that you may fit the criteria for this study. If you are interested in participating in this study, please complete and return the reply slip in the enclosed participant information sheet. If you have any further questions, please contact the study manager concerned, Mr Lee Kellingray on **01603 255308** or lee.kellingray@ifr.ac.uk as stated on the enclosed participant information sheet.

If, however, any of your details have changed or change in the future, or you would prefer to no longer remain on the database please could you inform the Human Nutrition Unit on 01603 255305.

Thank you.

Yours sincerely,

Aliceon Blair
HNU Senior Research Nurse

Invitation to take part in a Research Project

Effects of Brassica on human gut Lactobacilli (EBL study)

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen if you take part
Part 2 gives you more detailed information about the conduct of the study

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. This information sheet is yours to keep.
Thank you for reading this.

This study is funded through the NAT4LIFE project with the Danish Council for Strategic Research.
This study has been approved by a Local Research Ethics Committee.

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PART 1 of the information sheet

What is the purpose of the study?

Brassica vegetables (see list on page 11 of this information sheet) are an important part of our diet. Unlike other vegetables which form part of our daily diet, they contain compounds which have a protective effect associated with a reduced risk of many chronic diseases, such as cancer. The protective effect may be as a result of the breakdown of these compounds, which are called glucosinolates (GSLs).

In the vegetable, the GSLs are converted into active compounds called isothiocyanates (ITCs) by a protein called myrosinase. When we cook Brassica vegetables thoroughly the myrosinase in the vegetable stops working and the change from GSLs to ITCs is carried out by bacteria in our gut instead. It is the ITCs that are thought to be responsible for the protective effects against diseases.

Our gut contains trillions of bacteria, many of which help us to unlock extra nutrients from the food we eat. Some bacteria, called lactobacilli, are often referred to as 'good bacteria' and are added to probiotic products, such as yoghurts. These bacteria are naturally found in our gut and some foods are thought to be able to increase their numbers, and therefore may increase the health benefits associated with them.

In this study we will use the Brassica vegetables broccoli and cauliflower, as well as a broccoli and sweet potato soup. We are interested in whether eating Brassica causes an increase in lactobacilli in our gut.

What we aim to do:

We will look at whether a low or high Brassica vegetable diet causes a change in the number of gut lactobacilli.

Additionally, as it has been shown that people are able to change GSLs to ITCs at different rates and in different amounts, we would like to look at the rate at which you get rid of ITCs from your body by collecting urine samples.

Finally, we would like to look at your gut bacteria as an entire community to see what types we can find. We will do this by asking you to collect faecal samples. We will then extract the bacteria from these samples and use this to find out whether the different types of bacteria in your gut affect the rate and/or the amount of GSLs converted to ITCs by an individual.

What will I have to do:

Participants in the study will be asked to eat a low or high Brassica diet (one or nine portions of Brassica vegetables per week for 2 weeks), then following a 2 week break, they will be asked to consume the other Brassica diet. The order in which you eat the Brassica diets will be chosen by computer using a method called 'random allocation'. This means that neither you nor the scientists will be able to decide in which order you eat the Brassica diets.

During the study you will be asked to collect urine and faecal samples, and complete food diaries and stool charts (frequency and consistency of bowel movements).

Who <u>can</u> take part in the study?

We are aiming to recruit:

- ***Men and women***
- ***Aged between 18 and 50***
- ***Smokers and non-smokers***
- ***Live within 40 miles of Norwich***
- ***Have a body mass index (BMI, kg/m^2) between 19.5 and 30***

Who <u>cannot</u> take part in the study?

Please note: You **<u>will not</u>** be able to take part if you:

- are pregnant, have been pregnant in the last year or are lactating and/or breast feeding.
- are currently suffering from, or have ever suffered from, any diagnosed gastrointestinal disease, gastrointestinal disorders including regular diarrhoea and constipation (excluding hiatus hernia unless symptomatic), and/or have undergone

gastrointestinal surgery, or the study intervention/procedure is contraindicated.
- have been diagnosed with any long-term medical condition that may affect the study outcome (e.g. diabetes, haemophilia, cardiovascular disease, glaucoma, anaemia). These will be assessed on an individual basis.
- have been diagnosed with any long-term medical condition requiring medication that may affect the study outcome.
- regularly taking over the counter medications for digestive/gastrointestinal conditions.
- are on long-term antibiotic therapy. You may be able to participate if 4 weeks has passed from the end of a course of antibiotics (this will be assessed on an individual basis).
- regularly take laxatives (once a month or more).
- take certain dietary supplements or herbal remedies and are unwilling to stop taking them for one month prior to and during study period. This will be assessed on an individual basis.
- take pre- or pro-biotic drinks &/or yoghurts on an occasional basis, unless willing to abstain for one month prior to and during the study period. (if you regularly take pre &/or pro biotics (3+ times a week, and for more than one month) and will continue throughout the study then you will not be excluded).
- on or plan to start a diet programme that may affect the study outcome (e.g. 5:2 fasting diet) unless willing to abstain for 1 month prior to and during study period. This will be assessed on an individual basis.
- recently returned to the UK following a period abroad, and who have suffered gastric symptoms during the period abroad or on return to the UK. These will be assessed on an individual basis.
- regular/recent (within 3 months) use of colonic irrigation or other bowel cleansing techniques.
- are involved in another research project that includes dietary intervention and/or sampling of biological fluids/materials**.** Sampling of certain biological samples, such as saliva, may not affect the study and this will be assessed on an individual basis.
- record blood in your stools or have two or more episodes of constipation or diarrhoea (type 1, 2, or 7 stools) during the study.
- are unwilling to provide GPs contact details.
- are unable to provide written informed consent.
- are not suitable to take part in this study because of your eligibility screening results.
- are related to or living with any member of the study team.
- do not have access to a freezer.
- regularly consume more than 15 units (women) or 22 units (men) of alcohol a week.
- allergic to any of the foods/ingredients within the foods supplied.

Why have I been invited?

You have been sent this information sheet because:

- You responded to an advertisement about the study.
- Or your details currently held on the IFR Human Nutrition Unit (HNU) volunteers' database suggest you may fit the study criteria.

Do I have to take part?

It is up to you to decide. We have described the study in this information sheet. If, after reading it, you are interested in participating, and you live in or within 40 miles of Norwich please complete and return the response form (page 15 of this information sheet) using the pre-paid envelope enclosed. On receipt of the response form a member of the study team will contact you by telephone to arrange a pre-study talk with you at the Human Nutrition Unit (HNU) in Norwich, and give you the

opportunity to ask any questions you may have at this time.
You may also contact the study team by telephone, Lee Kellingray on 01603 255308; Joanne Doleman 01603 251477 or by email lee.kellingray@ifr.ac.uk or joanne.doleman@ifr.ac.uk if you have any questions before or during the study.
Please feel free to say no simply by not responding to this letter. Do not worry, no one will contact you or try to persuade you to join the study.

We aim to recruit a total of 10 participants to complete the study. If you register an interest in taking part, and we are near to the 10 participant point, we may ask you if you are happy to be placed on a standby list. If you are put on the list, there will be no guarantee that you will be required to take part in the study, but we will keep you informed and you are entitled to ask to be removed from the standby list at any time.

Expressing an interest in the study, having a discussion about the study, or attending the screening does not mean you have to take part.
You are entitled to withdraw at any time without giving a reason.

What will happen to me if I take part?
If you decide to take part, your involvement will last for approximately 11 weeks (dependent on whether you go on holiday, or a break is required for other reasons that will not affect the study), from first contact to the end of the study. During this period, the study team will keep in regular contact with you, or you can contact members of the study team, to answer any questions you may have about the study.

For this study, you will have an informal talk with one of the study team at the HNU in Norwich (see below for more information) and you will then need to visit the unit on **3** separate occasions, although additional visits may be necessary for sample delivery;

- Signing consent and the eligibility screening – Visit 1
- To bring your baseline urine sample and collect the first Brassica diet – Visit 2
- To bring a second baseline urine sample and collect the second Brassica diet after the 2 week washout period with no Brassica – Visit 3

Where possible, appointments for screening will be made at your convenience but will take place on a weekday.

Informal talk to discuss the study
An appointment will be made for a member of the study team to discuss the study with you at the HNU. This will last up to one hour. The study team member will go through this information sheet with you and answer any questions you may have.

After this conversation you will be given as long as you need but at least 72 hours (3 days) to decide whether or not you wish to take part in the study. During this time we will not contact you. After the 72 hours if you decide to take part you will need to contact a member of the study team to arrange an appointment for the eligibility screening visit.

After the talk, you will be given a copy of the Bristol Stool Chart and a small container. Should you decide not to take part in the study, you may dispose of these as you see fit. If you do join the study, we would like you to use the Bristol Stool Chart to identify what your typical stool type is, as you will be asked this at your screening visit by the HNU nurse. The container will be used for a urine sample, which you will need to bring to your screening visit. It is important for the urine dipstick test results and study analysis that you do not use any containers other than the ones given to you during the study to collect your urine. If you lose the pots we will provide you with another at your appointment and give you the opportunity to provide another urine sample.

Eligibility screening and informed consent – Visit 1

This visit will last approximately 1 hour, and you will need to bring with you a midstream urine sample, produced within two hours of your screening appointment. This is necessary for the test to work.

On arrival at the HNU you will be asked to sign a **consent form**, agreeing to participate in the

study. This form will also be signed by a member of the study team (study manager or study scientist) or the HNU Senior Research Nurse, and you will be given a copy to keep. Once you have signed the consent form you are still free to withdraw at any time without giving a reason.

After you have signed the consent form, the nurse will carry out a urine dipstick patch test on your urine sample. The results of this will be known immediately and the HNU nurse will discuss the results with you. Depending on your urine dipstick test results you may be excluded from the study or offered a re-screen and advised to speak to your doctor or surgery nurse about your results. The HNU nurse will then complete a medical declaration form, which you will be given a copy of, and a screening questionnaire with you. At this point, the HNU nurse will explain the Bristol Stool Chart and ask you which type reflects your typical stool, using the Bristol Stool Chart as a guide. The HNU nurse will then measure and record your:

- Blood pressure
- Pulse rate
- Height
- Weight
- Calculate your Body Mass Index (BMI, kg/m^2)

Your height and weight measurements are used to calculate your body mass index (BMI, kg/m^2). Body Mass Index is a measure of whether you are a healthy weight for your height. If your BMI (kg/m^2) is outside the range of 19.5 and 30kg/m^2 you will not be able to take part in the study.

We will send copies of all your clinical results (urine results, blood pressure, pulse, Body Mass Index (BMI (kg/m^2) and weight) to your GP. If any of your results are outside the study requirements you may not be able to take part in the study.

After your screening appointment

Following a successful screening appointment, a member of the study team will provide you with a faecal sample collection kit, along with a detailed sheet on how we would like you to collect and store your faecal sample until you can deliver it to us, or it can be collected by a member of the study team. Everything you need to collect the sample will be provided within the kit, including freezer blocks/ice cube bags as you will be required to freeze them to put into the insulated collection pot. This is so the sample can be kept cool until it is handed over to the study team/nurse.

After you have given us your faecal sample, we will arrange a time and date for you to bring in a urine sample and to collect the first Brassica diet (Visit 2). You will be asked to **stop eating any Brassica vegetables and ITC-containing foods, except those provided, for 8 weeks during your involvement on the study.** Should the study break be required, this will increase to 10 weeks. This includes 2 weeks before you collect the first Brassica diet (Visit 2). You will also be required to produce a second faecal sample, collected as before, following a minimum 2 week period of the Brassica vegetable and ITC-containing food diet restriction. Page 11 of this information sheet gives you a list of which foods you will not be able to eat during the study period, and if you are unsure whether you may eat a food item you can ask the study scientists. You will also be asked to complete **food diaries and stool charts for 21 days during the study**.

First Brassica diet collection - Visit 2

On the day you visit the HNU to pick up the first Brassica diet, you will be asked to:

- **Provide a faecal sample**, if you have not done so already, following a minimum of 2 weeks of the diet restriction.
- **Provide a small urine sample** on the morning of your visit to collect the Brassica diet. This will provide a baseline sample in which there should be no ITCs.
- **Continue your diet restriction** eating a diet that is free from Brassica vegetables and ITC-containing foods.

On the morning of the Brassica diet collection, when you arrive at the HNU a nurse or member of the study team will collect your urine sample which you will have brought with you. You will be

asked if you are happy to continue with the study, and whether you have had any changes to your health or medication since your screening appointment which may affect the study data. You will also be reminded to continue with the Brassica vegetable and ITC-containing food diet restriction.

If you are happy to continue, you will be given the first Brassica diet, cooking instructions, and a steamer to cook the vegetables with. Whilst steaming the Brassica we would appreciate it if you didn't add anything else to the steamer. You will be asked to consume the supplied Brassica diet as part of your normal diet across the 2 week period. When on the low Brassica diet, you will be supplied with one 84g portion of frozen broccoli and one 84g portion of frozen cauliflower, and you will be required to eat one of these portions in week one and the other in week two, with a minimum of five days between eating each portion. When on the high Brassica diet, you will be supplied with six 84g portions of frozen broccoli, six 84g portions of frozen cauliflower, and six 300g portions of broccoli and sweet potato soup (each soup contains 84g of broccoli). You will be required to eat three portions of broccoli, three portions of cauliflower, and three broccoli and sweet potato soups each week for 2 weeks, eating at least one portion per day across this 2 week period. During each Brassica diet period you will be asked to record your vegetable and fruit intake for 7 days and to keep a diary about your stool consistency for 7 days, and we will provide you with the Bristol Stool Chart as a guide for this. After eating the first portion of Brassica you will be asked to collect all the urine you produce for a full 24-hour period into the urine collection pot which we will provide, delivering it to the HNU once completed. Alternatively, transport can be arranged to collect it from you.

Please note that the urine container will contain a white powder called ascorbic acid (vitamin C) which is needed to preserve the urine. The urine container should be kept out of reach of children and pets, and away from any heat source or direct sunlight.

At the end of the 2 week Brassica diet intervention, you will be asked to collect a faecal sample in the faecal collection kit provided. When the sample has been produced, you will be asked to deliver it to the HNU, or collection from you will be arranged if required.

When you deliver the faecal sample you will be reminded that you will still need to follow the Brassica and ITC-containing food free diet during the 2 week washout period between the two diet phases. You will also be given a faecal collection kit and, after a minimum of two weeks, you will be asked to bring a faecal sample to the HNU, collected as before. An appointment will be made for you to visit the HNU with a urine sample, in a container provided, which will be used as a baseline sample, and to collect the second Brassica diet (Visit 3). There may be a study break at this point if requested by you, e.g. due to a seasonal holiday period,prior to starting the initial diet restriction. This will allow you to cease the dietary restrictions for a maximum of 6 weeks, providing you are willing to restrict your diet for 2 weeks and produce a faecal sample prior to continuing the study.

Second Brassica diet collection - Visit 3

On the day you visit the HNU to pick up the second Brassica diet, you will be asked to:

- **Provide a faecal sample**, if you have not done so already, following a minimum of 2 weeks of the diet restricted washout period.
- **Provide a small urine sample** on the morning of your visit to collect the Brassica diet. This will provide a baseline sample in which there should be no ITCs.
- **Continue your diet restriction** eating a diet that is free from Brassica vegetables and ITC-containing foods.

As before, you will be asked if you are happy to continue on the study and whether you have had any health problems or change of medication which may affect the study data, since the first intervention period. If you are happy to continue, you will be supplied with the second Brassica diet, which will be the alternate diet to that eaten in the first intervention period.

Again, you will be reminded to continue following the diet restriction and asked to use the steamer to cook the Brassica vegetables and to follow the cooking instructions provided. You will be asked to consume the Brassica diet as part of your normal diet in the same manner as explained earlier in this information sheet. Once again, you will be asked to record your vegetable and fruit intake for 7 days and to keep a diary about your stool consistency for 7 days, referring to the Bristol Stool Chart as a guide. After eating the first portion of the supplied Brassica you will be asked to collect all the urine you produce for a full 24-hour period into the urine collection pot which we will provide. You will be asked to deliver this to the HNU once completed, or collection from you will be arranged if required.

Once again, at the end of the 2 week Brassica diet intervention you will be asked to collect a faecal sample in the faecal collection kit provided. Once produced, you will be asked to deliver this to the HNU as before. On leaving the HNU you will be told that you no longer need to follow the Brassica-free diet. You will be given a faecal collection kit and, after a minimum of two weeks following your normal diet with no dietary restrictions, you will be asked to bring your final faecal sample to the HNU, or it will be collected as before. During the last week of this 2 week period you will be asked to record your vegetable and fruit intake for all 7 days and to keep a diary about your stool consistency for all 7 days, referring to the Bristol Stool Chart as a guide. When your final faecal sample is handed over we will also collect any outstanding food diaries and stool charts. This will signify the end of your involvement in the study.

We understand that there is a lot of information here but please don't worry, you will not have to remember every detail, we will provide you with step-by-step instructions throughout your involvement in the study. In addition, a brief summary of the study and a flowchart can be found on pages 12 and 13 of this information sheet.

Access to your personal information

When you are screened for the study, you will be given a code number. This code number is unique to you and will be used to protect your identity and make your samples anonymous. Access to any information about you will be restricted to the research team, nurses at the HNU, the HNU medical advisor and your GP. There is more information about this in Part 2.

Expenses and payments

Participating in these studies is on a voluntary basis. However, we do recognise that taking part can cause some inconvenience and there are associated travel costs. Thus, you will receive £335 as an inconvenience payment, however should the study break be required this will increase to £368; if you withdraw or are excluded from the study, *payment will be pro-rata*. Travelling expenses to and from the HNU will be reimbursed on presentation of a receipt for buses or trains, or the current IFR mileage rate for private cars, which is presently 45p per mile. If you require transport to and from the HNU, please let us know. We will arrange and pay for a taxi.

All payments are liable to tax and you are responsible for declaring your own payments for tax purposes.

Members of staff at IFR are free to participate in this study, however we would like to point out that their inconvenience payment will be taxed at source in accordance with BBSRC and IFR rules and HM Revenue and Customs (HMRC).

If you are in receipt of benefits this payment may affect your benefits.

What will I have to do next?

If you are interested in learning more about this study please complete and return the response form (page 15 of this information sheet) using the pre-paid envelope enclosed. When we receive the response form, a member of the study team will contact you by telephone to arrange a pre-study talk with you at the Human Nutrition Unit (HNU) in Norwich. If you wish to proceed, you will

then be able to book a screening appointment. Following a successful screening you will be recruited onto the study and you will be given a faecal collection kit. We will also arrange a date and time for the collection of the first Brassica diet. We will go over all the foods you will need to avoid for the two weeks before the study starts and a minimum of six weeks during the study (minimum of 8 weeks and a maximum of 10 weeks in total) discussed above. You will be reminded that you are free to withdraw from the study at any time without giving a reason, however a call (or email) to a member of the study team would be appreciated if at all possible.

What are the risks or side effects of participating in this study?

As with any pressure measurement (like blood pressure) the inflation of the blood pressure cuffs may cause slight discomfort and a reddening of the arm where the cuff is placed, but this affects some people more than others.
The HNU nurses will be happy to answer any questions you may have about any of the procedures involved.

What are the potential benefits of taking part?

For you, there are no direct benefits except receiving Brassica vegetables, which will contribute to your recommended 5-a-day. Our results will help us to understand:

- whether eating Brassica vegetables can change your gut bacteria and help to increase beneficial bacteria/bacterial products in our gut;
- whether the bacteria in your gut affects the rate at which you convert GSLs to ITCs;
- which bacteria in your gut are responsible for converting GSLs to ITCs.

The results from this study could also impact on future research into health benefits obtained from our diet, and the role our gut bacteria may play.

What if there is a problem whilst I am on the study?

Any complaint about the way you have been cared for during the study will be addressed. Detailed information about this is given in Part 2.

Will my taking part be kept confidential?

Yes – we follow Good Clinical Practice (GCP) and strict ethical and research governance rules. All information about you will be handled in confidence. More details about this are included in Part 2.

This completes Part 1 of the information sheet. If the information in Part 1 has interested you and you are considering taking part, it is important that you read the additional information in Part 2 before making any decision.

PART 2 of the information sheet

What if relevant new information becomes available or changes to the study are made?

If there are changes to the study or new information becomes available, we will tell you. If these changes are significant, you may be asked to sign another consent form.

What will happen if I don't want to carry on with the study?

If you withdraw from the study, we will analyse your samples collected up to the point that you leave the study with those we obtain from all the volunteers, unless you decide otherwise. You will receive payment pro-rata for any samples, diaries etc. you have contributed.

What if there is a problem?

If you have any concerns about the study, you should ask to speak to the study manager Lee Kellingray: 01603 255308 who will do his best to answer your questions. If you are still unhappy, and wish to complain formally, you can do this through the chairperson of the Human Research Governance Committee (HRGC) – Dr Linda Harvey.

The Institute of Food Research accepts responsibility for carrying out trials and as such will give consideration to claims from participants for any harm suffered by them as a result of participating in the trial, with the exception of those claims arising out of negligence by the participant. The Institute of Food Research has liability insurance in respect of research work involving human volunteers.

Please note that the Institute will not fund any legal costs arising from any such action unless awarded by a court.

Will my taking part in this study be kept confidential?

All information collected about you during the course of the study will be kept strictly confidential. Any information leaving IFR, such as bacterial DNA/RNA extracted from stool samples for phylogenetic analysis and metatranscriptomic analysis will be anonymous. Study information will be stored in locked filing cabinets at the Institute of Food Research (IFR). Personal data collected will be processed by computer, however only personal information that is essential for the study will be collected.

When you are screened for the study, you will be given a unique code number (volunteer code number). This number will be used to identify your samples and prevents anyone from working out whose samples are whose. Access to your personal records is restricted to the study team, the HNU nurses, the HNU medical advisor and your GP. Your data will be kept for 15 years in a secure archive, and will be destroyed after this time. Your personal information and the data collected will be handled to Good Clinical Practice standards.

All research is subject to inspection and audit. Although your records may be accessed for this purpose, any personal information remains confidential. IFR has CCTV cameras in use for security purposes. However, provision has been made that volunteers attending the HNU are not identified.

Will my General Practitioner (GP) be informed?

Yes, it is routine practice to inform your GP that you are taking part in a study at the IFR and we will send them:

- Details of the study
- Your eligibility screening results including the dipstick urine patch test, blood pressure, pulse, weight and BMI (kg/m^2)
- Any stool charts that indicate abnormal stools or the presence of blood

This is one of the things you agree to when signing the consent form. Any screening results which fall outside standard reference ranges will be assessed by the HNU nurses and medical advisor. We are unable to discuss test results with you, however you will be advised to speak to your GP about the results and sometimes the medical advisor may ask the HNU nurse to speak to you and/or your GP directly.

What will happen to the samples I give?

The urine sample at HNU Visit 1 (screening) will be used immediately for a urine dipstick patch test and then discarded.

The urine samples (including 24-hour collection) you provide during the study will be analysed for ITC concentration. This will be done using a liquid chromatography/mass spectrometry (LC/MS) and a cyclocondensation method.

LC/MS uses a method that can separate components of a mixture. It will allow us to work out how much ITCs your body has excreted.

The cyclocondensation method is a specific chemical reaction and will help us to find out the total amounts of ITCs in your urine samples.

The bacteria from your faecal sample will be collected and the different types of bacteria and number of lactobacilli present will be determined. The faecal samples will also be used to examine changes in gut metabolites using nuclear magnetic resonance spectroscopy (NMR). NMR is another method that is able to separate the components of a mixture. Finally, should the results indicate that it is worthwhile, we will use faecal samples to examine any changes in bacterial gene expression through metatranscriptomic analysis using a Next Generation sequencing platform, such as Illumina.

What will happen to the results of the research study?

As a volunteer you are valuable to us but we are unable to tell you any of your individual results. The data resulting from the study may be published in scientific journals or presented at meetings with our funders. At the end of the study we will provide you with some feedback about what we have found as a result of your help and what it may mean for future research.

Please note that data is presented as a whole and is anonymous. Your name will not appear anywhere in any of the results presented or published.

Who is organising and funding this study?

The study will be funded through the NAT4LIFE project with the Danish Council for Strategic Research.

Who has reviewed this study?

At IFR this research project has been reviewed by the Institute of Food Research Human Research Governance Committee (HRGC), as well as an external Local Research Ethics Committee. These are groups of independent people who review research to protect your safety, rights, well-being and dignity. This study has been reviewed by all committees, and given a favourable opinion.

Further information-what we need you to tell us

We need you to tell us some things for your safety and for the success of the study.

Please tell us if you:

- Have any episodes of illness, even if it is just a headache
- Are injured in any way
- Feel unwell during or after a visit to the Unit
- Become pregnant

Some medicines affect the information we are collecting. Please tell us if you take any medication including those you purchase at the chemist or supermarket (e.g. paracetamol).

You should bring details of any medication (i.e. name of the medicine and the dose taken) you are taking when you come for your screening visit (Visit 1).

Taking part in the research is entirely voluntary! You are free to withdraw from the study at any time without giving a reason.

Thank you for your attention.

Effects of Brassica on human gut Lactobacilli (EBL study)

A list of Brassica foods that are high in glucosinolates and ITCs that must be avoided:

- **Mustard:** all types sauces, leaves and powders including Ethiopian mustard, Indian mustard, Chinese mustard, red giant mustard, wrapped heart mustard cabbage, yellow mustard, black mustard, broad beak mustard, purple stem mustard, mustard spinach.

- **Broccoli:** all types including rapini or broccoli raab, alboglabra kai-ian (Chinese broccoli), romanesco broccoli, broccoli, broccoflower.

- **Sprouting broccoli**

- **Brussels sprouts**

- **Cauliflower**

- **Cabbage:** all types including white cabbage (drum), head cabbage, Savoy cabbage, red cabbage, green cabbage.

- **Kale:** all types including curly kale, Chinese kale, sea kale, pabularia siberian kale, acephala Kale.

- **Kohl rabi**

- **Turnip and turnip tops**

- **Spring greens and Collard greens**

- **Chinese cabbage; Pak Choi; Bok choy** and other Chinese brassica vegetables

- **Radish**

- **Salad rocket**

- **Horseradish**: sauces and vegetables

- **Cress:** all types, including watercress and garden cress

- **Papaya seeds**

- **Wasabi**

Brief summary of the study

Days 1-4:
Once you have discussed the study with a member of the study team, and should you wish to continue, you will be asked to bring a urine sample to the HNU Senior Research nurse. This will be tested as part of the screening appointment to determine whether you are suitable to take part in the study. If successful you will be given a container and you will be asked to return home and produce a faecal sample at your convenience.

Days 5-19:
Upon delivery of the faecal sample to the HNU, we will ask you to restrict certain foods from your diet (see page 11), which you will be required to maintain for approximately 8 weeks. After a minimum of 2 weeks on the restricted diet you will be asked to produce and deliver another faecal sample. You will then need to bring a urine sample to the HNU and collect the frozen foods that we would like you to eat for the next 2 weeks as part of your normal diet (with the exception of the foods we have asked you to avoid).

Days 20-34:
During the first 2 week diet period you will be asked to record the fruit and vegetables you consume in food diaries for 7 consecutive days and, during the same 7 days, complete stool charts noting the frequency and consistency of your bowel movements. After eating the first portion of the supplied diet, we would need you to collect all of your urine for the next 24 hours in a container that we will supply, and bring this to the HNU. At the end of this 2 week diet period you will be asked to produce and deliver another faecal sample to the HNU.

Days 35-49:
Next you will be asked to maintain the dietary restrictions but otherwise eat your normal diet for a minimum of 2 weeks (called a washout phase), at the end of which, you will be asked to provide a faecal sample.
There may be a study break at this point if requested by you, e.g. due to a seasonal holiday period, prior to starting the initial diet restriction. This will allow you to cease the dietary restrictions for a maximum of 6 weeks, providing you are willing to restrict your diet for 2 weeks and produce a faecal sample prior to continuing the study.

Days 50-64:
As before, you will be asked to produce a urine sample, and you will be given the foods required for the second 2 week diet period. Upon eating the first portion of the supplied diet you will be asked to collect all of your urine across the next 24 hours, and we will require you to complete both food diaries and stool charts for 7 consecutive days. On completing the second diet period we would like you to deliver another faecal sample to the HNU, and this will mark the end of the dietary restrictions.

Days 65-79:
Finally, we would require you to eat your normal diet for 2 weeks, completing further food diaries and stool charts for the last seven days, and producing a faecal sample at the end of the 2 week period. Upon delivery of the faecal sample, along with any outstanding food diaries or stool charts, you will have completed the study.

Flowchart of study

Day 1	• Study talk
Day 4	• Produce urine sample for Eligibility screening
Day 5	• Produce/deliver faecal sample to the HNU • Start diet restriction (see page 11)
Day 19	• Produce/deliver faecal and urine sample to the HNU • Collect first Brassica diet from the HNU
Day 20	• Start first 2wk Brassica diet, complete food diary and stool chart for 7 consecutive days within this time • Collect urine for 24hr after eating 1st portion
Day 21	• Deliver 24hr urine collection to the HNU
Day 34	• Last day of first Brassica diet • Produce/deliver faecal sample to the HNU
Day 35	• Start 2wk washout period
Day 49	• Last day of washout period • Produce/deliver faecal sample to the HNU
	• Study break if required (maximum of 6 weeks) **• Restart diet restriction** **• After 2 weeks of restricted diet, produce/deliver faecal sample to the HNU**
Day 50	• Produce/deliver urine sample & collect second Brassica diet from the HNU • Start second 2wk Brassica diet, complete food diary and stool chart for 7 consecutive days within this time, and collect urine for 24hr after eating 1st portion
Day 51	• Deliver 24hr urine collection to the HNU
Day 64	• Last day of second Brassica diet • Produce/deliver faecal sample to the HNU
Day 65	• Start 2wk habitual diet period
Day 72-79	• Complete food diary and stool chart for seven days
Day 79	• End of Habitual diet period • Produce/deliver faecal sample to the HNU & the study is completed

Effects of Brassica on human gut Lactobacilli (EBL study)

I am interested in taking part and/or finding out more information about this study (please complete the personal details below).

Name:
……
……..

Address: ……

…….....

Daytime telephone no.: ……

Evening telephone no.: ……

Mobile no.:
.....
.

I am happy for a message to be left via my daytime/evening/mobile number: **YES/NO**
**please circle as applicable*

Preferred number/time to call: .....

E-mail address: ……

Please return this form in the **FREEPOST** envelope provided, to:

Mr Lee Kellingray
Institute of Food Research
FREEPOST NC 252
Norwich Research Park
Colney
Norwich
NR4 7UA

Expressing an interest does not commit you to taking part in the study

IFR Institute of Food Research

WE NEED YOUR HELP ON

Effects of Brassica on human gut Lactobacilli

The EBL Study

There is evidence that regularly eating Brassica vegetables, such as broccoli or cauliflower, may improve your health.

Does it also increase the number of 'good' bacteria in your gut?

We need to recruit:
Men and women aged between 18 - 50 years old

You would have to:
- ☑ **Eat portions of broccoli, cauliflower, and broccoli and sweet potato soups over 4 weeks, which we will provide**
- ☑ **Eliminate Brassica vegetables from your diet for 8 weeks**
- ☑ **Provide biological samples such as urine**

We will: **Reimburse your expenses**
Provide recompense for taking part in the study

If you live within 40 miles of Norwich and would like further information on the study, please contact:

Mr Lee Kellingray
01603 255308
lee.kellingray@ifr.ac.uk
Dr Joanne Doleman
01603 251477
joanne.doleman@ifr.ac.uk

This study is funded by the Danish Council For Strategic Research and has been approved by a Local Research Ethics Committee.
An expression of interest does not commit you to participation.
Study duration, September 2014- September 2015.

Date

Dear_____,

Thank you for your interest in

Effects of Brassica on human gut Lactobacilli (EBL study)

at the Institute of Food Research.

I have sent you the details of this study, which is in progress at present, as you have responded to an advert about the study and you may fit the criteria for this study. If you have any further questions about the study, please contact, Mr Lee Kellingray on **01603 255308** or lee.kellingray@ifr.ac.uk as stated on the enclosed participant information sheet.

If you are interested in taking part or getting more information about the study, please fill out the reply slip on **page 15** of your participant information sheet, return it to the Institute of Food Research in the freepost envelope provided and a member of the study team will be in touch.

Thank you.

Yours sincerely,

Mr Lee Kellingray,
EBL study manager.

EBL APPOINTMENT CARDS

Screening / Rescreening @ HNU
Bring with you a midstream urine sample from within 2 hours of your appointment time.
Also bring with you the name and address of your GP and details of any medication and/or supplements taken.

Day _____
Date_____
Time_____

Start of Test phase 1 @ HNU
Bring with you a midstream urine sample.

Day _____
Date_____
Time_____

Start of Test phase 2 @ HNU
Bring with you a midstream urine sample.

Day_____
Date_____
Time_____

If you are unable to make the appointment please contact Mr Lee Kellingray on 01603 255308 or the HNU nurse on 01603 255305 Thank you.

.....

IFR Institute of Food Research

EBL APPOINTMENT CARDS

Screening / Rescreening @ HNU
Bring with you a midstream urine sample from within 2 hours of your appointment time.
Also bring with you the name and address of your GP and details of any medication and/or supplements taken.

Day _____
Date_____
Time_____

Start of Test phase 1 @ HNU
Bring with you a midstream urine sample.

Day _____
Date_____
Time_____

Start of Test phase 2 @ HNU
Bring with you a midstream urine sample.

Day_____
Date_____
Time_____

If you are unable to make the appointment please contact Mr Lee Kellingray on 01603 255308 or the HNU nurse on 01603 255305 Thank you.

IFR Institute of Food Research

EBL study

CONSENT FORM FOR RESEARCH STUDY

Volunteer please initial each box

I confirm that I have read and understand the information sheet **dated** ……; **version no**. ……; for the above study, and I have had the opportunity to consider the information, discuss the study and ask questions. ☐

I confirm that I have received satisfactory answers to my questions. ☐

I understand that my participation is voluntary, and I am free to withdraw from the study (1) at any time without giving a reason and (2) without my withdrawal affecting future participation in other research studies at IFR. ☐

With whom have you discussed the information for this research study?

Name: …….. Role: EBL Study Manager / Study Scientist / Nurse

I understand that any of my personal information and data collected during the study may be looked at by individuals at IFR, where it is relevant to participation in this study. ☐

I give permission for these individuals to have access to my information and data. ☐

I understand that my personal information and data will be held confidentially at IFR and that it will be destroyed after 15 years. ☐

I agree to my GP being informed of my participation in the study, and for my clinical results to be sent to my GP.

Name and address of your General Practitioner:
……
……
……

I understand that all research is subject to inspection and audit.
NB: although your records may be accessed for this purpose your personal information remains confidential

I agree to take part in the above study.

Signed: …….. (Name in BLOCK letters)

Date: …… **Date of Birth**: ……

I confirm that the participant above has been given a full verbal and written explanation of the study.
Signed: …….. (Name in BLOCK letters)
Role: …….. (BLOCK letters) **Date**: ……

CONFIDENTIAL Participant Eligibility Screening Questionnaire EBL

Participant code number…….. **Sex**: Male / Female

Date of birth: ……. **Age**: **years**

Height:……**cms** **Weight**: ……**Kg** **Body Mass Index** (BMI, kg/m^2)……

Blood Pressure: **Right arm**…… **Left arm**…… **Pulse**: ……

Urinalysis: see page 4

Have you ever had any of the following? If yes give details below each relevant section.

Angina/heart disease: Y N
……
……

Thrombosis: Y N
……
……

High Blood Pressure: Y N
……
……

High Cholesterol: Y N
……
……

Chest problems: Y N
……
……

Diabetes: Y N
……
……

Depression or anxiety: Y N
……
……

Digestive/Gastrointestinal disorders: Y N
……
……

Skin conditions: Y N
……
……

Inflammatory disease: E.g. rheumatoid arthritis Y N
……
……

Liver problems: Y N
……
……
……

Other medical conditions: Y N
……

Kidney/Renal problems: Y N
……
……
……

Are you currently on any of the following?
If yes, give details below each relevant section of brand, dosage, frequency, when started etc.

Prescribed medication: Y N
……
……
Exclude if on medication for gastrointestinal problems

Colonic irrigation/bowel cleansing/ laxative/diarrhoea treatments: Y N

……

……

Exclude if on medication/treatment for diarrhoea, constipation, bowel discomfort/disorder (includes over the counter medications).

Dietary Supplements: Y N **Herbal remedies: Y N**

…… ……

…… ……

…… ……

If taking supplements/herbal remedies which may affect the study data and the participant is not willing to discontinue use for one month before and during the study, please exclude. Check supplements/Herbal remedies with scientist

WOMEN ONLY SECTION

Are you/could you be pregnant? Y N

Have you been pregnant within the last 12 months? Y N

Are you breast-feeding? Y N

End of women only section

Have you had a major physical injury/operation? If yes give details below: Y N

……

……

Are you currently suffering from any illness/injury? If yes give details below: Y N

……

……

SMOKING:

Are you currently a: **Non-smoker / Current smoker / Ex-smoker / Lifelong smoker (circle appropriate)**

What do/did you smoke? (E.g. cigarettes, roll ups, cigars, pipe etc.)……

If a non-smoker/Ex smoker, have you ever smoked? Y N

If yes, how long since you stopped smoking?.....How many did you smoke each day?...

If currently a smoker/lifelong smoker: How many years have you been smoking?…

DRINKING:

Do you drink alcohol: Y N How many units do you drink per week? ……

A unit of alcohol is approximately half a pint of beer or lager, a single pub measure of spirit e.g. gin/vodka or a small glass of wine (125mL).
Exclude participants who appear to binge drink or regularly consume >15 units (women) or >22 units (men) per week.

DIETARY QUESTIONS:

Are you a vegan or vegetarian? Y N

Do you have any special dietary requirements: Y N

If yes state:…… ……

Have you any known allergies: Y N

Food:…… **Drugs**: ……

Other: ……

Are you currently on/or plan to start a diet programme? Y N

If yes state which:……

If on a diet programme which may affect the study data and the participant is not willing to discontinue for one month before and during the study, please exclude. Check diet programmes with scientist

BLOOD DONOR:
Are you a regular blood donor? Y N
If yes, when did you last donate blood? …….

Do you take prebiotics or probiotics? These may be in a drink, yoghurt or other products.
If yes, which products?.....
How frequently?.....
If taking regularly (3+ times a week) and willing to continue throughout the study then include.
If taking occasionally (<3 time a week), participant will have to discontinue use for at least 1 month before starting the intervention and throughout the study. If participant not happy to do so, please exclude from study.

GUT FUNCTION:
On average, how many times do you open your bowels per day? ……..
Do you typically need to strain when opening your bowels? Y N
What is the typical colour of your stools? ……..
What is your typical stool type? (refer to Bristol Stool Chart):
Type**: 1 2 3 4 5 6 7 (circle appropriate)**
If participant typically passes stool types 1, 2, or 7, exclude from study.
Do you typically suffer from any pain/discomfort when passing stools? Y N
If yes, would you describe it as typically being:**Mild/Moderate/Severe (circle appropriate)**
Have you ever seen blood in your stools? Y N
If yes, when did you last see blood in your stools? ……..
If the participant did not seek medical advice on blood in their stools, exclude from study.

GENERAL PRACTITIONER:
Do you agree to us informing your General Practitioner of your participation in the study or of any results found?
Y N
If you have answered NO to this question then we are unable to accept you on this study.

Name and Address of your General Practitioner:
……
……
……Telephone number:……..

As far as you know are you related to or living with any member of study team? Y N

Are you currently participating in another research study? Y N
If yes check with participant whether it involves dietary intervention– refer to HNU Senior Research Nurse or scientist

Are you currently undergoing any GP/Hospital investigations? Y N

Form completed by (print name):…… ……..Signature:…… ……

Designation:…… ……. Date: ….....……..

URINE DIPSTICK TEST RESULTS
Attach to screening questionnaire to be kept at Study centre

Study Title:

Participant code number **(NOT NAME)**:……. Date of Birth:…… …… Male/Female (circle)

Date of sample:…… …… Time of sample:…….

Dipstick urine test reults:

Protein:…… Glucose:…… Ketones:…… Bilirubin:…….. Urobilinogen:……... Blood:……

Specific Gravity…… pH: ……

Test performed by:…… Signature:……

Date:…… Time:……..

Menstruating: Y N N/A (circle as appropriate) **If menstruating do not refer to GP repeat urine test 5 days after finishing Menstruation. If blood indicated on this occasion refer to GP as flagged urine.**

Repeat urine dipstick test results.

pH……..Protein:……Glucose:……Ketones:…… Bilirubin:…….

Urobilinogen:…….. Blood:……. Specific Gravity…….

Test performed by:…… Signature:……Date:……Time:……

Abnormal results referred to HNU protocol 'for the referral of abnormal urinalysis results at screening': Y N

Comments:……
……
……
……

Please note a copy of these results must be sent to the participant's G.P on the Eligibility Screening Results Form.

Date

Dear Doctor ……

This is to inform you that your patient ……..date of birth…….has consented to participate in a human nutrition study at the Institute of Food Research. The study,

Effects of Brassica on human gut Lactobacilli (EBL study)

has been approved by a Local Research Ethics Committee and the EBL Study Manager,
Mr Lee Kellingray, can be contacted on **01603 255308** or **lee.kellingray@ifr.ac.uk** if you require further information.

It is our policy to forward to the participant's GP, copies of all screening results obtained during the study.

We anticipate your patient will complete this study by……..

Yours sincerely,

Mr Lee Kellingray
EBL Study Manager

IFR Institute of Food Research

Effects of Brassica on human gut Lactobacilli (EBL study)

- You are receiving this study summary as one of your patients has consented to participate in the EBL study and has undergone screening (results enclosed). As their GP, you will be sent the results from their screening, which includes dipstick urine test results, BP, pulse, weight and BMI. In addition, copies of stool charts used during the study will be sent to you, should any significant changes to your patients stool type be recorded throughout the study period.

- **The main aim of this study** is to examine whether eating Brassica vegetables can modulate the gut microbiota and cause an increase in the lactobacilli bacteria in our gut.

- Additionally, we are interested in what role the gut bacteria may play in the metabolism of glucosinolates contained within these vegetables.

- Lactobacilli are a normal part of our gut bacteria and have been linked with health benefits in humans. Some foods can increase their number and, therefore, increase these health benefits. We aim to determine whether Brassica vegetables can increase the numbers of these 'good' bacteria.

- Glucosinolates (GSLs) are found in plant tissues of Brassica crops, such as broccoli, cauliflower and watercress. GSLs accumulate in these foods with glucoraphanin being the predominant glucosinolate found in broccoli. When the tissue is disrupted the glucosinolates are converted to ITCs by the plant enzyme myrosinase.

- When cooked, the myrosinase has been inactivated. So when cooked Brassica vegetables are consumed, it is the myrosinase-like enzyme that the microflora in the colon produce that are responsible for converting glucosinolates to ITCs.

- ITCs have been shown to exert diverse biological effects. Sulforaphane (SF) is the predominant ITC obtained from glucoraphanin in broccoli. SF has been linked to health benefits in humans, such as cancer chemoprevention and helping to maintain a healthy heart.

- It has been shown that the extent of conversion of GSLs to ITCs by the human gut microbiota varies greatly between individuals. We aim to assess whether the differences in GSL metabolism rates can be explained by differences in the gut microbiota populations by faecal bacteria phylogeny analysis.

- Your patient will be asked to eat a low Brassica diet (1 portion a week) for 2 weeks and a high Brassica diet (9 portions a week) for 2 weeks, in a random order, with a 2 week washout period separating these diets. During their involvement in the study, your patient will be asked to provide a total of 6 urine samples and 7 faecal samples for ITC & gut microbiota analysis, respectively.

- This project is funded by the Danish Council for Strategic Research and has been approved by a local research ethics committee.

Study Title: **Effects of Brassica on human gut Lactobacilli (EBL study)**

Participant's Name:……

Date of Birth:……. Male/Female:……..

Dipstick urine test reults: Multistix SG test strips used – Leucocytes and Nitrites not tested

Tested as per test strip guide times

pH……Protein:……Glucose:……Ketones:…… Bilirubin:……..
60 seconds 60 seconds 30 seconds 40 seconds 30 seconds

Urobilinogen:……... Blood:…….. Specific Gravity……
60 seconds 60 seconds 45 seconds

Menstruating: Y N N/A (circle as appropriate)

Test performed by (print name):…….. Signature:……. Date:…… Time:……

Repeat test required at surgery prior to re-screen: Y N N/A (circle as appropriate)

Repeat urine dipstick test results (HNU). Tested using Urine dipstick test strips/guide times as above

pH…….Protein:……Glucose:……Ketones:……
Bilirubin:…….

Urobilinogen:…….. Blood:…… Specific Gravity…….

Test performed by (print name):…….. Signature:……. Date:……
Time:……

Observations:

Blood Pressure: Right arm:……Left arm:……. Pulse: Rt…… Lt…….

Weight(kg): …… BMI(kg/m^2):…… Reference range for study: ≥19.5 - ≤30

Copies of screening blood results attached: Yes No Not applicable (circle as appropriate)

The ……. (above/1st urine/repeat urine /BP/ BMI (kg/m^2) –insert as appropriate) result(s) will / will not exclude your patient from this study.

Date:…….. Signature:……
Designation:……..

Abnormal results are referred to the HNU Medical Advisor for comments regarding participation in the study.

Date

Dear Doctor ……..

Your patient, ……..., date of birth …….. has consented to take part in a human nutrition study at the Institute of Food Research entitled,

Effects of Brassica on human gut Lactobacilli (EBL study)

Following consent it is our standard practice to screen the volunteers to exclude any health factors which may affect the study data or whose screening results may indicate an issue which may require further investigation. We are looking for healthy people who have no chronic illness and are not taking any prescribed medication which may affect the study data.

Some/none of your patient's results fell outside the standard reference range on this occasion.

These results **will/will not** affect the study data.

Your patient **will/will not** be able to participate in the study.

Yours sincerely,

Mr Lee Kellingray
EBL Study Manager

IFR Institute of Food Research

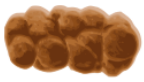
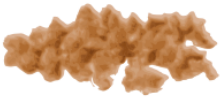
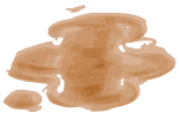
Effects of Brassica on human gut Lactobacilli (EBL study)

Stool chart

- This stool chart will allow us to monitor and record any changes to gut function during the study period.
- Vegetables are a good source of fibre and this is fermented in the colon by your gut bacteria. This releases nutrients which we can use, however much of the fibre is bulky and not of use to us.
- This fermentation process includes the release of gases, which are in turn released by us through flatulence (wind).
- Increased fibre intake can sometimes lead to a larger number of bowel movements, discomfort and increased flatulence.
- Therefore, during the periods outlined below we would like you to keep a record of **each stool** using the Bristol stool chart on page 2 to identify the type of stool. During these periods, we would also like you to record whether you see any blood or mucus on/in your stool and rate any experience of abdominal discomfort/pain, abdominal bloating, straining when opening your bowels (constipation), and/or flatulence for **each day**, leaving a gap to separate each day (example on page 2).
- Ladies who are menstruating at the time entries are added to the stool chart should mark M in the box referring to stool type as this may help to interpret the data.
- We would like you to keep this record for 7 days whilst consuming each of the supplied diets, and then for the 7 days which comprise the final week of the study.
- You can choose which days to complete, but **ideally it must be 7 consecutive days**. If this is not possible, then the 7 days should include one weekend.
- Key to gastrointestinal sensations:
  None – no discomfort.
  Mild – minimal discomfort but not interfering with everyday activities.
  Moderate - causes interference with everyday activities.
  Severe – prevents normal everyday activities.

Please call Lee Kellingray on 01603 255308
or Joanne Doleman on 01603 251477
OR the study mobile on 07542 124238
during normal working hours for any help.

Bristol Stool Chart

Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on the surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. **Entirely Liquid**

Towards the bottom of the stool chart, which we would like you to complete, you will notice questions relating to any changes in medication. Should you have any changes to your medication, or start taking any medication of any kind, we would appreciate it if you could record what is being taken, any changes such as increased/decreased dose, date the medication was taken/changed, and the reason for taking/changing the medication.

The HNU Senior Research Nurse may contact you by telephone to discuss information on the stool chart, and might advise you to speak to your GP should it be deemed necessary, as well as advising on your continuation or exclusion from the study.

If you suffer from diarrhoea during the study, we would like you to contact a member of the study team and you will be asked to not collect a faecal sample for a minimum of 48 hours after the last episode of diarrhoea. Should the diarrhoea persist for more than 72hrs, you may be advised by the HNU Senior Research Nurse to speak to your GP and will be excluded from the study.

EBL study stool chart example:

Date	Time	Type (refer to Bristol stool chart)	Abdominal discomfort (none, mild, moderate, severe)	Abdominal pain (none, mild, moderate, severe)	Type of abdominal pain (dull ache, sharp pain, cramping)	Bloating (none, mild, moderate, severe)	Constipation (none, mild, moderate, severe)	Flatulence (none, mild, moderate, severe)	Colour (pale cream, light brown, dark brown, black)	Blood Y/N If yes, what colour (bright red, dark red)	Mucus Y/N
01/06/14	09:05	3	none	none	none	none	none	none	Pale cream	N	Y
01/06/14	14:25	2	none	none	none	mild	none	moderate	Light brown	Y Bright red	N
02/06/14	10:50	4 M	none	none	none	none	none	none	Dark brown	N	N
02/06/14	17:15	2 M	mild	none	none	none	none	mild	Light brown	N	N
03/06/14	All day	none	moderate	moderate	dull ache	mild	severe	severe	n/a	n/a	n/a

Have there been any changes to your medication? Y (N)

What is the medication? **Senokot**……

How has your medication changed? **Took 2 tablets**……

When did your medication change? **Tuesday 3rd June 2014 at 10pm**……...

For what reason did your medication change? **I was constipated and experiencing abdominal pain**.....

EBL FOOD DIARY

KEY INFORMATION FOR RECORDING FOOD AND DRINK CONSUMPTION

- Food diaries are a very important part of the study because they help us to interpret the information we get from your study periods. It is however, **essential that you eat as normal, apart from the foods we have asked you to avoid**.
- We would like you to record what **fruits and vegetables** you are eating during the periods outlined below.
- Start **each day on a new sheet**. Use as many pages as you need for each day
- Circle the day of the week and date at the top of each page.
- You can choose which days to complete, but **ideally it must be 7 consecutive days**. If this is not possible, then the 7 days should include one weekend.
- Try to **write down what you eat at the time you eat it** rather than from memory at a later date. Remember to add all fruits and vegetables eaten as snacks and nibbles as these can be easily forgotten.
- Take a notebook with you if you eat out and try to include as much detail as possible. A **portion size guide has been included** to help you (please write which photo you are referring to by each portion of food). **Keep the labels of unusual foods containing fruits or vegetables** if they are clean and put in the back of the record book.
- Be as **specific as possible**, for example, Sainsbury's ham and pineapple pizza Finest range. Use brand names and include details about food types such as Porcini mushrooms or button mushrooms.
- For items that are rehydrated, e.g. dried apricots, it is important to state whether you are recording dry or soaked weight, if you do choose to weigh it out.
- If you spill anything or don't finish the fruit or vegetable, add this to the remarks section and include how much was spilt or left uneaten.
- For any meals cooked from a recipe containing fruit or vegetables, complete the recipe sheet provided (example on page 8). State how much you ate as a proportion, e.g. one quarter, or half.
- Record any fruit or vegetable supplements taken during diary recording days.
- For items that are eaten and drunk on a very regular basis such as orange squash, record as much detail as possible the first time i.e. amount of water, amount of orange squash, brand of orange squash and then record 'as usual' for these items in future.
- Record how you cooked your food (boiled, steamed, fried, grilled etc). If you fried the food, don't forget to record what you fried it in (olive oil; butter; lard, beef dripping etc).
- Record where you buy your food and drink when you eat away from home e.g. bacon, lettuce and tomato sandwich from Marks and Spencer. Break down foods like salads, and sandwiches into individual ingredients.
- If the weight is on the packaging of the food item, record that, noting down the individual ingredients and amounts. Write the weight of the portion that you actually ate on your food recording sheets.
- An example of a record sheet is shown on page 7.

Please call Lee Kellingray on 01603 255308 or Joanne Doleman on 01603 251477 at IFR OR the Study mobile on 07542 124238 during normal working hours for any help.

The table below lists common foods and drinks and gives examples of the words that you can use to describe these items. This list is an example, you will only need to do this for foods and drinks containing fruits or vegetables.

(Note: You may wish to use other relevant words that are not on this list)

Food / Drink	Examples of Descriptions
Alcoholic drinks	Red wine / white wine / rosé wine / sparkling wine, alcopop, low alcohol. If measured in a pub: pint / half-pint, single / double, large / small/bottle.
Soft drinks	No added sugar, diet, diluted / undiluted volume. If measured in a pub: pint / half-pint.
Biscuits	Varieties: shortbread / digestive / rich tea / custard cream / etc. With / without chocolate / cream-filled.
Bread	Wholemeal / brown / white / 50:50 / seeded / granary / fruit. Not pre-sliced, medium / thick slice, small loaf, toasted. Crusty / soft, baguette / French stick, bagel, pitta.
Breakfast Cereals	Include details about milk and sugar (type & quantity). Porridge: instant / homemade, quantity of raw oats, toppings.
Butter	Hard / spreadable, salted / unsalted Check butter & not buttery spreads like I Can't Believe It's Not Butter
Cakes & Buns	Type: Victoria / chocolate / fruit / carrot, muffin / sponge / etc. Filling: frosting / royal icing / marzipan / buttercream / jam.
Cheese	Variety: Cheddar (mature, mild) / parmesan / brie / etc. Hard / soft / cream / cottage / processed / triangle. Half-fat / light.
Cream	Double / clotted / single / sour / long-life / spray / crème fraiche. Half-fat / light.
Coffee & Tea	Include details about milk and sugar / sweetener (type & quantity). Cup / mug. Fruit / herbal / green. Filter / instant / decaffeinated / cappuccino / espresso.
Eggs	Medium / large. Scrambled (include details for milk & butter) / boiled / fried (include details for oil).
Fish & Shellfish	Type: salmon, cod, sardines, king prawns. Fresh / canned (oil, brine or water) / frozen. Breadcrumbs / batter / cakes / fingers.
Fruit, Vegetables & Fruit Juice	Varieties: e.g. apples - braeburn, cox, gala. State If peeled and add the weight of the peeled item, e.g. apples, potatoes, kiwi, banana. Canned (juice or syrup) / fresh / stewed / dried / frozen. Fruit juice: freshly squeezed / fresh (e.g. from chilled section) / from concentrate (e.g. long-life).
Gravies & Sauces	Instant / sachet / jar / fresh. With meat juices / milk (type & amount), thick / thin.
Meat & Poultry	Type: pork, chicken, beef, lamb. With / without fat or skin, well-done / rare. Cut: chop / steak / leg / drumstick / mince (lean) / sausages (pork or beef, thick or thin, low-fat) / bacon (streaky or back).
Milk	Skimmed / 1% fat / semi-skimmed / whole (or full-fat).

	UHT / soya / rice / flavoured / condensed.
Nuts & Seeds	Salted / unsalted / honey roasted / dry. Please state type of nuts & seeds, e.g. walnuts, peanuts, sesame seeds.
Oils	Varieties: olive oil / extra-virgin olive oil / vegetable oil / sunflower oil / rapeseed oil / sesame oil.
Pasta, Rice, Noodles & Couscous	Wholemeal / brown / white / pilau / egg-fried. It is important to state whether weight is for the **dried or cooked** ingredient.
Pies & Quiches	Short crust / puff pastry / suet. Individual / family-sized / party-sized. Provide details on filling, e.g. steak and kidney.
Pizza	Size: 8" / 10" / 12". Takeaway / chilled / frozen. Thick / thin / stuffed crust. List toppings.
Potatoes	Chips: frozen / fresh, oven-baked / fried, thick-cut / crinkle cut / French fries. Mash: remember to include butter, milk, cheese, etc. Roast: remember to include type & amount of fat used. New & Baked: with / without skin, butter.
Sandwiches	Spread / no spread / mayonnaise. List all fillings.
Spreads	Varieties: buttery (e.g. I can't believe it's not butter, Clover), olive oil-spread (e.g. Bertolli), vegetable oil spread (e.g. Flora), sunflower spread, soya spread. Low fat / full-fat.
Yoghurts	Creamy / full-fat / low fat / fat-free. Natural / Greek / fruit / toffee / fromage frais

Recipes

What to do if you make a dish containing more than one portion, e.g. lasagne, casserole, meat pie, stir fry, apple crumble (please see the attached example).

In the **recipe section**:

- **Name** of dish
- **Date** of when the meal was cooked and then eaten
- **Cooking method**
- **Portion** of recipe consumed, e.g. ¼.
  - Please add this as a fraction, not a weight.
- **Leftovers**
- **Ingredients**
  - List all ingredients-if you are able to weigh things, that would be very useful
  - It is important to include cooking oils/fats in your ingredients

In the **main diary**:

- Record the **recipe name**, e.g. Lasagne - recipe.
- It is not necessary to re-write the quantity consumed or leftovers in this section

Food portion sizes

Rice

A | B | C

Spaghetti

A | B | C

Boiled potatoes

A | B | C

Chips

A | B | C

Breakfast cereals/cornflakes

A | B | C

Cheese

A | B | C

Stew

A | B | C

Battered fish

A | B | C

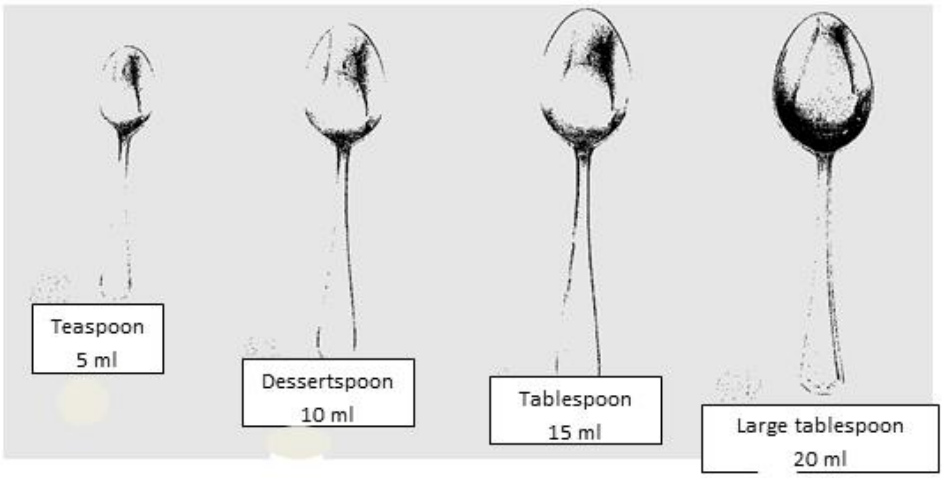
Quiche / Pie

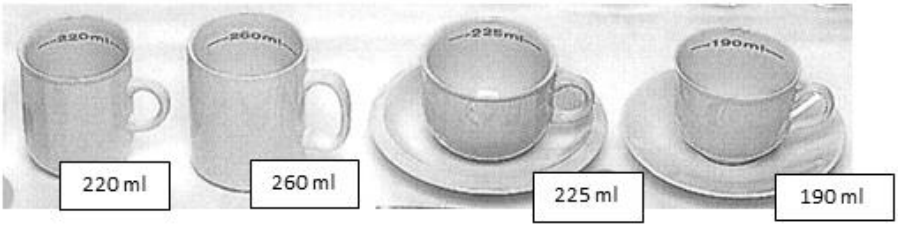
A | B | C

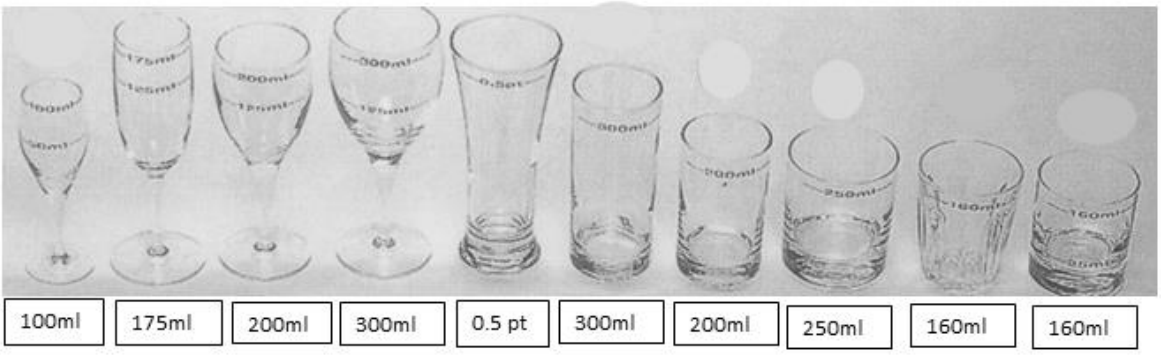
Cake

A | B | C

Commonly used spoons, cups and glasses sizes







FOOD DIARY EXAMPLE

EBL STUDY

Volunteer Code:…EBL123…….

7 days during the 2 week period of eating the Brassica diet in test phase 1

7 days during the 2 week period of eating the Brassica diet in test phase 2

The 7 days in the last week of the habitual diet phase

(Circle as appropriate)

FOOD DIARY **EBL STUDY**

Food Intake example Page

Volunteer Code: EBL123 **Day**: Mo Tue We Th Fr Sa Su (please circle) **Date**: 17/06/14

TIME	BRAND NAME	FOOD/DRINK (description; how cooked; where bought; brand and any comments)	AMOUNT SERVED	LEFT OVER	CODE	AMOUNT
08.00	Kelloggs	Sultana bran	Small bowl (A)			
	Tesco	Orange squash	~100ml			
	Robertson's	Marmalade orange (thick cut)	1 tablespoon			
10.30		Coffee (from machine), milk, no sugar	small cup (190ml)	teaspoon		
13.00	Local grocer	Cherry tomato	small portion (about 70g) (A)			
		Orange	large	Peel & pips		
	Tesco	Fanta	1 can (330ml)			
15.30	PG Tips	Green tea with lemon - see recipe sheets	medium mug (220ml)			
18.00		Chilli con carne – see recipe page	medium portion 300g (B)			
		Carrots, boiled in salted water	small portion (about 70g) (A)			
		Potatoes, roasted in vegetable oil	small portion (about 70g) (A)			
	Ski	Low fat Strawberry yoghurt	1 small yoghurt			
20.00	Blossom hill	Red Wine – Italian Shiraz	1 small glass (175ml)			

Columns in green for official use only.

EBL STUDY

Recipe Sheet - EXAMPLE

Please use this sheet for any recipes that you use whilst recording your intake for us. In the "portion served" box tell us how much of the recipe you actually ate. Use this sheet also to tell us how you would usually take your tea/coffee on a regular basis.

FOOD DIARY

Volunteer Code: EBL123 **Day**: Mo [Tue] We Th Fr Sa Su (please circle) **Date**: 01/07/14

NAME of RECIPE AND INGREDIENTS (description, brand etc)	BRAND NAME	AMOUNT IN RECIPE (g)	PORTION SERVED
Green tea (medium cup)			220ml
water		200ml	
semi skimmed milk	Tesco	30ml	
Tea bag	PG Tips	strong infusion	
Chilli con carne			Medium portion (300 g) (B)
1 large onion, diced		Large onion	
6 cloves of garlic, finely chopped		6 fat cloves	
tin of tomato puree	Tesco	small tin	
tin chopped tomatoes	Napoli	Large can (440g)	
tin red kidney beans, drained and washed	Tesco	Large can (440g)	
1 large red pepper, deseeded and chopped		1 large pepper	
A little vegetable oil - (enough to cover the bottom of the casserole dish)	Waitrose	2 tablespoons	

Effects of Brassica on human gut Lactobacilli (EBL study)

Faecal Collection Kit:

In order to collect your faecal sample we have provided you with a faecal collection kit. The kit should include:

- An insulated container with a label on it
- A plastic pot
- A plastic bag
- A nappy sack
- Freezer blocks or ice cube bags
- A plastic clip to close the plastic bag
- When necessary, a urine sample collection bottle will be included and we would advise you to put this somewhere separate from the faecal collection kit until it is required.

Please can you check your kit to ensure that you have all the items. Although we have checked the kit, if anything is missing please contact a member of the study team on the **study mobile on 07542 124238**, **Lee Kellingray on 01603 255308** or **Joanne Doleman on 01603 251477** before you collect your sample.

The instructions as to how we would like you to collect your sample are on the other side of this piece of paper.

Please ensure you have read and understood the instructions in advance of trying to collect your sample. If you have any questions please get in touch with a member of the study team.

Instructions for faecal collection:

Day before sample collection

Place the freezer blocks in the freezer so that they are ready to place into the bottom of the insulated container on the day of the sample collection. If you have ice cube bags please fill the bags with water and place in the freezer overnight.

Day of the sample collection

1. Place the labelled plastic bag into the plastic pot as though you were lining a bin, roll the excess bag down over the outsides of the plastic pot (note pot will be white not black as in picture).

2. Just prior to collecting your sample, place the frozen freezer blocks or bags of ice cubes into the bottom of the insulated container (may not be as pictured).
3. Collect your faecal sample directly into the labelled plastic bag in the pot. The pot is only used to make it easier to hold the bag. Please do not place any toilet paper into the pot and avoid collecting any urine in the pot if possible.

4. Once the faeces is inside the labelled plastic bag, roll the top of the bag back up and seal the bag, close to but not touching the sample, using the plastic clip provided. Remove the sealed bag from the plastic pot and throw the plastic pot away in your normal domestic waste.

5. Place the sealed bag inside the nappy sack and tie the nappy sack shut using the bag handles.
6. Place the tied nappy sack with the freezer blocks/ice cubes into the insulated container. Close the lid of the insulated container and clip it shut to secure it. Wash your hands.

7. Please write your volunteer number, and the date and time of collection on the label on the outside of the insulated container.
8. Please call the study mobile on 07542 124238, or a study scientist Lee Kellingray on 01603 255308 or Joanne Doleman on 01603 251477 before you deliver your sample so that we can be ready to receive it.
9. If for any reason a study scientist is unable to take your call, you can contact the Human Nutrition Unit (HNU) on 01603 255305. If you call the HNU and the answer phone is on it means there is no one at the HNU and you will need to contact one of the study scientists on the numbers above.
   Thank you very much.

IFR Institute of Food Research

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A list of foods that are high in glucosinolates and must be avoided:

- **Mustard** : all types including ethiopian mustard, indian mustard, chinese mustard, red giant mustard, wrapped heart mustard cabbage, yellow mustard, black mustard, broad beak mustard, purple stem mustard, mustard spinach.
- **Broccoli:** all types including rapini or broccoli raab, alboglabra kai-ian (chinese broccoli), romanesco broccoli, broccoli, broccoflower.
- **Sprouting broccoli**
- **Brussels sprouts**
- **Cabbage:** all types including white cabbage (drum), head cabbage, savoy cabbage, red cabbage, green cabbage.
- **Kale:** all types including curly kale, chinese kale, sea kale, pabularia siberian kale, acephala Kale.
- **Kohl rabi**
- **Rutabaga:** Swede, neep, turnip and turnip tops
- **Collard greens and spring greens**
- **Chinese cabbage; Pak Choi; bok choi** and other Chinese brassica vegetables
- **Radish**
- **Salad rocket**
- **Horseradish**: sauces and vegetables
- **Cress:** all types, including watercress and garden cress
- **Papaya seeds**
- **Cauliflower**
- **Wasabi**

Brassica vegetables to avoid

Kohlrabi

Collard greens

Collard greens

Spring greens

Kale

Wasabi

Cauliflower

Cabbage

Brussels Sprouts

Chinese cabbage

Pak Choi cabbage

Bok Choy cabbage

Savoy cabbage

Broccoli

Salad rocket

Sprouting Broccoli

Turnip and turnip tops

Radish

Horseradish

Mustard/mustard seeds

Cress

Watercress

Watercress

Papaya seed

MEDICATION/MEDICAL CONDITIONS DECLARATION AGREEMENT

Certain illnesses and medication may affect the outcome of research studies. Therefore, we would like you to inform the study organisers if you

- start taking medication
- suffer from any illness

OR

- become pregnant

Please sign below to confirm that you have agreed to this request.

I…….consent to inform the study organiser of the commencement of any medication/medical changes whilst participating in the study

OR

If I think I may be pregnant whilst participating on the study.

Signature of volunteer…….

Date……..

Signature of scientist…….

AB/Vers.2/Rev. Nov.04

REPORT ON ADVERSE EVENT (AE)/REACTION (AR)/SERIOUS ADVERSE EVENT (SAE)
(SAE/SUSAR-internal use only as the official NRES form will be used and signed off by Chief Investigator)

Adverse Event / Adverse Reaction (circle as appropriate)

Study Title: EBL

REC reference:……..

Participant Code Number:…….. Date of Birth: ……... Age: …….

Male/Female (delete as appropriate)

Date/Time of occurrence: …….. Date/Time reported: ……....

Reported to (initially): …….. Place of occurrence: ……

Description of AE/AR/SAE/SUSAR:

Extent of Adverse Event (delete as appropriate)**:** Mild Moderate Severe

Treatment/Action:……

……

……

Outcome (delete as appropriate)**:** Recovered Not yet recovered Unknown

Description of Trial Material:

Drug Reaction (delete as appropriate)**:** Certain Probable Possible Unlikely Unclassified

Reporter details (print name):……**Title**:……

Signature:……..
Date:……..

Professional Address: HNU, IFR, Colney, Norwich**,** NR4 7UA **Tel. Number:** 01603 255 305

Chief Investigator **Professor Richard Mithen**

Signature: **Date:**

Professional Address: HNU, IFR, Colney, Norwich**,** NR4 7UA **Tel. Number:** 01603 255 305

Comments by IFR Medical Advisor:

……..

……..

……..

……..

……..

……..

Participant's screening questionnaire attached (circle as appropriate): Yes No

If 'No' state reason:……..

N.B. please ensure any relevant paperwork is attached

IFR Medical Advisor

Name:.....

Professional Address:

……..

……..

……..

Telephone No.: …….

Signature:…… **Date:**……

Based on Version 4/Aliceon Blair/Rev. Mar 2006

IFR Institute of Food Research

EBL

Cooking Instructions

- Cook the vegetable portions from **frozen**; **DO NOT DEFROST**
- Fill the base of the steamer to the low fill level with water
- Plug in and switch on. Set the timer to 10 minutes
- Wait until steam can be seen rising from the vent at the top.
- **Use the top tier to steam your broccoli or cauliflower**

Brassica florets: Steam Brassica florets for **4-5 minutes**

We ask that you do not cook the Brassica vegetables any more, but feel free to incorporate your portions of Brassica vegetables into your meal at the end of its cooking time.

Should the steamer stop working, please contact
Lee Kellingray on 01603 255 308
Joanne Doleman on 01603 251 477
or the study mobile on 07542 124238
during normal working hours.
THANK YOU

Soup storage and cooking guidelines

Storage instructions

Frozen soups collected by/delivered to you should be stored in the freezer on receipt. Please DO NOT re-freeze soups once they have thawed.

Cooking instructions

Hob: Empty chilled contents into a pan. Heat gently, stirring occasionally for 4 minutes. Ensure soup is piping hot.

Microwave: Can be heated from chilled or frozen. Remove lid. Lay lid back on the pot and place pot on microwaveable plate and heat for the times below, stirring halfway through the cooking time. Allow to stand for 2 minutes after heating.

Microwave	B. 650W	D. 750W	E. 850W
Chilled	6 minutes	5 minutes	4 ½ minutes
Frozen	9 minutes	8 ½ minutes	8 ½ minutes