The biotransformation of glucosinolates

A bacterial perspective

A thesis presented in partial fulfilment of the

requirements for the degree of

PhD

in Food Technology

At Massey University, Manawatu

New Zealand

Jane Adair Mullaney

2013

Abstract

Epidemiological studies have shown an association between the consumption of cruciferous vegetables and a reduced risk of certain types of cancers, in particular, colon, bladder and bowel. This is thought to be due to the conversion of glucosinolates present in the vegetables into bioactive isothiocyanates which in turn stimulate a host response involving detoxification pathways. Conversion of glucosinolates is catalysed by the enzyme myrosinase, which is co-produced by the plant but stored in separate tissue compartments and brought together when the tissue is damaged. Myrosinase activity can be reduced or lost during storage of vegetables and is often inactivated by cooking. However, in the absence of active plant myrosinase, bacteria are capable of carrying out a myrosinase-like activity on glucosinolates producing isothiocyanates or nitriles.

This thesis examined the bacterial biotransformation of glucosinolates by two lactic acid bacteria and *Escherichia coli* Nissle 1917, all three considered beneficial bacteria. They were compared with a known glucosinolate-metabolising gut bacterium *Enterobacter cloacae in vitro, in vivo* and *ex vivo* to determine the bacterial responses to glucosinolates and what the products of their glucosinolate metabolism might be. Exposure of the host to beneficial bacteria and glucosinolates resulted in induction of the host detoxification enzyme quinone reductase which was elevated in bladder tissue for all dietary intervention groups consuming glucosinolates and beneficial bacteria, alone or combined.

In vitro, Nissle reduced alkylsulfinyl glucosinolates and their hydrolysis products through redox to alkylthiols and *in vivo*, the host microbiota responded similarly. *In vivo*, the host response to alkylthiol nitriles was to oxidise these back again to alkylsulfinyl nitriles and oxidise further resulting in some nitriles being irreversibly oxidised to the sulfone.

The association between consumption of cruciferous vegetables and reduced cancer of the colon, bladder and bowel is only that; an association. However, the results of this thesis demonstrated that bladder tissue was affected by beneficial bacteria and glucosinolates alone or together, which suggests that both exert a protective effect that could be measured by elevated quinone reductase, a biomarker for cancer chemoprevention.

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There is a saying that “once you know something you cant not know it any more”. I believe in the health benefits of combining broccoli with beneficial bacteria.

I would like to express my gratitude to Massey University who awarded me a doctoral scholarship and also to Riddet Institute who in collaboration with AgResearch and Plant and Food Research chose me for this project.

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This work is dedicated to

Graeme and Daphne Brockelbank

and I did it all because of Paul

Abbreviations

Allyl isothiocyanate	AITC
Antioxidant response element	ARE
Benzyl isothiocyanate	BITC
Broccoli seed powder	BSP
Cytochrome P450	Cyp450
de Man Rogosa and Sharpe media	MRS
Dichloromethane	DCM
Glucose 6-phosphate dehydrogenase	G6PH
Glucosinolate	GSL
Glutathione	GSH
Glutathione S Transferase	GST
Histone deacetylases	HDAC
Glycoside family 1	GH1
Isothoicyanate	ITC
Kelch-like ECH-associated Protein 1	Keap1
Nicotinamide adenine dinucleotide phosphate	NADP
Nicotinamide adenine dinucleotide phosphate-oxidase	NADPH
Nuclear Magnetic Resonance spectroscopy	NMR
Nuclear response factor 2	Nrf2
Quinone reductase	QR
Reactive oxygen species	ROS
Reinforced clostridia media	RCM

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Ethics and ERMA approval was obtained for work within this thesis as follows:

- Gene cloning and expression: ERMA No. 200814
- Animal trial: Animal ethics approval No. AE12354

ONE

1.0 Glucosinolates

Epidemiological studies have shown an association between the amount of cruciferous vegetables in the diet and a reduced risk of certain types of cancers, in particular, pancreatic, bladder and colorectal[1-12]. A possible reason given for this is that these vegetables contain the bioactive compounds known as glucosinolates (GSLs). GSLs are secondary plant metabolites which have a biological effect on plants, fungi, insects and bacteria[13]. Originally named mustard oil glycosides, they occur in dicotyledons; in almost all species of the families Cruciferae, Resedacea, Moringaceae and Capparidaceae. They have also been identified in species of Euphorbiaceae, Phytolaccaceae, Tropaeolaceae, Caricaceae and Rubiaceae[14,15].

Some of the edible vegetables from the Cruciferae include cabbage, broccoli, kale, Brussels sprouts, mustard, radish and rapeseed (canola)[4,16]. GSLs are thought to provide plants with resistance to non-adapted pathogen and insect pests, and structural variations between GSLs are known to affect the plant's fitness in response to attack by pathogens or animal predators[21-23].

Knowledge about the health benefits of eating cruciferous vegetables has been around for centuries. Hippocrates (460-356 BC) wrote about eating cress, Horatius (65-68 BC) wrote poems about the flavour of cabbage, Pliny the Elder (23-79 AD) wrote about the benefits of cruciferous vegetables in the "*Natural History*" as did Dioscorides (40-90 AD) in the "*De Materia Medica*" pharmacopoeia [17]. Although not understood then, it is now thought that the active compounds responsible for this health benefit are the sulfur-containing GSLs. Prior to the discovery that GSLs might provide health benefits, much more attention was given to their anti-nutritional properties and toxicity when fed to animals as fodder crops [18-20].

1.0.1 The structure of glucosinolates

Genes that determine the variation have been identified, are known to be conserved across most Brassicaceae and seem to vary depending on the type of insect pest[21-23]. It has been suggested that the evolutionary changes are due

to reactions to the fluctuating insect populations and the diversity of GSLs reflect their differing biological actions. Another suggested possible function for GSLs has been as a nutrient storage system for sulfur and nitrogen [13,22,23].

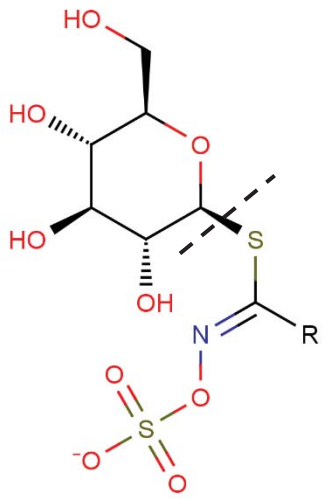


Figure 1.1. General structure for all glucosinolates, the dashed line showing the site where the glucose molecule is cleaved during hydrolysis

GSLs are *β*-D-thioglucoside-(*Z*)-*N*-hydroxyiminosulfate compounds that contain an amino acid-derived side chain (see Figure 1.1). The discovery that allyl glucosinolate (sinigrin) was degraded to glucose, potassium hydrogen sulfate and isothiocyanate (ITC) or “schwefelcyanallyl” was a significant step forward leading to a proposed structure in 1897. This structure was incorrect as it had the side chain attached to the nitrogen rather than the carbon atom of the ‘NCS” group [4,24] (Figure 1.2). Left unchallenged for years it failed to account for the formation of allyl cyanide along with allyl isothiocyanate (AITC) from allyl glucosinolate. Eventually the correct structure was proposed in 1956[25], and the first chemical synthesis of a GSL achieved a year later [26]. The structure of allyl glucosinolate was confirmed in 1970 by crystallisation and X-ray analysis[27] .

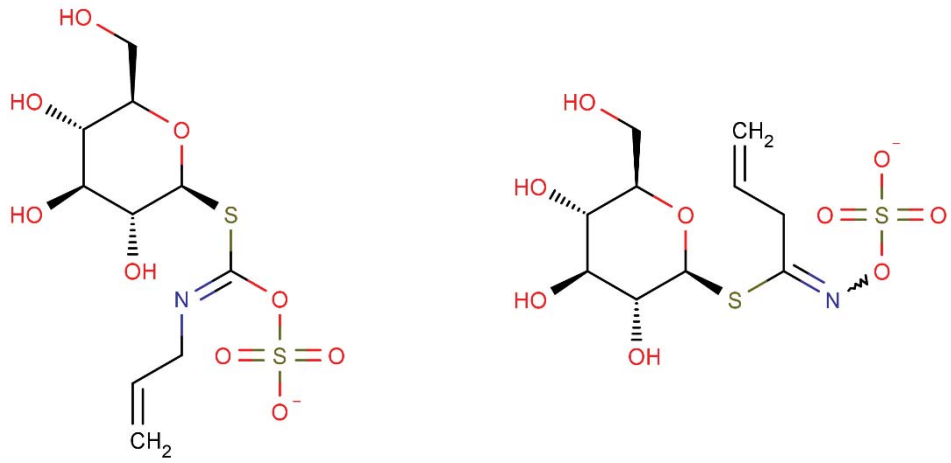


Figure 1.2. Left: Original proposed structure of allyl glucosinolate (sinigrin) showing the R group attached to the nitrogen which was revised in 1956[25] and later verified by chemical synthesis[24] to be the structure on the right

1.0.2 Classification of glucosinolates

The number of reported GSLs described in literature are reaching two hundred [28] and are classified according to their structures which alkyl, aromatic, benzoate, indole, multiple glycosylated and sulfur containing side chains. (for a comprehensive description see the reviews[4,28]. There are also small groups of benzyl GSLs containing another glycosidic-linked sugar such as rhamnose or arabinose to the aromatic ring. While the significance of this is not clear, it is interesting that they are present in plants exploited for pharmacological properties[4].

Although there are other non-Brassicaceae including some Chinese herbal plants which also contain GSLs[15], interest has been focused on the Brassicaceae family, which contains around 350 genera and 3000 species because every member of this family tested to date has been found to contain GSLs[4]

1.0.3 Hydrolysis of glucosinolates yields nitriles, thiocyanates and isothiocyanates

Hydrolysis of GSLs occurs by enzymatic attack on the thioglucoside bond releasing D-glucose, a sulfate ion and an unstable aglycone which undergoes spontaneous rearrangement to form any one of several products as shown in figure 1.3[25,26,29]. The estimated activation energy for thermal degradation of allyl glucosinolate is 22.6 Kcal/mole so under natural environment conditions, spontaneous hydrolysis is unlikely to occur. GSLs will form nitriles, thiocyanates or isothiocyanates (ITCs) if the sulfur-linked glucose molecule is removed either enzymatically, or through acid hydrolysis, or by non-enzymatic thermal degradation [29].

As Figure 1.3 shows, the end product is determined by several factors. Generally, hydrolysis at neutral pH leads to the formation of ITCs whereas acid hydrolysis in the presence of ferrous ions and or nitrile specifier proteins drives the reaction in favour of the formation of nitriles[22,29,30]. Other associated proteins referred to as ‘epithiospecifier’ proteins or ‘epithiomodifier’ proteins can direct the formation of epithionitriles[22,31-34] while oxazolidine thiones (goitrin for example) are formed from cyclised hydrolysis products from GSLs such as progoitrin (2-hydroxy-3-butenyl glucosinolate).

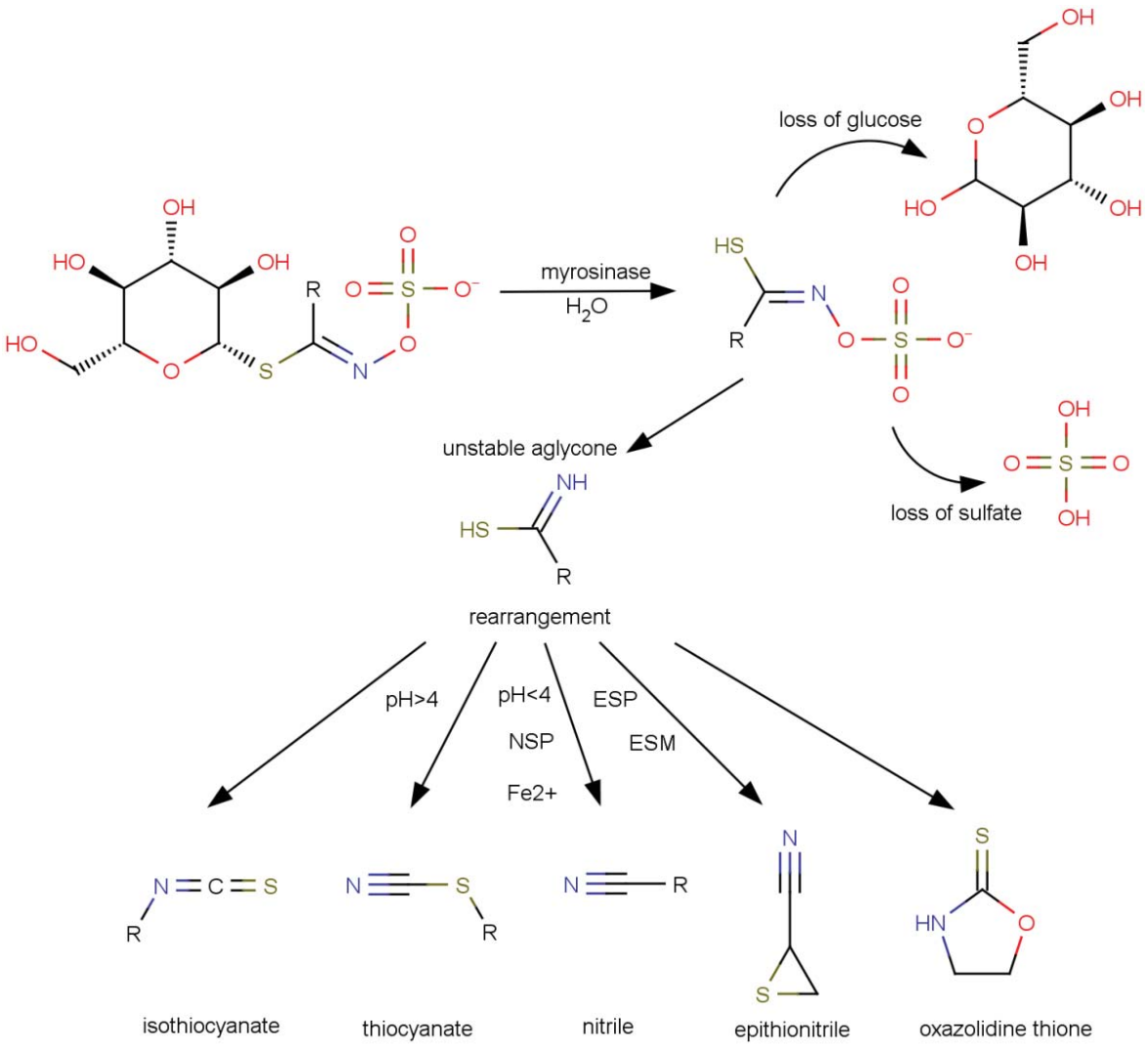


Figure 1.3. Hydrolysis of a glucosinolate liberates a glucose molecule and an unstable aglycone intermediate shown in the centre. Rearrangement then releases a sulfate group and generates different products depending on the R-group characteristics, pH, ferrous ions (Fe^{2+}), and the presence of epithiospecifier (ESP), epithiomodifier (ESM) or nitrile specifier proteins (NSP)

1.0.4 Myrosinase

Enzymatic removal of the glucose is performed by myrosinase, an S-glucosidase enzyme[29]. Myrosinase demonstrates broad substrate specificity, and unlike O-glycosidases which are very common in nature, myrosinase is the only known S-glycosidase[16]. Glycosidases (or glycoside hydrolases) are classified according to whether they either retain or invert the end product and myrosinase is known to be a retaining enzyme consistent with its sequence similarity with family 1, the O-glycosidases[16]. Myrosinase is stored in plant tissue but compartmentalised away from GSLs which prevents it from

hydrolysing GSLs until the plant tissue becomes damaged[36,37]. Damage by crushing or chewing breaks down the compartments and allows GSLs and myrosinase to come together.

The structures of native myrosinase from white mustard seed (*Sinapis alba*) have been solved at 1.6Å resolution[16,22] and the protein is known to fold into a (*β/α*) 8-barrel structure, very similar to that of the cyanogenic *β*-glucosidase from white clover. Myrosinase forms a dimer stabilised by a Zn2+ ion and is heavily glycosylated. A hydrophobic pocket enables binding of the hydrophobic side-chain of the GSLs, and two arginine residues interact with the sulfate group of the substrate. With the exception of the replacement of the general acid-base glutamate by a glutamine residue, the catalytic machinery of myrosinase is identical to that of the cyanogenic *β*-glucosidase. The structure of the glycoside enzyme intermediate shows that the sugar ring is bound via an *α*-glycosidic linkage to Glu409, the catalytic nucleophile of myrosinase[16].

1.1 Glucosinolates to isothiocyanates

1.1.1 Isothiocyanates and toxicity

GSL-containing oilseed crops for many years have been produced as a supplementary feed for livestock (and more recently as fuel)[4,35]. Cabbage feeding was first attributed to goiter in 1928 [36] and goitrin identified as the cause [37], while myrosinase had earlier been identified as “myrosin, a thioglycosidase which hydrolyses mustard oil” [38]. At this time inactivation of myrosin was thought be all that was required to make the food ‘safe’, however it was discovered that even in the absence of active myrosinase, when brassica plants were fed to animals, goitrin was still being produced[36].

A solution for this was to reduce the toxicity of glucosinolate hydrolysis products by selective breeding of low glucosinolate cultivars. Canola (*Brassica napus L*.) for example, is just one cultivar that has intentionally had it’s GSL content reduced through selective breeding in order to increase palatability as well as decrease toxicity[31].

1.1.2 Isothiocyanate antimicrobial properties

Extracts from members of the Brassicaceae family are able to inhibit growth of phytopathogenic fungi, due to the action of the hydrolysis products of their GSLs, the ITCs. These volatile compounds within the plants that harbour

antibacterial and antifungal properties have been characterised[29,39]. For example, allyl and phenyl ITCs have antifungal properties which inhibit wheat fungal pathogens and post-harvest fruit pathogenic fungi[18,40-43].

In vitro, ITCs show promise as protectants of food from pathogens and spoilage[44,45]. AITCs are also used as preservatives in the food industry and have been shown to be cytotoxic to some pathogenic bacteria including *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica*[18,46-49].

ITCs have even been proposed as dietary agents for controlling pathogens: results to date show that they do have some biocidal activity[50]. The volatiles from two Brassicaceae species, including ITCs, have been reported to be 25% as effective as antibiotics against *E. coli*, 33% as effective against *Bacillus subtilis*, and equally effective against the non- *Lactococcus lactis*[51].

The ITC, sulforaphane is so well known it even has its own Wikipedia page; entering 'sulforaphane' into Google's search engine as of February 2013 generated over 537,000 entries. Formed from hydrolysis of glucoraphanin sulforaphane has shown biocidal activity towards *Helicobacter pylori in vitro* and *in vivo*[52] and studies of people (and mice) who took oral doses of broccoli sprouts found infections from *H. pylori* were reduced or eradicated[52,53].

1.2 The host response to isothiocyanates

1.2.1 Phase II inducing, apoptosis inducing and anti-proliferative compounds

The body's detoxification system is responsible for the inactivation and elimination of toxins and xenobiotics and is a two-step process, usually referred to as the phase I and phase II system. Phase I and II enzymes which catalsye the elimination of drugs and xenobiotics are grouped according to their mode of action. Oxidative (phase I) enzymes include the dehydrogenases, oxidases and oxidoreductases while the conjugative (phase II) enzymes include the reductases and transferases. A review of the molecular mechanisms of phase I and phase II enzymes was published in 2007[54]. The detoxification system is dynamic, surveying everything that passes through the liver and responds when necessary by producing enzymes to target and detoxify any molecules requiring elimination.

This has relevance to ITCs which have been investigated extensively for their role in cancer chemoprevention[55-63]. ITCs from Brassicaceae exert either a chemo preventative (delaying or reversing damage) or a therapeutic effect by promoting cancer cell death (apoptosis), or both. They do this by modulation of metabolic pathways involved in the elimination of foreign compounds from the host, namely histone deacetylation systems, apoptotic pathways, antioxidant response pathways and the phase I and phase II enzymes.

Phase II enzymes may be induced, for example, one consequence of this induction by ITCs is that phase II enzymes are able to directly and independently activate signalling pathways which may have been switched off by developing cancer cells. One of these pathways is to apoptosis (programmed cell death) and once induced, cancer cells are destroyed[6,7,64-70] Another consequence of phase II induction is that it is known to stimulate the antioxidant response pathways as well which are also protective[71].

1.2.2 Sulforaphane and the phase I and phase II inducing potential of isothiocyanates

While repeated intake of broccoli does not appear to be toxic[72] some have shown that sulforaphane is mutagenic to cells in cell culture, but for reasons that may be more due to GSH depletion than cytotoxicity[65,73]. Others show however, that sulforaphane is protective not mutagenic, elevating the concentration of nuclear response factor 2 (Nrf2) dependent enzymes and GSH[74]. In another cell culture study Nrf2 and GSH levels remained elevated in astrocytes for more than 20 hours after stimulation[75].

Due to their electrophilic properties, ITCs can damage DNA but the threshold for a damaging response depends on the reactivity of the species of ITC and the dosage. For example, when administered at between 10 and 20 μM, sulforaphane can elicit the formation of intracellular reactive oxygen species (ROS) inducing single stranded DNA breakage, leading to apoptosis[76] whereas single stranded DNA breakage, generated by AITC has been found to be only temporary and is quickly resolved by the DNA excision systems[77]. The phase II induction and subsequent antioxidant capacity have also been assessed recently with sulforaphane trialled as a protective agent against UV light–induced skin cancer, with some success[78-80].

Although considered inactive, GSLs may be able to generate cellular responses just as ITCs do[81-83]. For example, it has been shown already that GSLs can be absorbed intact [84] and *ex vivo*, glucoraphanin and glucoerucin have been found to elevate *o*-dealkylations of methoxy- and ethoxyresorufin cytochrome which are markers for cytochrome P450 (Cyp450) proteins CYP1A1, CYP1A2 and CYP1B1. Both glucoraphanin and glucoerucin were also shown to elevate if only 'modestly', phase II enzymes, quinone reductase (QR) and GSTs[81].

1.2.3 Null genotypes of glutathione-S-transferase

A person's genotype also determines the phase I and phase II response. Two common null genotypes are found in the human population for glutathione (GSH) transferase genes *GSTM1* or *GSTT1* and can affect the concentration of GSL metabolites in the blood and the rate of clearance from the body. In a 2005 study, it was found that while *GSTM1*-null genotypes did have significantly higher levels of sulforaphane metabolites in plasma, they also had a greater rate of urinary excretion of sulforaphane metabolites during the first six hours after broccoli consumption, and also had a higher percentage of sulforaphane excretion twenty four hours after ingestion[85].

In another study, it was shown that there was a positive association between ITC levels and lung cancer for *GSTM1*-null genotypes among women who had never smoked[86]. While the causes of this are as yet unknown, the authors suggested that this could be related to increased exposure to indoles leading to an unintended increased activation of phase I enzymes and ROS.

1.2.4 Apoptosis

Programmed cell death or apoptosis involves a cascade of signalling molecules. Briefly there are two major pathways involved either extrinsic (Fas and TNFR superfamily members and ligands) or intrinsic (mitochondrial associated), both of which are found in the cytoplasm. The extrinsic pathway once triggered, initiates a signalling cascade mediated by the activation of the cysteine-dependent aspartate-directed proteases (caspases) which are essential for apoptosis and necrosis of cells including cancer cells[87]. Caspases also initiate DNases, inhibiting DNA repair enzymes and breaking down structural proteins in the nucleus[88].

1.2.5 Antioxidant response element, Nrf2 and Keap1

The antioxidant response element (ARE) involves the transcriptional induction of a number of genes that make up the chemoprotective response system[89]. Induction of the cellular transcriptional factor Nrf2 alone will in turn, activate the ARE for induction of phase II enzymes[90-95]. Nrf2 is usually maintained in the inactive state through its binding to the Kelch-like ECH-associated 1 (Keap1) dimer complex which has a number of cysteine residues that are known to be involved in the interaction with ARE inducing compounds. The role of Keap1 and how its oxidation status affects the stability and subsequent induction of Nrf2 has been elucidated [96-98] and has resulted in a greater understanding as to how these cruciferous derived bioactives might be activating phase II cytoprotective enzymes.

Sulforaphane is already known to activate Nrf2 by forming thionoacyl adducts within Keap1 and sulforaphane can apparently accomplish this alone or as a sulforaphane-GSH conjugate[99,100]. There are multiple cysteine residues available for interaction within the Keap1 dimer complex, but the reactive cysteine of interest is found at amino acid position 151 (Cys151). Inducers can therefore be categorised into preferential Cys151 inducers and independent Cys151 inducers with sulforaphane falling into the preferential group[96-99,101]. A review of the mechanisms by which glucosinolate breakdown products are thought to inhibit carcinogenesis including the ARE elements has been published recently[102].

1.2.6 Gene expression, regulation and damage

The silencing or un-silencing of genes can occur via changes in the DNA methylation state as well as through epigenetic modifications at the level of the histones, the proteins responsible for the packaging and order of DNA into nucleosomes.

Histone acetylation results in a more open DNA structure, which permits the transcription of genes, while deacetylated histones cause the DNA to be condensed into chromatin and silenced. The acetylation state of the histones is dynamic, modified continuously by histone acetyltransferases or histone deacetylases[103]. HDAC inhibitors can also trigger cell cycle arrest and apoptosis by disrupting the cell cycle in G2, causing the cells to enter M phase early.

This seems to have more effect on cancer cells than normal, and although the mechanisms are not well understood, recent studies have implicated several candidates including ITCs that can cause accumulation of reactive oxygen species and also induction of apoptosis proteins TRAIL, DR4 and DR5[63,70,71,107,108].

1.2.7 Sulforaphane as an angiogenesis inhibitor

There is some evidence that sulforaphane could act as an angiogenesis inhibitor. This would make it another tool in the fight against cancers which often require more vascular tissue (angiogenesis) in order to direct blood and nutrients to the growing mass. ITCs are known to interfere with angiogenesis, and this occurs because sulforaphane modulates both the expression and function of Hypoxia Inducible Factor and vascular endothelial growth factor both of which are positive regulators of angiogenesis[104].

1.3 How the microbiota contribute to health

The microbiota which largely occupy the distal regions of the intestinal tract become established in succession from birth onwards and there can be in excess of a thousand species found within adults[105] As the intestinal tract is the first point of contact for the host immune system and microorganisms, the microbiota has an important role in health. A recent review has been published which describes the modulation of the microbial fermentation in the gut by fermentable carbohydrates[105].

1.4 The biotransformation of glucosinolates

The health benefits of dietary vegetables are well known. Apart from providing essential nutrients and fibre, cruciferous vegetables such as broccoli contain as their major GSL, glucoraphanin, and this can be transformed by enzymatic hydrolysis into bioactive sulforaphane. As discussed, sulforaphane is a potent inducer of phase II enzymes in the host.

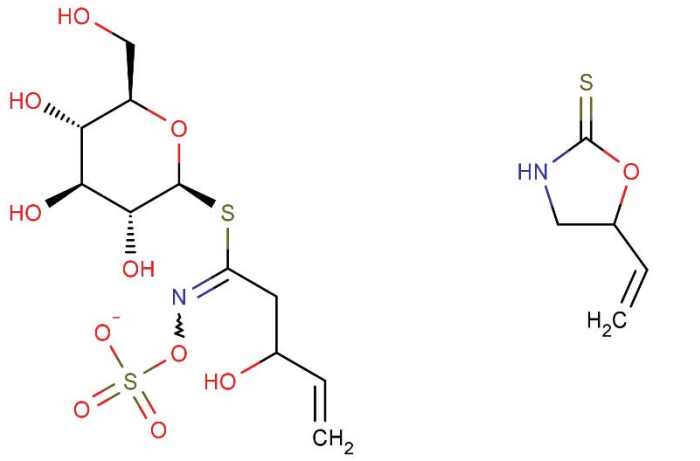
When broccoli is chewed or the tissue damaged, the broccoli myrosinase enzyme is released from within the cell compartment and is available to hydrolyse any GSLs it finds. However, myrosinase may be inactive due to storage or cooking[32,110-114], so for GSLs to be transformed into ITCs requires

other means of hydrolysis. That other means could be by the bacteria which inhabit the gut. Their contribution to the production of bioactives is the key aspect of this thesis because while it has been shown that bacteria are able to transform GSLs, combining beneficial bacteria with dietary GSLs *in vivo* has never been investigated. Nor has the relationship between these beneficial bacteria, the host microbiota and the GSLs been studied.

In order to understand what leads us to this point, first it is necessary to go back in time and describe the work that has been done in the role of bacteria in the bioconversion of GSLs.

1.5 The role of bacteria in the bioconversion of glucosinolates: a timeline from the past to the present

Goitrin, an ITC formed from the hydrolysis of the GSL progroitin, is a compound that reduces the production of thyroid hormones, which may lead to development of goitre. In 1949, the anti-thyroid GSL progoitrin was identified and isolated[37] and in 1959 Greer and co-workers were surprised to find that even after inactivating myrosinase, progoitrin was still being transformed to goitrin when eaten.



Progoitrin Goitrin

Figure 1.4. Glucosinolate progoitrin and its isothiocyanate goitrin.

They conceded that the current view (crucifers would be made safe by cooking) would “need some revision” but did not speculate or comment why this

hydrolysis might still be occurring[19]. In 1965, Oginsky and co-workers linked bacterial activity to the conversion of progoitrin to goitrin, introducing the bacterial myrosinase concept[35]. *Aspergillus niger* was added to the list of myrosinase producing organisms in 1969[106]. Then, in 1974, bacteria found growing in a sinigrin solution were isolated, further characterised and taxonomically identified as *Enterobacter cloacae*[116]. Following this, a bacterial myrosinase enzyme was purified and its myrosinase activity compared with the plant[107]. The following year myrosinase activity was discovered in the yeast *Geotrichum candidum*[108] and in soil dweller *Bacillus cereus* in 1983[109]. Between 1988 and 1993, germ-free or gnotobiotic rats were used by researchers to demonstrate that intestinal bacteria possessed myrosinase activity. Whether colonised with whole human microbiota or mono-associated, myrosinase activity could be observed[20,110-112].

In 1995 lactic acid bacteria were screened for sinigrin degrading potential: one strain, later identified as *Lactobacillus agilis* R16, demonstrated "considerable" sinigrin degrading activity producing AITC as one of the end products[113].

By 1998, the Nugon-Baudon group, had followed their gnotobiotic rat studies with a study of the changes in Cyp450 enzymes resulting from intestinal microbiota in the presence of GSLs from myrosinase-free apeseed[110]. They found that some phase I Cyp450 enzymes were modulated by GSLs and that microbiota were a prerequisite for a decrease in total Cyp450 to be observed. However, they identified that other unknown factors were involved because there were isoforms of Cyp450s that were elevated or depressed only in germ-free rats. Their results highlighted the complex interactions occurring within the host.

Also in 1998, Shapiro and co-workers showed that when the bowel microbiota was reduced by mechanical cleansing and antibiotics, the conversion of GSLs became negligible[3], confirming the important role of bacteria in the biotransformation of GSLs. In 1999, GSLs were shown to be converted to ITCs even when plant myrosinase was completely inactivated. Getahun and co-workers incubated cooked watercress juice with fresh human faeces under anaerobic conditions and found 18% of GSLs were hydrolysed to

ITCs within 2 hours: they attributed the hydrolysis to the microbiota (enteric bacteria) present[114].

In 2001, the types of GSLs and bacteria being investigated were extended. Gnotobiotic rats were colonised with a human digestive strain of *Bacteroides thetaiotaomicron* and dosed with pure allyl glucosinolate which yielded AITC or allyl cyanide after hydrolysis[115]. In this case, the appearance of AITC or ACN could only be due to bacterial degradation and these were found only in the gastrointestinal tracts of the rats' monoassociated with *B. thetaiotaomicron*. This year also, Nuclear Magnetic Resonance Spectroscopy (NMR) was employed to enable more sensitive analysis and identification of the degradation products that ally glucosinolate and glucotropaeolin were converted to by human microbiota[116].

In 2002, an *in vitro* large intestine model was used in combination with pooled human colonic microbiota containing species of *Enterococcus, Enterobacter*, *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Clostridium* to demonstrate the conversion of allyl glucosinolate to AITC although not all of the ITC was accounted for[117]. Gnotobiotic rats were used again in 2003 to determine the influence of plant and bacterial myrosinase activity: the fate of allyl glucosinolate and benzyl GSL as they passed through the gastrointestinal tract was compared, with or without microbiota[118]. They concluded that the bacterial contribution was minimal, so the bacterial status of the host was irrelevant and plant myrosinase was the key to the generation of ITCs. In rats colonised with whole human microbiota, the recovery of ITCs in urine was less than the germ-free animals. It was theorised that not all ITCs were able to be accounted for because in the colonised hosts the bacteria were able to transform ITCs into other products that were not ITCs. Supporting this hypothesis was the observation that benzyl isothiocyanate (BITC) which was added to the GSLs and fed as a biomarker or internal standard to enable quantitative analysis of the recovery of ITCs had 'disappeared' during the process. The authors commented that it was hard to quantitatively detect anything due to background excretion products. As *E. cloacae,* a commensal gut bacteria found in humans is known to degrade BITC into benzyl amine and hydrogen sulphide and was almost certainly present in the + flora group, this may explain the disappearance of BITC[119]. In the bacterial transformation of

GSLs, not all of the products are accounted for, and those that are detected may not be the expected products. In a study of intestinal bacteria using human strains of *Bifidobacterium* (*B. pseudocatenulatum* JCM 7040, *B. adolescentis* JCM 7045 and *B. longum* JCM 7050), allyl glucosinolate and benzyl glucosinolate were added and resulted in the production of their corresponding nitriles 3-butenenitrile and 2-phenylacetonitrile, rather than the expected ITCs AITC and BITC which were barely detectable in the broth[120].

1.5.1 Is bacterial bioconversion significant?

So is the role of the bacterial bioconversion of GSLs a significant one? Martijn Vermeulen and co-workers downplayed the bacterial contribution[121] because they found from their human trials that sulforaphane "bioavailability" from raw broccoli was 37% compared with cooked broccoli at 3.4%. The cooked broccoli presumably had no active myrosinase and was reliant on bacterial hydrolysis. Although they determined the GSL concentration of the broccoli and verified that no glucoraphanin remained after "crushing and incubation", they also acknowledged that not all the glucoraphanin had converted to sulforaphane. An unexpected finding was that the raw broccoli hydrolysed by myrosinase, had less sulforaphane generated than expected when compared to the glucoraphanin content of the same amount of cooked broccoli. This they conceded could be due to production of other metabolites such as sulforaphane nitrile but did not consider this product for analysis presumably because it is not known to induce phase II enzymes. So they only looked for sulforaphane and sulforaphane conjugates and found that there was a lower concentration of sulforaphane mercapturic acid conjugates present than expected.

1.5.2 Bacterial bioconversion produces erucin nitrile as the major product

Lactobacillus species (*L. gasseri, L. acidophilus, L. casei*, and two *L. plantarum*) also contribute to GSL metabolism in rats[122]. A degradation capability of up to 49% after 24 hours incubation was seen, with the major hydrolytic product a nitrile, although one group colonised with caecal microbiota generated some sulforaphane as well as the nitriles. When glucoraphanin was introduced directly into the caecum of live rats, sulforaphane could be detected in the portal blood stream within two hours. However, while conversion in the

caecum by caecal microbiota led to absorption, at the same time the conversion yielded erucin nitrile rather than sulforaphane or sulforaphane nitrile[134] Glucoraphanin was becoming glucoerucin which was being transformed into erucin nitrile and Lai and co-workers suggested that the reduction-oxidation of GSLs and their nitriles could explain this[123]. A schematic of the reactions possible is given in Figure 1.5 and 1.6.

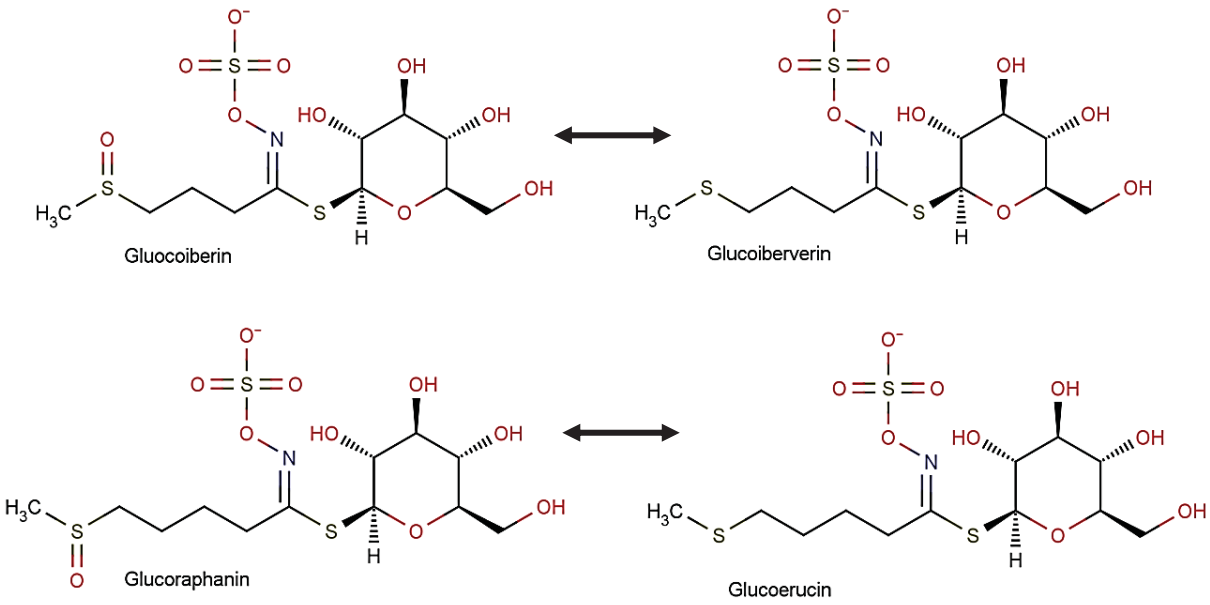


Figure 1.5. Top from left to right: Glucoiberin and glucoiberverin are the same molecules in different states of redox. Below from left to right: Similarly, but with one extra carbon in their alkane chain, glucoraphanin and glucoerucin are a redox pair. Arrows indicate that molecules can shift between redox states

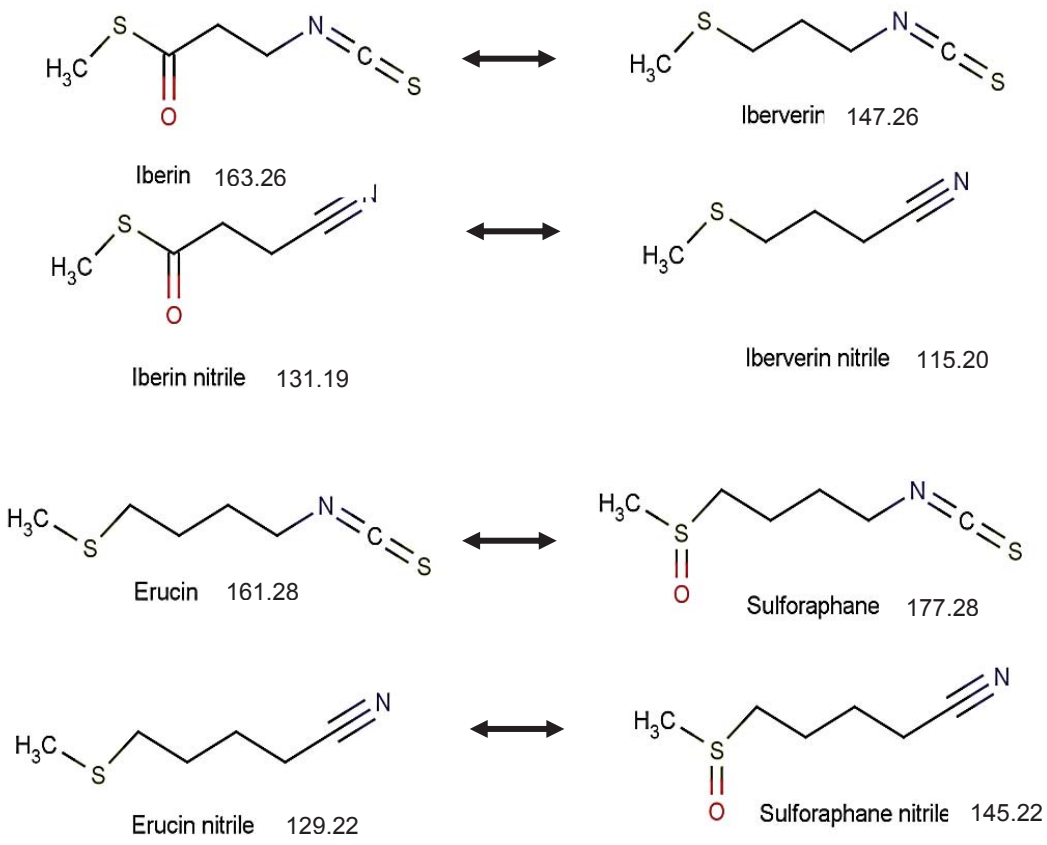


Figure 1.6. The glucosinolate hydrolysis products of glucoraphanin, glucoerucin, glucoiberin and glucoiberverin are also redox pairs. The masses are shown beside the names

1.6 Bacterial conversion of glucosinolates into good, bad or indifferent compounds?

The bioactivity of the hydrolysis products of GSLs is affected not only by how much bioactive compound is available for absorption but also its degree of bioactivity. While nitriles are thought to be less bioactive than ITCs, each one has some level of bioactivity as determined by its ability to induce a phase II host response. Substitution of an atom within a reactive group may impact on bioactivity; for example, when selenium becomes substituted for sulfur. In this instance fertilising broccoli with selenium had an impact on phase II induction which was thought to be due to selenium becoming incorporated into GSLs[124]. In this study, it was found that seleno-GSLs could produce isoselenocyanates that were more bioactive than ITCs[125]. However

producing seleno-GSLs reduced the concentration of all of the glucosinolates in a dose-dependent manner; further, modification to the GSLs seemed to occur at the expense of GSLs suggesting that the quality of the product may have been at the expense of quantity[125]. The structure is given in Figure 1.7.

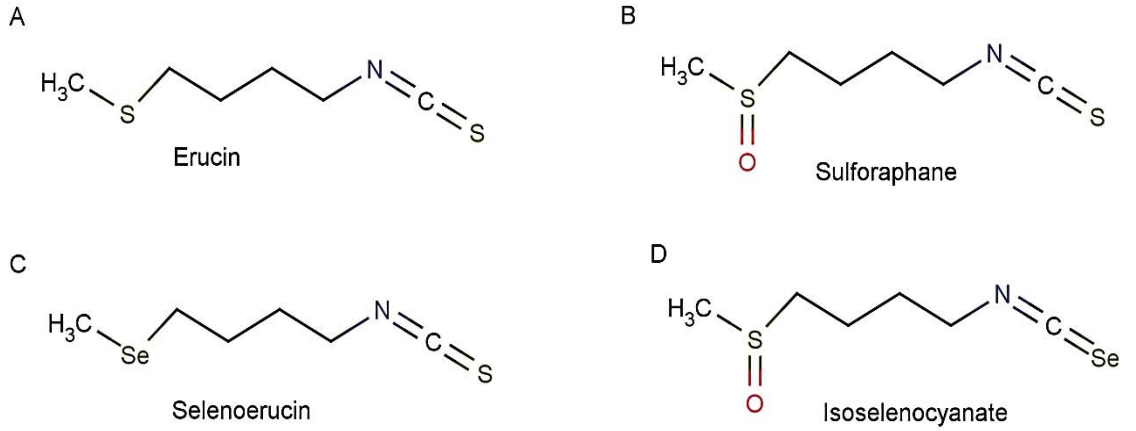


Figure 1.7. A: Erucin, the ITC derived from glucoerucin, B: Sulforaphane the ITC of glucoraphanin. C: the selenoisothiocyanate produced by plants shows that the sulfur-selenium substitution is at the methyl end of the molecule. There is no oxidation of selenium making it more like erucin (A) than sulforaphane (B). D: the chemically synthesised isoselenocyanate places selenium at the cyanate end and has the sulfinyl group similar to sulforaphane

The position the selenium occupies matters. Recently, chemically synthesised isoselenocyanates were found to have more potency as inducers of phase II enzymes than sulforaphane[94,126]. However, the synthesised isoselenocyanates were synthesized from 1-amino-4-(methylsulfinyl)butane and the selenium added to the isothiocyanate group as shown in Figure 1.7 and it has been since shown that this is not the way selenium is replaced for sulfur in the plant GSL biosynthesis[137]. Matich and co-workers have recently analysed selenium fertilized broccoli using NMR to confirm the position selenium occupies in seleno-GSLs and their hydrolysed products either nitriles or ITCs[127]. As Figure 1.7 shows, there are two main differences between the chemically synthesized sulforaphane analogue and the plant biosynthesis product. First as (D) shows, the chemically synthesised species is similar to sulforaphane (B) in that it has a sulfinyl group, but differs in that selenium is incorporated into an isoselenocyanate group. Second, the plant-derived seleno-isothiocyanate (C)

has selenium replacing sulfur at the sulfinyl group, and the selenium is not oxidised making it more like a thiol group similar to erucin (A). Recently comparison of phenylalkyl isoselenocyanates with phenylalkyl isothiocyanates suggests that the thiol groups may be contributing to the reactivity of ITCs[124]. Further studies are required to determine the degree of bioactivity of phase II inducing plant synthesised seleno-GSLs and their hydrolysis products. As selenium is taken up by plants and incorporated into methionine (selenomethionine), cysteine (selenocysteine) and other organic molecules, these may also be exerting an effect independent of any seleno-GSLs and their derivatives[124,125,128].

1.7 Do nitriles possess any of the bioactive properties of isothiocyanates?

Of The ITCs from GSLs that have been tested, all have shown potent phase II inducing capability and many have shown promise as therapeutic treatments against cancers but ITCs are not the only transformation product resulting from hydrolysis of GSLs, nor are they the predominant species[30,32,34,123,129-131]. It is mostly nitriles which are produced when faecal bacteria are incubated with GSLs. Nitriles are considered poor inducers of phase II enzymes[1,132-134] so how does the consumer benefit from consumption of dietary GSLs if nitriles are the main product from GSLs; and are cell culture and *in vitro* systems valid when considering GSL to nitrile metabolism *in vivo*? Keck and co-workers found that their results may have cast doubt of the validity of *in vitro* systems. They noted that in cell culture, the doses of crambene needed for induction of QR were ~100-fold greater than effective doses of sulforaphane and yet when administered to Fischer 344 rats, crambene performed nearly as well as sulforaphane (1.5 and 1.7 fold induction respectively)[135].

Crambene (1-cyano-2-hydroxy-3-butene), formed from the cruciferous GSL progoitrin, has apoptotic properties similar to sulforaphane[136]. Crambene's major metabolite is the N-acetyl-cysteine conjugate derived from GSH with two other minor metabolites identified but as yet unassigned which may also be bioactive[136,137] (Figure 1.8). Crambene elevates GSH and GSTs which serves to demonstrate that for crambene, the detoxification pathway to excretion is via the

phase II pathway and GSTs. Other nitriles such as 4-hydroxybenzyl cyanide and 1-isothiocyanato-3-methylsulfonylpropane (cheirolin nitrile) have demonstrated antiproliferative properties in an *in vitro* cell culture model with an inhibitory concentration value (IC_{50}) of 104 μM and 43 μM respectively[135,138].

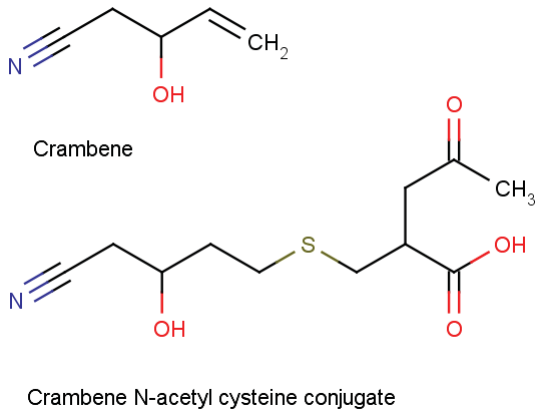


Figure 1.8. Chemical structure of (a) 1-cyano-2-hydroxy-3-butene (crambene) and the N-acetyl-cysteine conjugate of crambene (2-(acetylamino)-3-(4-cyano-3-hydroxybutyl(sulfinyl)propionate)

1.8 Bacteria may be the key to biotransformation of glucosinolates in the intestine

Previous studies have shown that the enzyme myrosinase not only loses its activity through cooking, but its activity also declines over time with the rate of loss dependent on temperature and the ambient storage conditions of the vegetables[139-144]. In the absence of active myrosinase, our gut microbiota are evidently able to carry out this process, and bacteria possess genes encoding glycoside hydrolases which may have specificity to GSLs.

There is some evidence that the myrosinase enzyme is likely to be cell-associated as previous experiments using *Lactobacillus* bacterial cells found no GSL transformation capability unless the cells were intact[113] but as yet, a GSL uptake mechanism has not been identified. It is not known whether the bacteria are actually using the GSLs as a source of glucose in the gut environment (taking them in through an unknown transporter system) or whether breakdown of GSLs with the liberation of glucose and sulfate is simply a consequence (by-

product) of metabolic activity by bacteria. The order in which bacteria remove key components of the GSLs might provide insight as to whether it is the glucose that is targeted and also might explain why nitriles are always the preferred products over ITCs. As ITCs exhibit antimicrobial properties[5,44,46-48,50-52], bacteria may recognise ITCs as toxic and be directing the transformation in favour of nitriles instead.

1.9 The bacterial metabolism of glucosinolates may be beneficial to our health

Dietary GSLs in combination with intestinal bacteria may offer health benefits through the bacterial metabolism of these GSLs into bioactives. The degree of bioactivity will vary depending on several factors: the population dynamics and metabolic activity of the gut microbes, the GSLs that are in the food, the hydrolysis products from their transformation and the presence or absence of plant-based myrosinases. Something one can be confident about is that it is desirable for cruciferous vegetables to contain plenty of GSLs so choosing cultivars high in this compound is desirable as long as it doesn't lead to a lack of other nutrients in the process (sometimes over production of one nutrient in a cultivar leads to underproduction of others, e.g., selenium fertilised broccoli has already been shown to have a reduced GSL profile). Another approach is to increase the ITC component of our food by combining GSL supplements with fresh vegetables containing active myrosinase or mixing GSLs together with ITCs[145-147].

Because ITCs are so much more potent as phase II inducers, it may be preferable that GSLs are converted into ITCs rather than nitriles. However, indications are that some of the most potent phase II inducing ITCs are not actually produced *in vivo* from the corresponding GSL. For example, studies into the fate of glucoraphanin have shown that *in vivo* glucoraphanin does not translate into equivalent molecules of sulforaphane or sulforaphane nitrile but much is interconverted to glucoerucin[87], and then transformed from this into erucin and erucin nitrile[123,129].

Nitriles derived from GSLs are still biologically relevant because, due to their increased stability over ITCs, the effects observed could be due to accumulation which is associated with potency. In general (and crambene is an

exception) nitriles don't seem to elevate GST enzymes and GSH attachment is associated with clearance (a state that is in a constant state of change, involving supply and demand), possibly due to nitriles not having the same binding sites for GSH to attach to[148].

There are several paths GSLs can take via intermediaries to nitriles. Thus, there may be varying degrees of bioactivity, related to redox capacity, and there may also be a relationship between inducer potency and the length of time the weak inducing product is in contact with the host tissue[86,148,155-157]. The nitrile end is a cyanide group and the best characterised cyanide is hydrogen cyanide which is known to be metabolised to thiocyanate by the addition of a sulfur donor[149]. If this is occurring, then if GSH conjugates are not possible, perhaps nitriles are able to be converted to thiocyanates which are able to be conjugated with GSH and induce phase II enzymes just as ITCs do.

There is also evidence for microbial thiocyanate degradation which, if occurring, would bypass this route entirely[150,151]. This author was unable to find any published work that describes and characterises host metabolic degradation of GSL nitriles to date. Aliphatic nitriles, on the other hand, have been studied for toxicity; this is related to their conversion to cyanide and causing GSH depletion[152].

Understanding how bacteria metabolise glucosinolates is important because in the absence of myrosinase, GSLs pass through the body intact[123]. However, if our microbiota possesses myrosinase activity, GSLs can still be transformed into GSL hydrolysis products which are bioactive, where bioactivity is defined as inducing phase II enzymes and or apoptosis inducing. Also, the products of bacterial GSL metabolism may differ from the products of plant myrosinase-mediated hydrolysis, and the effects of the bacterial products have not yet been studied.

For example, it is becoming apparent that the by-products of bacterial metabolism, the short chain fatty acids (SCFAs) have significant effects on our health[153-158]. Some metabolic diseases have their own "signature" of microbiota population dynamics and the ratios of the top three, acetate, butyrate and propionate correlate with the individual's health status[159,160]. Dietary beneficial bacteria are associated also with an increase of propionate and butyrate while

acetate levels are reduced[161]. Butyrate from bacterial SCFAs has also been associated with phase II induction capacity[156].

Even though nitriles do not have the phase II QR or GST-inducing power of the ITCs in cell culture, this does not mean they do not make a difference, especially considering that nitriles are the main product from hydrolysis of GSLs in the gut.

Even knowing which GSLs are eaten does not necessarily allow us to be certain which products will be produced from the process of GSL biotransformation when microbiota are involved. Nor does it allow us to predict how bioactive they will be because the interactions that occur between host and microbiome are so complex.

1.10 Aims of this thesis

The consumption of cruciferous vegetables is associated with a decreased risk of certain cancers. While not evidence on its own, ITCs show promise as cytoprotective agents and ITCs are formed through hydrolysis of GSLs which are in every crucifer studied to date. Linking crucifer consumption through to a decrease in cancer is not a straight forward connection because of the complex nature of the interactions between host, microbiota and food. For example, the species of GSL is determined by the variety or species of crucifer and different GSLs have different bioactivity potentials.

For bioactivity, GSLs must be hydrolysed, and myrosinase is the plant enzyme that performs this task as long as the activity is not lost through cooking or storage conditions. Chewing and crushing brassica during eating will release myrosinase and allow it to hydrolyse any GSLs available. Bioactive compounds in this instance would form in the upper digestive tract such as the oesophagus, stomach and duodenum. In the absence of myrosinase activity, the gut bacteria also hydrolyse GSLs but this occurs in the lower digestive tract, such as the caecum and colon. Perhaps where hydrolysis occurs is as important as what is produced because after hydrolysis, an unstable molecule is left behind which spontaneously rearranges to form thiocyanates, ITCs or nitriles based on the presence of ferrous ions, nitrile specifier proteins, pH and apparently, bacteria.

Just as genetic variations impact on the host's phase II responses to ITCs, the microbiota within the host also respond to the GSLs as part of their

metabolism determined in part by the composition and diversity of bacteria present, the metabolic activity of individual groups and the food that makes it all happen.

It is known that ITCs are potent inducers of phase II enzymes, activating pathways for inhibiting cell division, promoting apoptosis, inducing antioxidant response pathways and killing tumours in general, yet when bacterial GSL degradation has been studied, nitriles are the preferred hydrolysis product over ITCs. Nitriles are chemically stable and this makes them non-reactive which in general (crambene is one exception) makes them poor as ITC substitutes.

So this thesis aims to answer the following questions. It aims to identify beneficial bacteria which survive transit through the gut, are efficient GSL transformers from those which can be added as a supplement safely in a diet.

It aims to discover whether these bacteria can adapt to GSLs in the diet becoming more efficient at GSL transformation and how these beneficial bacteria metabolise GSLs.

It aims to discover what the hydrolysis products of GSL metabolism by bacteria are, whether these products might confer health benefits and whether these benefits are systemic or tissue specific because going back to the beginning of this chapter, epidemiological data associates the amount of cruciferous vegetables in the diet with a reduced risk of certain types of cancers in particular, pancreatic, bladder and colorectal.

It may turn out that the schema we have from past studies showing that GSLs transform into ITCs while in the gut, then activate host responses before being excreted while still active, is accurate, or it may not be. There is a puzzle that this thesis hopes to solve. The product going in is well characterised, the bioactivity of some products coming out is known, but what goes on inside intestinal walls has not been fully explored.

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2.0 The bacterial strains, culture conditions and chemicals

A complete list of the strains used is given in Table 2.1 with the culture conditions given at Table 2.2. For the glucosinolate (GSL) assays, bacteria were grown at 37°C to simulate body temperature and started as a 1% inoculum into the appropriate medium; either “de Man, Rogosa and Sharpe” (MRS) or reinforced clostridial media (RCM) (both from Oxoid, Auckland, NZ). The chosen medium was used with or without additional broccoli GSL extract. A minimal medium was also used for testing sugar preference for GSL degradation. Media compositions are given at 2.0.5.1, 2.0.5.2 and 2.0.5.3.

2.0.1 Aerobic cultivation

For cultivating bacteria to extract plasmids or for competent cells, or for oxygen experiments, a 0.1% inoculum of bacteria were added to a conical flask containing the appropriate media and incubated shaking at 300 RPM for ~16 hours.

For GSL consumption experiments, a 2% inoculum was added to a 500 mL conical flask containing 100 mL of RCM media with or without GSLs and incubated shaking at 300 RPM for the appropriate time period.

2.0.2 Anaerobic cultivation

For anaerobic cultivation, 0.1% inoculum of bacteria were added to a 10 mL polypropylene cell culture tube containing 5 mL of appropriate media then the tube was incubated in an anaerobic container containing CO_2 sachets for ~16 hour.

The contents of the tube were gently mixed by inversion and 2% inoculum added to the appropriate size tube for GSL degradation experiments incubated in the anaerobic container. For time course experiments, separate containers were used for each time point so as not to disturb the anaerobic environment of the other samples.

Table 2.1. List of bacterial strains used in this thesis

Bacteria	Strain	Reference
Bifidobacterium adolescentis	NCTC11814	ESR Culture collection NZ
Bifidobacterium animalis subsp. *lactis*	HN001	Fonterra strain unpublished
Bifidobacterium longum	NTCC3937	ESR Culture collection NZ
Lactobacillus plantarum subsp. *plantarum*	KW30	[1]
Lactobacillus plantarum	WCFS1	BAA-793
Lactobacillus plantarum	ATCC8014	ATCC 8014
Lactobacillus plantarum subsp. *plantarum*	ATCC14917	ATCC 14917
Lactobacillus plantarum	NC8	ATCC BAA-793
Lactobacillus rhamnosus	NH0016527/DR20	[2]
Lactobacillus brevis	LMG 11437	ATCC 367
Lactobacillus sakei	LB790	[3]
Lactococcus lactis subsp. *lactis*	KF147	[4]
Lactococcus lactis subsp. *lactis*	KF152	[4]
Lactococcus lactis subsp. *lactis*	KF282	[4]
Escherichia coli	Nissle 1917	DSM 6601
Escherichia coli	XL1-Blue	Stratagene
Escherichia coli	BL21 (DE)3	New England Biolabs (NEB)
Enterobacter cloacae	ATCC13047	ATCC 13047

Table 2.2. Cultivation conditions for the bacterial strains

Bacteria	Temperature °C	Media
Bifidobacterium	37	RCM
Lactobacillus	37	MRS
Escherichia coli Nissle 1917	37	RCM
Escherichia coli XL1-Blue or BL21(DE)3	37	LB
Enterobacter cloacae	30	RCM
Lactococcus lactis subsp. *lactis*	28	MRS

2.0.3 Glucosinolate consumption by bacteria

For GSL consumption experiments, bacteria were precultured in the appropriate medium supplemented or not supplemented with GSL extract (0.3 (w/v)) depending on whether GSL-adaption or GSL-naive conditions were being followed. From an overnight culture, a 1% inoculum was added to the appropriate growth medium supplemented with 0.3% (w/v) GSLs for 24 h and/or up to 48 h and then the spent medium was clarified by centrifugation at 10,000 *x g* for 10 minutes, the supernatant removed, filtered and the products of the GSL consumption extracted and assessed by GC-MS and LC-QTOF-HRMS.

Growth was determined by measuring optical density at 600 nm (OD_{600}) and for some experiments growth was also verified by the most probable number method (MPN)[5,6] which is described next.

2.0.4 Enumerating Bacteria: Most Probable Number Method (MPN)

Bacteria were serially diluted across microplates in their appropriate media until theoretically none remained (see Figure 2.1). By knowing the starting dilution and the number of dilutions, it is possible to calculate the most probable number of viable bacteria in the starting culture because the assumption is made that the lowest concentration resulting in growth was from a single cell. The difference between this method and optical density is that MPN takes into account cell viability not just cell presence.

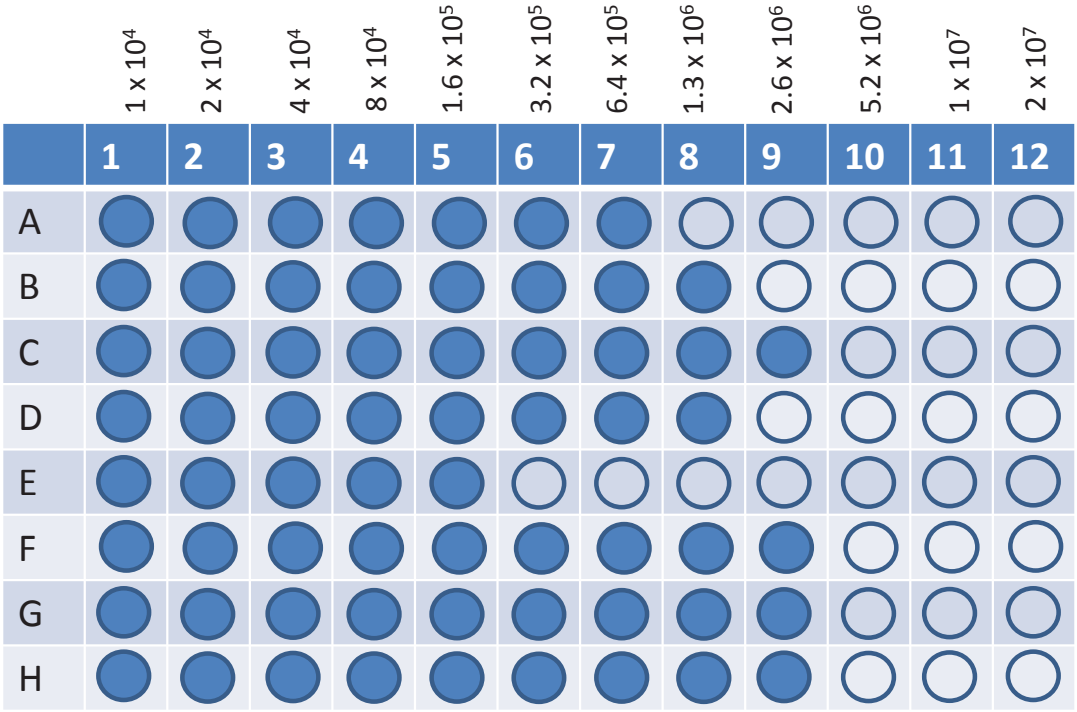

Figure 2.1. Schematic of 1:1 serial dilutions from left to right

2.0.5 Liquid media

2.0.5.1 Reinforced Clostridial Media (RCM)

Tryptone	10 g
Beef extract	3 g
Yeast extract	5 g
K_2HPO_4	2 g
NH4 acetate	3 g
Tri-Na-Citratex2H_2O	3 g
$MgSO_4$ 1M	0.8 mL
$MnSO_4$ 0.4M	1 mL
Tween80 0.1%	1 mL
pH to 6.8 using HCl then autoclave	
Add 0.5% cysteine before autoclaving or 0.05% sterile afterwards	
For 2x concentrate make up to 500mL with water	

2.0.5.2 de Man, Rogosa and Sharpe media (MRS)

Casein peptone, tryptic digest	10 g
Meat extract	10 g
Yeast extract	5 g
K_2HPO_4	2 g
$(NH_4)_2$ citrate	2 g
Sodium acetate	5 g
Dipotassium hydrogen phosphate	2 g
Tween-80	1 g
$MgSO_4$-$7H_2O$	0.2 g
$MnSO_4$-H_2O	0.05 g
Glucose (when included)	20 g
Adjust pH to between 6.2 and 6.5 with HCl	
Add 0.5% cysteine before autoclaving or 0.05% sterile afterwards	
For 2x concentrate make up to 500mL with water	

2.0.5.3 Minimal media

Trypticase peptone	20 g
Sodium acetate	1.0 g
Ascorbate	0.5 g
$MgCl_2$	2.0 g
$MnCl_2$	0.2 g
Cysteine	5.0 g
Potassium phosphate buffer	50 mM pH 7.2
Make up to 1 Litre with water and autoclave	

2.0.5.4 Luria-Bertani (LB)

Luria-Bertani (LB) media (Life Technologies) 20 g per litre of H_2O and then autoclaved at 121°C for 20 minutes.

2.0.6 Solid Media

2.0.6.1 LB agar

10 g of Luria-Bertani (LB) media and 8 g of agar were dissolved in 500 mL H_2O. The medium was autoclaved at 121°C for 20 minutes.

2.0.7 Antibiotic stock solutions and final concentration

The antibiotic solutions were prepared according to laboratory protocols[5], sterilised by filtration through 0.22 µm filter and stored in aliquots at -20°C for further use. After autoclaving the nutrient media and subsequent cooling to 50°C, the antibiotics were added at the concentrations given below (Table 2.3).

Table 2.3. Antibiotic stock solutions and respective concentrations in mg/mL

Antibiotic	Concentration of stock solution (mg/mL) and diluents	Final concentration (µg/mL)
Tetracycline	12.5 in EtOH (70%)	12.5
Ampicillin	100 in H_2O	100

2.0.8 Preservation of strains

Strains were incubated overnight in the appropriate liquid media as given in Table 2.2. 1 mL of the culture was transferred to a 2 mL cryovial tube and 60 µL of sterile dimethylsulfoxide (DMSO) added to give a final concentration of 7.5% (w/v). The tube was stored at -70°C for future use. For resuscitation of the strain, the tube was allowed to thaw on ice then 5 µL was added to 5 mL of the appropriate liquid media in a polypropylene 10 mL culture tube which was incubated at the appropriate temperature either aerobically on a shaking platform or anaerobically depending on the requirements of the strain.

2.0.9 Preparation of cells for long term storage

As previously described by Hanahan[7,8], the *E.coli* strain was cultivated in 50 mL LB liquid medium at 37°C until OD_{600} reached 0.3. After 10 -15 minutes incubation on ice, the cells were harvested by centrifugation at 2250 *x g* in a Jouan C4 centrifuge, (Thermofisher Scientific, NZ), resuspended in 18 mL RF1 solution and incubated on ice for a further 30 minutes. Cells were then again centrifuged for 15 minutes at 2250 *x g* and resuspended in 4 mL of RF2 solution. The resulting competent cells were then aliquoted to 1.5 mL microfuge tubes (200 µL per tube) and stored at -70°C.

RF1 solution	
100 mM	RbCl
50 mM	$MnCl_2$
30 mM	Potassium acetate
10 mM	$CaCl_2.6H_2O$
Adjust the pH to 5.8 with acetic acid.	

RF2 solution	
10 mM	RbCl
10 mM	MOPS
75 mM	$CaCl_2.6H_2O$
15% (v/v)	Glycerol
Adjust the pH to 5.8 with NaOH.	

2.0.10 Transformation of *E. coli* cells

200 µL of competent cells were mixed thoroughly with 1-2.5 µL of plasmid DNA and incubated for 60 minutes on ice to allow the DNA to become adsorbed at the surface of the competent cells. Then the cells were heated at 42°C for 90 seconds and then placed back on ice for 5 minutes. For regeneration of the cells and for expression of the plasmid-encoded antibiotic resistance, 800 µL of LB was added and the cells incubated at 37°C for one hour. For isolation of the recombinant clones, the cells were pelleted by centrifugation for 2 minutes at 5000 *x g* Jouan C4 centrifuge, (Thermofisher Scientific, NZ), and resuspended in 350 µL fresh LB, 50-100 µL were spread onto solid media plates containing the appropriate antibiotic and the plates incubated overnight at 37°C.

2.0.11 Isolation of plasmid DNA

Strains containing the respective plasmid were incubated in 20mL LB medium with the appropriate antibiotics at 37°C overnight. 1 mL of culture in a microfuge tube was centrifuged at 10,000 *x g* for 3 minutes (Biofuge Pico, Heraeus, Germany), the supernatant was removed, 100 µL ice-cold GET solution was added to the pellet and the pellet resuspended. This was followed by the addition of 200 µL freshly prepared SDS-NaOH solution. After gently inverting the microfuge tube a few times, a clear solution was obtained indicating cell lysis was complete. The protein was precipitated by addition of 150 µL of HSS solution.

After centrifugation at 10,000 *x g* for 30 minutes in a Jovan C4 centrifuge, the supernatant was transferred to a new microfuge tube and centrifuged at 10,000 *x g* for a further 15 minutes.

The supernatant was transferred to a new microfuge tube containing 350 µL ice-cold isopropanol and centrifuged at 10,000 *x g* for 30 minutes.

After the supernatant was poured off, 300 µL of 70% ice-cold ethanol was added and the pellet resuspended by vortexing followed by centrifugation of the tube at 10,000 *x g* for a further 30 minutes.

The supernatant was poured off, the tube allowed to dry at room temperature and the dried plasmid DNA dissolved in 30 µL of TER buffer and stored at -20°C. The recipes for the solutions used are given in Table 2.4.

Table 2.4. Reagents for DNA isolation.

GET solution:	
25 mM	Tris/HCl, pH 8.0
10 mM	EDTA
50 mM	Glucose
SDS-NaOH solution:	
200 mM	NaOH
1% (v/v)	SDS
HSS solution	
3M	Potassium acetate
1.8 M	Formic acid pH 4.8
RNase solution for TER buffer	
150 mM	NaCl
1% (v/v)	RNase A
T E Buffer for TER buffer	
10 mM	Tris/HCl
1 mM	EDTA, pH 8.0
TER Buffer	
10 µL RNase solution: 990 µL TE buffer	

2.0.12 Isolation of genomic DNA

2 mL of an overnight culture was pelleted and 100 µL TE buffer added. The pellet was resuspended and microwaved for up to one and a half minutes in 20 second bursts with 20 seconds resting on ice in between. Next 50 µL of 10% SDS was added and the mixture incubated at 75°C for 15 minutes. Next 300 µL of phenol-chloroform-isoamylalcohol (25:24:1 (w/v)) pH 8.0 saturated in

Tris-HCl was added to the tube and the microfuge tube shaken vigorously by hand for 1 minute.

The tube was centrifuged at full speed for 2 minutes and the supernatant decanted. 300 µL 70% ethanol was added, the pellet resuspended by gently flicking the tube and the tube centrifuged for 15 minutes at full speed. This step was repeated once again, then the liquid was carefully aspirated off, and the pellet dried.

To remove any remaining contaminating phenol, the pellet was resuspended in 100 µL of diethyl ether and centrifuged for 5 minutes at full speed.

Next the top layer of diethyl ether (containing any phenol contaminant) was pipetted off leaving the pellet which was air-dried before resuspension into 100 µL of TE buffer. DNA was assessed for purity and quantitation with the Nanodrop (see section 2.1.4) and/or by loading 5 µL onto an agarose gel and electrophoresing (see section 2.1.6).

2.0.13 Determination of the size of DNA fragments

The purity and concentration of the DNA solution was determined by at least one of two methods. The first was by agarose gel electrophoresis and/or measuring the absorption at 260 nm and 280 nm using the Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA).

2.0.14 Determination of the DNA concentration

The agarose gel electrophoresis was performed in horizontal slabs. For routine analysis of a DNA sample, 1 – 2 % agarose in TBE buffer was used. DNA samples containing the respective stop-mix (Table 2.5) was loaded into the wells in the gel. The gel electrophoresis conditions were 100-160 V for 30 – 60 minutes using a Biometra gel electrophoresis unit (Biometra, Germany). After electrophoresis, the gel was stained in SYBR® Safe DNA gel stain (Life Technologies S33102) for approximately 15 minutes, rinsed briefly in distilled water and then detected by UV transilluminator at 254 nm. An image was generated using GELDOC software (BioRad, Auckland NZ).

2.0.15 Agarose gel electrophoresis (AGE)

The agarose gel electrophoresis was performed in horizontal slabs. For routine analysis of a DNA sample, 1 – 2 % agarose in TBE buffer was used. DNA samples containing TBE buffer and stop-mix was loaded into the wells in the gel. The gel electrophoresis conditions were 100-160 V for 30 – 60 minutes using a Biometra gel electrophoresis unit (Biometra, Germany). After electrophoresis, the gel was stained in SYBR® Safe DNA gel stain (Life Technologies S33102) solution for approximately 15 minutes, rinsed briefly in distilled water and then detected by UV transilluminator at 254 nm. An image was generated using GELDOC software.

TBE buffer and stop mix.

TBE Buffer	Stop-Mix (6 X)
50 mM Tris/HCl	4 M Urea
50 mM Boric Acid	50 mM EDTA
2.5 mM EDTA	50% (w/v) Sucrose
approx pH 8.5	0.1% Bromophenol Blue

2.0.16 DNA A-tailing procedure

To obtain A-tailed DNA from blunt-end PCR products a purified fragment generated by a proofreading polymerase (Pfx polymerase, Life Technologies) was used.
A typical reaction mixture contained the following:

1-6 µL PCR fragment

1 µL Taq polymerase (Life Technologies)

1 µL 10 x Taq reaction buffer without $MgCl_2$ (Life Technologies)

1 µL 50 mM $MgCl_2$ (Life Technologies)

1 µL dATP (0.2 mM) (iNtRON)

The final reaction volume was 10 µL which was incubated at 70°C for 30 minutes. 3.5 µL of this reaction was used in a ligation mixture containing the following:

3.5 µL A-tailed PCR product

1 µL l 10 x ligase (Roche)

5 µL pLUG 2 x ligase buffer (iNtRON)

0.5 µL pLUG vector (iNtRON)

The final reaction volume was 10 µL. This was incubated at 4ºC by floating tubes in water (temperature approximately 10ºC) then transferring the container to a fridge and leaving overnight.

2.0.17 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed for the amplification of DNA fragments of template DNA which was flanked by specific oligonucleotide primers. The reaction was performed by repeated cycles of denaturation, annealing and extension or elongation in a GeneAmp PCR9700 (Applied Biosystems, NZ). Platinum Pfx polymerase (Life Technologies) was used and

the manufacturer's instructions followed accordingly. The reaction mixture is listed in Table 2.5.

Table 2.5. PCR reaction mixture.

In a 100 µL volume	Final Concentration
10 µL amplification buffer	1 x
5 µL 50 mM $MgSO_4$	2.5 mM
5 µL DMSO	5%
10 µL each of forward and reverse primers	0.1 µM of each
10 µL dNTPs (dATP, dTTP, dCTP and dGTP)*	0.1 mM of each
1.5 µL Template DNA	up to 300ng
0.5 µL Pfx polymerase (1 unit)	0.5 µL = 1 unit p
58 µL Autoclaved distilled water	

* *(Thermofisher Scientific, NZ)*

2.0.18 DNA sequencing

Sequencing of recombinant plasmids or amplified PCR products was performed by Allan Wilson Centre Genome Service using a capillary ABI3730 Genetic Analyzer, from Applied Biosystems Incorporated. Each sequencing reaction tube contained 300 ng of DNA and primers were added at a concentration of 3.2 pM in 15 µL. Results were provided in ABI format and analysed using Vector NTI version 10 (Life Technologies) software.

2.1 Protein methods

2.1.1 Protein extraction from bacteria

Bacteria were cultivated with and without glucosinolates according to methods previously described at section 2.0 for 24 hours. After this time the bacteria were harvested by washing 3 times in 50 mM potassium phosphate buffer then protein obtained from the cells with SMART^{TM} bacterial protein extraction solution used according to the manufacturer's instructions (iNtRON Biotechnology, Ngaio Diagnostics, NZ). The protein was extracted into 50 mM Tris HCl pH 7.2 and stored in microfuge tubes at -80°C until required.

2.1.2 General methods for protein analysis

2.1.2.1 Protein concentration measurement (Bradford 1976)

200 µL of Bradford reagent was added to 10 µL of the sample to be measured (0.05 - 0.4 µg/mL) and the colour absorbance change measured at 595 nm by a SpectraMax Plus 190 Microplate Reader (Molecular Devices, Biostrategy, NZ). A standard curve was prepared with BSA in concentrations ranging from 0.05 µg – 0.4 µg mL^{-1}. Bradford reagent was purchased from Biorad Laboratories (California, USA) and was used according to the manufacturer's instructions.

2.1.2.2 Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE)

For characterisation and evaluation of proteins, SDS-PAGE was performed in an XCell SureLock® Mini-Cell SDS-PAGE vertical slab gel electrophoresis apparatus (Life Technologies EI0001) according to techniques described previously[9]. Precast NuPAGE® Novex® 10% Bis-Tris Gels were used (Life Technologies NP0301PK2) with NuPAGE® MOPS SDS Running Buffer (Life Technologies NP0001).

SDS denaturing buffer

SDS	8.0 g
β - mercaptoethanol	20 mL
EDTA	37.2 g
Glycerol	40 mL
Bromophenol blue	5 mg
Make up to 100 mL with distilled water.	

2.1.2.3 Preparation of protein samples for SDS-PAGE

2 volumes of the protein solution were mixed with 1 volume of the SDS denaturing buffer and incubated for 15 minutes at 95°C. The electrophoresis conditions were 15 mA in the stacking gel and 26 mA in the separating gel layer. Relative molecular weights of proteins were estimated by comparing the mobility of the proteins with the mobility of molecular weight standard proteins. The Novex® Sharp Pre-stained 3.5 - 260 kDa Protein Standard (Life technologies LC5800) was used.

2.1.2.4 Protein staining

The SDS-PAGE gel was stripped from the gel plate, transferred to SimplyBlue™ SafeStain solution (Life Technologies LC6060), and stained on a slow shaker for 20-30 minutes. Then the gel was washed thoroughly with water until the background colour had gone.

2.1.3 Determination of protein activity - hexokinase glucose 6-phosphate dehydrogenase coupled assay

Protein activity for bacterial myrosinase was determined by use of the hexokinase glucose 6 phosphate dehydrogenase coupled assay[10]. For the determination of hydrolysis of glucosinolates, 96 well Greiner µClear plates (GR 655095) were used. In the presence of active myrosinase, glucose is liberated from the glucosinolate molecule. The free glucose is modified into 6-phoshate by the hexokinase/Glucose 6-phosphate dehydrogenase enzyme in the buffer in the presence of ATP which reduces nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate oxidase (NADPH). As NADP has a maximum absorbance at 280 nm whereas NADPH has a maximum absorbance at 340 nm, this reaction can be measured as a decrease in absorbance at 280 nm with an increase in absorbance at 340 nm as shown in Figure 2.2.

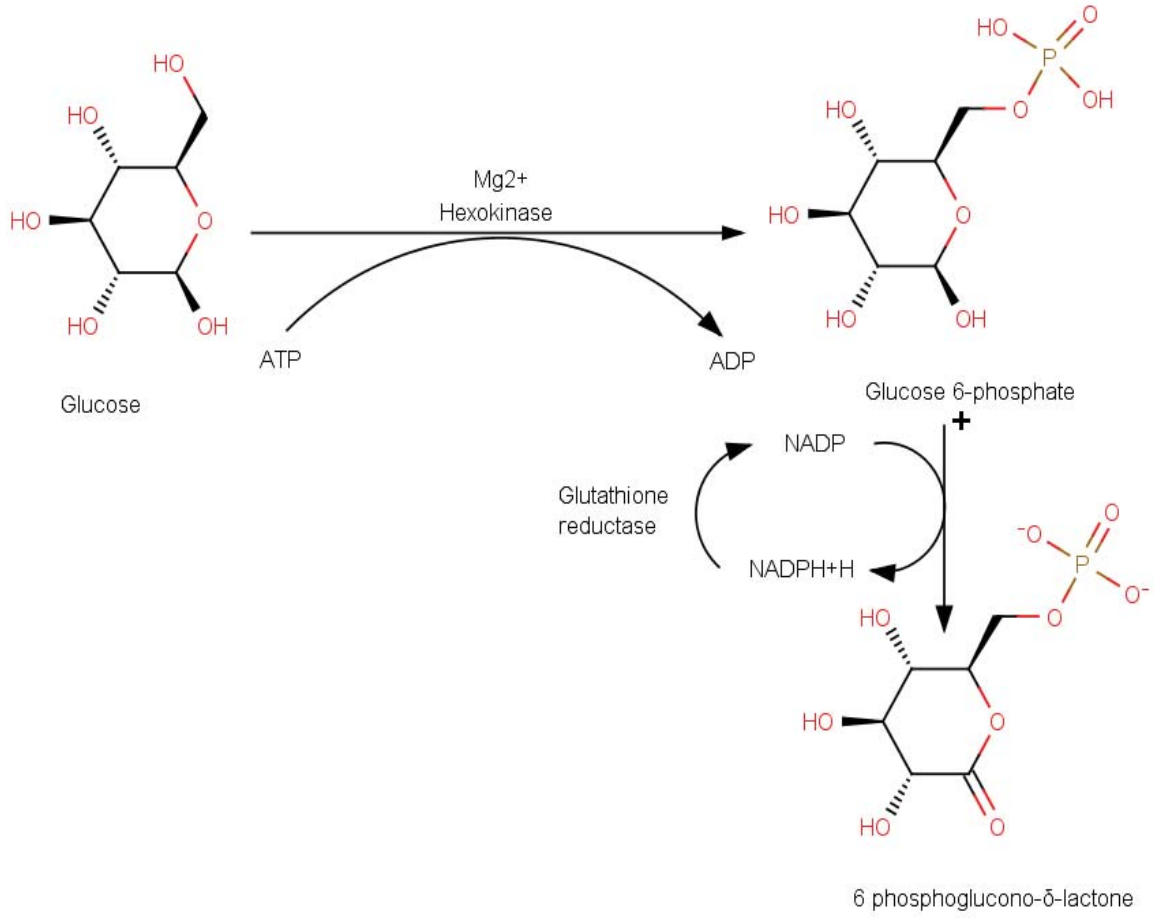

Figure 2.2. The principle of the hexokinase glucose 6-phosphate dehydrogenase coupled assay

The reaction requires glucose 6-phosphate dehydrogenase (G6PH), hexokinase, NADP and ATP. When glucose is released by the activity of myrosinase, it becomes converted to glucose 6-phosphate by hexokinase and glucose 6 phosphate dehydrogenase (G6PH) converts this to the lactone recycling NADPH back to NADPH+H in an ATP dependent reaction. The buffer for this reaction is given in Table 2.6.

Table 2.6. The coupled assay buffer

	Stock solution	**200 µL**
Tris buffer 50mM pH6.8		108 µL
3 mM $MgCl_2$	60 mM	10 µL
0.25 mM ascorbate	5 mM	10 µL
0.55 mM ATP	20 x	10 µL
0.75 mM NADP	20 x	10 µL
0.5U hexokinase/Glucose 6 Phosphate	1U/µL	1 µL
5 mM sinigrin	25 mM	40 µL
“Myrosinase”	0.1U/µL	1 µL
Or bacterial lysate	mg/mL	10 µL

2.1.4 MALDI-TOF mass spectrometry

Mass spectrometric analyses of tryptic peptides were performed as a service by the Centre for Protein Research Department of Biochemistry, University of Otago, Dunedin, NZ. Excised protein spots/bands were subjected to in-gel digestion with trypsin using a robotic workstation for automated protein digestion (DigestPro Msi, Intavis AG, Cologne, Germany). The protocol for automated in-gel digestion is based on the method of others[11]. Eluted peptides were dried using a centrifugal concentrator. The protocol for tryptic digestion can be downloaded at http://biochem.otago.ac.nz/cpr/protocols.html.

Peptides were re suspended in 30% [v/v] acetonitrile and 0.1% [v/v] TFA (trifluoroacetic acid) in water. One µL of peptide solution was premixed with 2 µL of matrix (10 mg per mL alpha cyano-4-hydroxycinnamic acid (CHCA) dissolved in 65% [v/v] aqueous acetonitrile containing 0.1% [v/v] TFA and 10 mM ammonium dihydrogen phosphate). 0.8 µL of sample/matrix mixture were spotted onto a MALDI sample plate (Opti-TOF 384 well plate, Applied Biosystems, MA) and air dried.

For mass spectrometry, samples were analysed on a 4800 MALDI tandem Time-of-Flight Analyser (MALDI TOF/TOF, Applied Biosystems, MA). All MS spectra were acquired in positive-ion mode with 800-1000 laser pulses per sample spot. The 15 - 20 strongest precursor ions of each sample spot were used for MS/MS collision-induced dissociation (CID) analysis. CID spectra were acquired with 2000-4000 laser pulses per selected precursor using the 2 kV mode and air as the collision gas at a pressure of 1*E-6 torr.

For protein identification MS/MS data was searched against the UniProt/SWISS-PROT amino acid sequence database (downloaded in May 2010) using the Mascot search engine http://www.matrixscience.com). The search was set up for full tryptic peptides with a maximum of 3 missed cleavage sites. Carboxyamidomethyl cysteine, oxidized methionine, pyroglutamate (E, Q) were included as variable modifications. The precursor mass tolerance threshold was 75 ppm and the max. Fragment mass error 0.4Da.

2.2 Broccoli glucosinolates: Preparation, extraction, separation and characterisation

2.2.1 Defatting of freeze dried powdered seed

Freeze dried broccoli seeds were kindly provided by Comvita NZ Ltd., Paengaroa, NZ. Defatting was performed using a method as previously described[12] but adapted as follows:

300 mL of n-hexane (Mallinckrodt) was added to 40 g of powder then the mixture was stirred for 30 minutes at room temperature. Next the liquid was decanted and replaced with another 300 mL of n-hexane and stirred for a further 30 minutes. Following this, the liquid was removed and the residue evaporated to dryness under a fume hood at room temperature (approximately 2 hours).

2.2.1.1 Broccoli sprouts

These were obtained from Living Foods (Auckland, NZ) fresh and were freeze dried in an industrial custom made kiln-like freeze drier (Cuddon Engineering, Blenheim) then because the thermal inactivation of myrosinase occurs after 60°C[12], processed as follows:

The dried sprouts were separated into two groups. The first group, untreated, was vacuum-packed and stored at -20°C. The other half were blanched in boiling water for 6-8 minutes, drained, then vacuum packed and frozen at -20°C.

2.2.1.2 Broccoli heads

These had been harvested prior and frozen at -80°C. Pieces were added to boiling water, microwaved for 3 minutes to maintain the temperature, then the container was placed in a water bath preheated to 80°C for 45 minutes and the mixture allowed to cool.

2.2.1.3 Broccoli seed

Seed was defatted as described at 2.3.1. The dried residue was dissolved in 50 mL 50 mM potassium phosphate buffer (pH7.2) that had been pre-heated to 80°C and held at that temperature for 45 minutes in a water bath.

After this time the mixture was allowed to cool and then the proteins were removed as described next at 2.2.2.

2.2.2 Removal of proteins

The liquid portion of the broccoli slurry was collected by filtering through Whatman No. 1 paper. Next Zinc/barium acetate (1:1) to a final concentration of 20 mM was added and this was left to precipitate for 30 min at 4°C. The material was then centrifuged at 4000 *x g* for 30 min at 4°C and the supernatant collected.

2.2.3 Purification with solid phase extraction columns (SPE)

Further purification for the broccoli seed was performed using a method previously described by Rochfort *et al*[13]. SPE columns C_{18} and aminopropyl (NH_2+) were activated with methanol (MeOH), then the C_{18} conditioned with water (C_{18}) and the NH_2+ with 1% acetic acid. The supernatant collected previously was diluted 1:5, then with the C18 connected in series to the NH2, the supernatant was loaded onto the top column (C_{18}), washed with water then with methanol. Elution was by adding 1% ammonia (NH_4OH) in methanol and a schematic for this is shown in Figure 2.3.

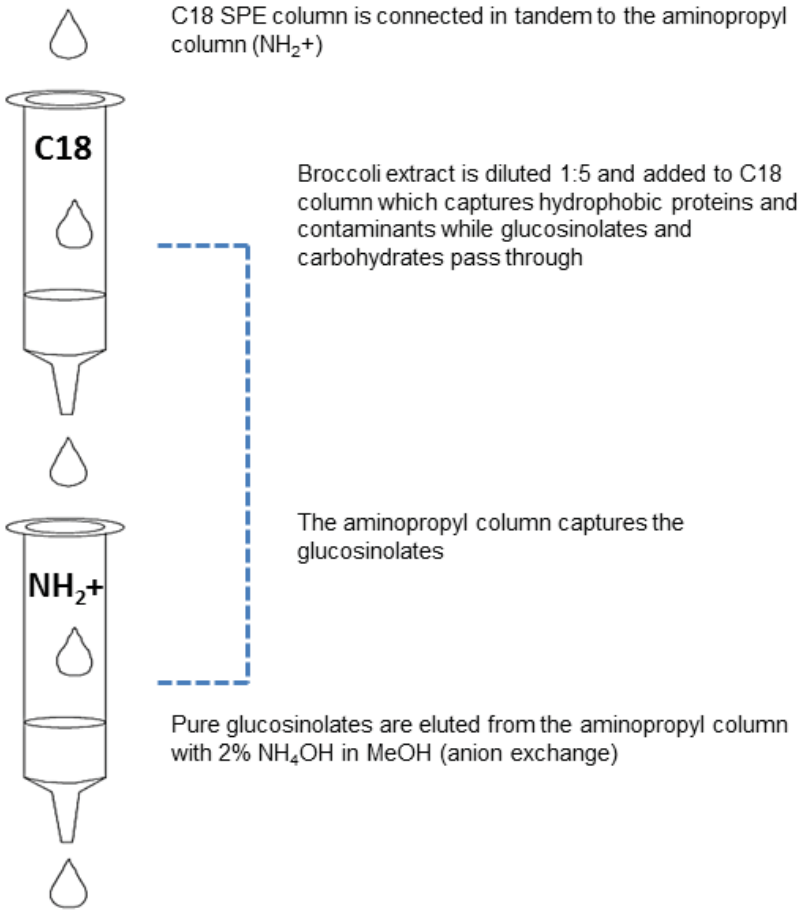

Figure 2.3. Anion exchange solid phase extraction (SPE) for purification of glucosinolates from broccoli

The eluted solution was evaporated under vacuum at 40°C until a yellow clear residue remained and this residue was dissolved in MilliQ water. Quantification of the GSL solution was obtained by reading the absorbance at 229 nm using allyl glucosinolate also known as sinigrin (Sigma S1647) as a reference standard. All reagents were purchased from Thermo Fisher Scientific New Zealand Ltd except for the zinc acetate which was purchased from Merck Ltd, New Zealand.

2.3 Analytical

2.3.1 Spectrophotometric assay for total glucosinolate concentration estimation

The SpectraMaxPlus 190 plate reader (Biostrategy, Auckland NZ) equipped with Softmax Pro analysis software 3.0 and Greiner 655801 UV transparent plates (Raylab Ltd, Auckland NZ) were used.

2.3.2 The Nanodrop for total glucosinolate concentration estimation

Using a Nanodrop (ThermoScientific), UV was selected in the 229-230 nm range after scanning for maximum absorbance. Allyl glucosinolate was used to generate a standard curve for concentration, following a standard method[14].

2.3.3 Separation and identification of intact glucosinolates: HPLC-MS and LC-QTOF-HRMS

2.3.4 HPLC-MS Method – Christchurch

Broccoli extract (~1mg/mL) was analysed by Nigel Joyce (Plant and Food Research, Lincoln Christchurch NZ) as a service:

The LCMS system consisted of a Thermo Electron Corporation (San Jose, CA, USA) Finnigan Surveyor MS pump, Finnigan MicroAS auto-sampler, Finnigan Surveyor PDA detector and a ThermaSphere TS-130 column heater (Phenomenex, Torrance, CA, USA). A 2 µL aliquot of each prepared extract was separated with a mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) by reverse phase chromatography (Luna guard cartridge 4 x 2 mm, 10 µm and Luna C18 (2), 3 µ, 80 Å, 150 x 3 mm, Phenomenex, Torrance, CA, USA) maintained at 30°C with a flow rate of 200 µL/min. A gradient was applied: t_{min}/A%/B% as 0/98/2, 3/98/2, 15/85/15, 20/60/40, 25/50/50, 30/2/98, 33/2/98 and returned to start condition.

The eluent was scanned by PDA (190-390nm) and Atmospheric Pressure Ionization mass spectrometry (API-MS) (LTQ, 2D linear ion-trap, Thermo-Finnigan, San Jose, CA, USA) with electrospray ionisation (ESI) in the negative mode. Data were acquired for parent masses from m/z 250–1000 amu with PQD fragmentation at 40 arbitrary units. Parallel Database Query (PQD) fragmentation using mass spectra file format 2 (MS2) allows collection of lower

m/z data (<1/3 of the parent m/z) thus 96[SO4]- and 97[SO4H]- m/z ions indicating glucosinolates could be obtained in a similar screening approach as Mellon et al. Data were processed with the aid of Xcalibar®2.05 (Thermo Electron Corporation).

2.3.5 HPLC Method - Palmerston North

Broccoli extracts (~3 mg/mL) were processed by Tony McGhie (Plant and Food Research, Palmerston North NZ) as follows:

The HPLC instrument was an Alliance 2690 (Waters, Milford, MA, USA) liquid chromatography. The column was a Synergi 4u Hydro-RP 80A column 250 x 4.6 mm. The mobile phase consisted of 50% 50 mM ammonium acetate (A) and 80% 50 mM ammonium acetate in methanol (B) by reverse phase at a flow of 1 mL/min. The gradient was: 100% A. 0% B, 0 - 20 min; linear gradient to 70% A, 30% B, 0.5 - 8 min; linear gradient to 75% A, 25% B, 8-13 min; linear gradient to 0% A, 100% B, 20 - 25 min; composition held at 100% B, 25 - 27 min; linear gradient to 100% A, 0% B, 27 - 32 min; to return to the initial conditions before another sample injection after 32 min.

The wavelength for detecting glucosinolates in all instances was 229 - 235 nm using a Waters 996 diode array detector (Waters, Milford, MA, USA) and data was processed on Chromeleon (Dionex V6.80 SR7 build 2528).

2.3.6 LC-QTOF-HRMS - Palmerston North

Samples were processed by Tony McGhie who developed and optimised the method. All samples were prepared by the author and all data analyses performed by the author. LC-MS grade acetonitrile was from Fischer Scientific, methanol (ChromAR) was from Mallinckrodt Chemicals, and ethanol (95%) was from LabServ.

LC-MS grade acetonitrile was from Fischer Scientific, methanol (ChromAR) was from Mallinckrodt Chemicals, and formic acid (0.1%) was from LabServ. The LC-MS system was composed of a Dionex Ultimate® 3000 Rapid Separation LC system and a microTOF QII mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an electrospray source operating in positive mode. The LC system contained a SRD-3400 solvent rack/degasser, HPR-3400RS binary pump, WPS-3000RS thermostated autosampler, and a

TCC-3000RS thermostated column compartment. The analytical column was a ZorbaxTM SB-C18 2.1 x 100 mm, 1.8 µm (Agilent, Melbourne, Australia) maintained at 50 °C and operated in gradient mode.

Solvents were A = acetonitrile, and B = 0.1% formic acid at a flow of 400 µL/min. The gradient was: 1% A, 99% B, 0 - 0.5 min; linear gradient to 70% A, 30% B, 0.5 - 8 min; linear gradient to 75% A, 25% B, 8 - 13 min; linear gradient to 100% A, 0% B, 13 - 15 min; composition held at 100% A, 15 - 17 min; linear gradient to 1% A, 99% B, 17 - 17.2 min; to return to the initial conditions before another sample injection at 21 min. The injection volume for all samples and standards was 1 µL. The microTOF QII source parameters were: temperature 200 ºC; drying N_2 flow 8 L/min; nebulizer N_2 1.5 bar, endplate offset -500 V, capillary voltage -3500 V; mass range 100-1500 Da, acquired at 2 scans/s. Post-acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis. GSL components were quantified using QuantAnalysis (Bruker Daltonics) by extracting ion chromatograms with mass windows of 10 mDa.

Caecal samples were prepared for analysis by centrifugation to precipitate solids, transferring 100 µL of the supernatant to a vial containing 900 µL of water that had added to it the internal standard epicatechin at a concentration of 5 µg/mL. Urine samples were prepared for analysis by making a 1:3 dilution of the urine with methanol/ H_20 (20:80), then the sample was centrifuged to precipitate any particulates and 100 µL of supernatant transferred to a vial. Plasma samples were prepared by treatment with 100 mM acetic acid[15]. Briefly, 0.5mL of plasma was treated with 100 mM acetic acid in methanol (1 mL), left on ice for 30 min, and centrifuged at 2000 *x g*, for 5 minutes at 4°C. 100 µL of supernatant was added to a vial containing 100 µL of 100 mM acetic acid in methanol. Quantitation was based on allyl glucosinolate; using reported methods[14].

2.3.7 GC-MS

GC-MS was processed by Martin Hunt, Plant & Food (Palmerston North NZ). Samples were prepared by the author, with method development and all analyses also performed by the author.

GC–MS analyses employed a Shimadzu 17A GC coupled to a Shimadzu 5050A quadrupole mass detector spectrometer with a source temperature of 240 °C. One µL injections were made into the injection port at 220 °C. Standard separations of compounds used a 30 m x 0.25 mm ID x 0.18 µm film thickness Rxi-5ms (Restek) capillary column with a helium flow of 1 mL min^{-1}. The oven temperature program was 60 °C for 1 min, 10 °C min^{-1} to 300 °C, and held for 1 min. Scan mode was 33 m/z to 500 m/z (urine) and SIM mode conditions are listed in Table 2.7. Samples were prepared for analysis as follows. One volume of sample was added to 2 volumes DCM (Sigma-Aldrich 650463), the tube tightly capped, shaken and mixed by inversion for 15 min at room temperature. The samples were centrifuged for 5 min at 2000 *x g* to separate the phases and the organic phase (bottom layer) carefully transferred to a vial using a glass Pasteur pipette.

To generate the individual compounds, broccoli extract was hydrolysed with and without myrosinase, at pH 9 and pH 4. For myrosinase hydrolysis, the 50 mM potassium phosphate buffer was adjusted to either pH 4 or 9 and contained 0.5 mM ascorbic acid. Fe_3Cl_2 (100 mM) was also added to the pH 4 reaction to help promote GSL conversion to nitriles. For acid hydrolysis the broccoli extract was added to pure water and the pH adjusted to 4 with hydrochloric acid (HCl). Compounds were identified by matching peaks to pure standards sulforaphane (Sigma S6317), BITC (Sigma W510548) and from the author's library constructed from published data for individual compounds.

Retention times were checked against previously published data to validate results when there were few differences separating compounds which may have differed only by the number of carbons in the alkyl chain[12,15-25]. To verify retention times the broccoli hydrolysis products or pure standards were added to urine or blood. The areas under the peaks identified were normalised to the internal standard and the partitioning efficiency validated empirically using triple solvent extraction (2.4.8). Based on the efficiency and where possible, sulforaphane was used to generate a standard curve for estimating nitrile concentration. Internal standards were used to ensure that the variation between replicates was within normal tolerances. This was determined empirically for each experiment and where sulforaphane could not be used for

estimation of concentration, the ratio of the peak to the internal standard is reported instead.

The limit of detection was determined for each experiment at three times the signal to noise ratio (6.0×10^3 for urine analysis and 5.0×10^3 for blood).

Table 2.7. The conditions for single ion monitoring (SIM) and the 14 masses (m/z) selected

SIM conditions	
start time (min)	6.2
end time (min)	26.0
Volt=Abs	2kV
Threshold	1000
Interval	0.5 sec
Solvent cut time	6.1 min
microscan width	0 u
m/z	55.1, 61.0, 64.0, 68.1, 72.0, 82.1, 101.0, 115.1, 129.0, 131.0, 145.1, 147.1, 160.1, 161.1

2.3.8 Triple extraction

1 mL of aqueous supernatant containing the glucosinolate hydrolysis product was added to 2 mL DCM, the tube tightly capped, and shaken vigorously for 2 minutes. Then the tube was centrifuged at 1500 *x g* for 3 minutes to separate the phases. The lower phase containing the DCM was removed carefully using a glass Pasteur pipette and filtered into a 2 mL GC vial for analysis. All of the solvent was carefully removed leaving the aqueous phase behind. A further 2 mL DCM was added and the whole process repeated twice more (in triplicate).

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THREE

3.0 Separation and characterisation of glucosinolate-containing broccoli material

Broccoli contains a number of glucosinolates with the type and concentration varying depending on the plant tissue and the age of the plant. Estimates vary but the total glucosinolate content has been determined to be low at less than 15 μmoles per gram of dry mass and the major glucosinolate, glucoraphanin, at ~7.1 μmoles per gram[1-5]. Broccoli seed is known to contain concentrated amounts of glucoraphanin (45-114 μmoles per gram), but equally, broccoli sprouts are known to contain high levels of glucosinolates and not much else[6,7]. A source of glucosinolates was sought, preferably high in glucoraphanin the precursor of sulforaphane. It needed to be a vegetable crucifer that one could include in the diet. Finally, it needed to be in sufficient quantity to enable large-scale extraction generating enough material to allow multiple experiments while maintaining the same glucosinolate profile and quality throughout.

3.1 Detection of glucosinolates

As glucosinolates have no chromophore, detection can be problematic. Detection techniques generally rely on HPLC although there have been spectrophotometric assays reading UV absorbance at 227 nm[8]. Alternatively, detection can be accomplished with a spectrophotometer by reading the change in absorbance at 340 nm by the coupled assay described at section 2.1.3. Glucosinolates absorb maximally at ~229 nm but their products, the isothiocyanates absorb at ~240 nm[9]. However in any solution that is less than pure, for example, when glucosinolates are mixed with other compounds such as proteins, background interference from these compounds prevents accurate determination of the glucosinolate concentration. Preliminary experiments to determine the maximum absorption between 190 nm and 250 nm confirmed that for allyl glucosinolate, the maximum absorption peak was at 231 nm (Appendix A, Figure A1). The bacterial lysate of extracted protein in Tris buffer (shown as lysate) gave a maximum absorption at 210 nm. While the allyl

glucosinolate peak could easily be distinguished among the lysate at a concentration of 0.5 mg/mL, detection sensitivity was lost when the allyl glucosinolate concentration fell under 0.03 mg/mL. The only way to increase sensitivity using a spectrophotometer was to further purify the samples by removing the proteins. This could be accomplished by the use of solid phase extraction (SPE) C18 cartridges which when activated with methanol and conditioned with pure water, capture protein and hydrophobic particles yet allow hydrophilic material including glucosinolates to run through. The only downside to this method is the amount of processing and number of SPE cartridges required. Also the samples require serial dilutions across the microplates to ensure that the concentration is within the linear range of the standard curve of concentration. In other words, hundreds of SPE cartridges, many plates and many hours with potentially low sensitivity, loss of analyte (recovery from SPE cartridge is not 100%) and variations in the results due to the method generating systemic errors that confound the results.

A better method is liquid chromatography with mass spectrometry (LC-MS) by reverse phase chromatography (RP) with anion exchange and there are efficient methods available to accomplish this[9]. Glucosinolates can be difficult to separate from one another because of they interact only weakly with RP (Figure 3.1). They are a highly charged molecule; with a sulfate group which gives them a pKa of -9 so they are almost always in their anionic state[10]. Also the side sugar group (thioglucopyranoside) makes them strongly hydrophilic so it is only the R group that differentiates them for separation.

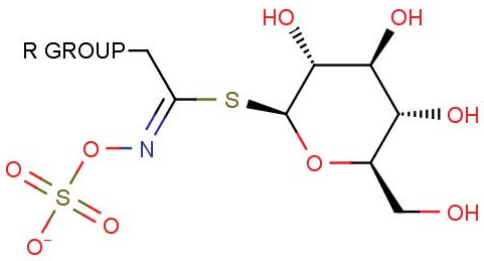


Figure 3.1. The basic structure of glucosinolates impacts on their affinity for reverse phase separation

At the beginning of this project direct access to HPLC or LC-MS on site was not available. However, a simple, rapid calibration technique was needed to quantify the semi purified glucosinolates (section 2.5) to validate the

purification methods and also so that the right concentrations could be added to media during ongoing experiments.

3.1.1 Nanodrop enabled estimation of total glucosinolates

Because the spectra for glucosinolates are in the UV range, the Nanodrop 1000 (Thermofisher Scientific, NZ) could be used on its UV/VIS setting provided the sample was pure and in aqueous solution.The Nanodrop enabled estimation of the recovery efficiency of total glucosinolates from purification by SPE columns and could be used with stored extract to check for glucosinolate concentration and purity. Prior to use, any degradation of glucosinolates could be seen by the shift in absorbance of a single peak at 229 nm to a double peak, the second at 240 nm. This provided an inexpensive, easy and simple method for calculating and having the optimal concentration of glucosinolates when adding to media for experiments. Not only could the concentration now be calculated, but during the purification steps it was possible to monitor the recovery and optimise this process by adjusting the method so that the SPE cartridges were used to their maximum efficiency. If overloading occurred, the excess could be captured and recycled back through the next SPE cartridge during the washing steps.

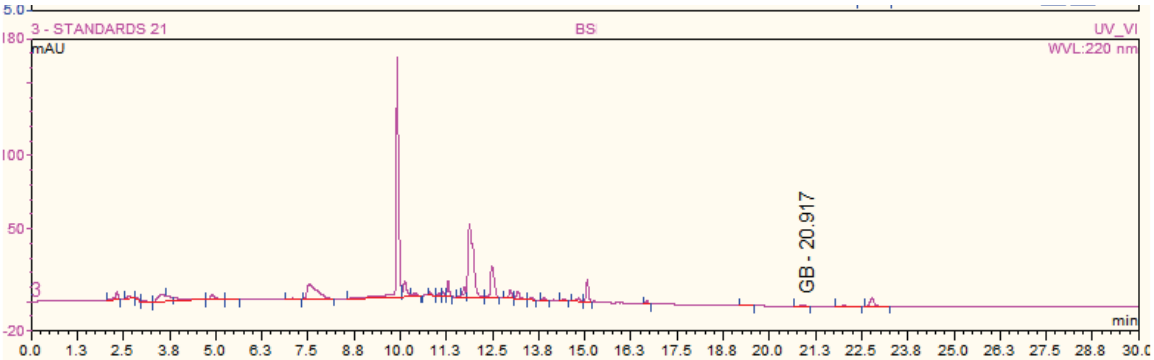
Appendix A, Figure A2 shows at A, pure allyl glucosinolate at 2.5 mM with a single peak at 230 nm. B is broccoli extract diluted 1:10 having three peaks, the largest at 390 nm. C shows the eluted fraction to contain a single peak at 230 nm. By using a range of allyl glucosinolate concentrations, a standard curve could be generated enabling quantification of the total glucosinolates in the broccoli extract. The limitations of this method were that it was a measure of the total glucosinolates present and could not distinguish individual glucosinolates.

3.1.2 HPLC

The system available to us in Palmerston North (2010) was high performance liquid chromatography (HPLC) which did not have mass spectrometry. Without the benefit of mass spectrometry, pure compounds of the analyte of interest are necessary and at the time of this study the only commercially available analytical glucosinolate standard was allyl glucosinolate (Sigma S1647). At the Plant & Food site in Christchurch which had full

LCMS/MS facilities, a number of broccoli cultivars had already been analysed for their glucosinolate composition. A sample of freeze-dried, powdered, quality control (QC) broccoli cultivar was obtained* that had already been characterised by HPLC-MS. Samples were run on the available HPLC system at Palmerston North, and then glucosinolate peaks were annotated with the peaks we found based on the retention times and the UV absorbance data supplied† (Appendix A, Figure A3). Next this was compared against freeze-dried ground broccoli sprouts (obtained from Living Foods, Auckland NZ) and freeze-dried powdered broccoli seed (BSP)‡.

The Comvita seed extract contained GSLs at the highest concentration giving a signal five times that of the blanched sprouts, while the QC (labelled PRN), had the lowest (Appendix A, Figure A3). As blanching destroys myrosinase activity, it was expected that the concentration of glucosinolates in these samples would be higher than in the non-blanched. The QC broccoli contained the least and this was expected also, because the lowest concentrations of GSLs are known to be found in mature plant material, the highest in seed[1,11]. From these results it was decided to use the powdered seed as it met all the requirements of this study being available in large quantities, being from a dietary source, the composition was known and it was certified as containing 12.9% glucoraphanin (w/v) (see Appendix A, Figure A4). Analysis by HPLC agreed with this as the largest peak from the seed was glucoraphanin (Figure 3.2 and Appendix A, Figure A5).



* Author acknowledges Paula Rippon Christchurch Plant & Food Research, Lincoln for sharing LC-MS data and providing samples of high-glucosinolate broccoli material
† Author acknowledges Tony McGhie, Phytochemistry team, Plant & Food Research, Palmerston North for technical assistance, processing and help with compound identification
‡ Author acknowledges Comvita, Pangeroa, NZ for providing the broccoli seed extract used for this work

Figure 3.2. Chromatogram of broccoli seed powder gave a major peak at ~10 min that was identified as glucoraphanin. The only other identified peak was glucobrassican at 20.9 min

Having the identities of the main glucosinolate peaks, it was hoped that it would be possible to demonstrate their transformation by bacteria. After the first experiments with bacteria incubated in glucosinolate extract, it was apparent that some of the bacteria were changing some of the glucosinolates differently to others (Appendix A, Figures A6-A9). Furthermore, identification had not been carried out on all glucosinolate peaks. As the mass spectrometry data had not yet been obtained which would identify all the peaks (these differed a little from the QC sample) a sample of BSP extract was sent to Lincoln Plant and Food site for analysis (see section 2.3.4). It was hoped that from this it would be possible to able to align all of the glucosinolates allowing identification of all glucosinolate peaks. These data confirmed that the other major glucosinolates present were glucoiberin and glucoerucin with minor component glucosinolates progoitrin, allyl glucosinolate and glucoiberverin (Appendix A, Figure A10). Even having this information it was not possible to extrapolate those peaks to the system used in Palmerston North because of variations in the analytical column used and the variation across systems.

3.2 LCQTOF-HRMS enabled sensitive analysis and quantification of individual glucosinolates

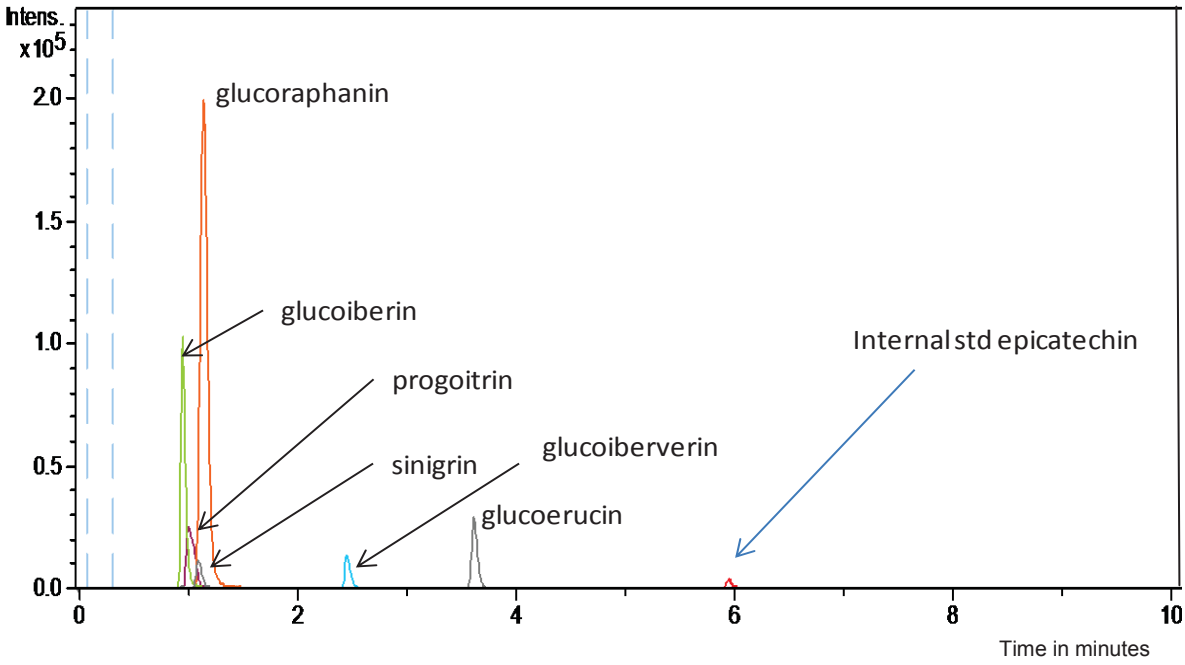


Figure 3.3. LCQTOF-HRMS separation of individual glucosinolates of interest from broccoli seed

The acquisition of a new LC QTOF-HRMS system for Plant & Food, Food and Innovation group, (Palmerston North) resolved this problem. This system clearly separated out the major glucosinolates from the BSP extract even when their retention times overlapped (Figure 3.3). A preliminary test was made with a sample of BSP extract as shown and the method was optimised to find the best resolution and separation. F3.4urther samples were run to identify the GSLs found in the extract based on the known mass spectra data whch are listed in Table 3.1. and the BSP extract was now characterised for the individual glucosinolates and could now be assessed for changes.

Table 3.1. LC QTOF-HRMS analysis of the glucosinolates in the broccoli seed extract

Chemical name and common name (italics)	MW	Relative abundance (%)	retention time (min)
3-methylsulfinylpropyl glucosinolate (*Glucoiberin*)	422.025	17.2	1.2
2-hydroxy-3-butenyl glucosinolate *(Progoitrin)*	388.042	4.8	1.5
2-propenyl glucosinolate *(Allyl glucosinolate)*	358.026	2.0	1.7
4-methylsulfinylbutyl glucosinolate *(Glucoraphanin)*	436.041	48.0	1.8
5-methylsulfinylpentyl glucosinolate *(Glucoalyssin)*	450.056	0.4	2.9
3-butenylglucosinolate *(Gluconapin)*	372.044	1.3	3.0
3-methylthiopropylglucosinolate *(Glucoiberverin)*	406.032	1.7	3.7
4-methylthiobutyl DS glucosinolate*(Desulfoglucoerucin)*	341.092	1.4	4.3
4-methylthiobutyl glucosinolate *(Glucoerucin)*	420.046	15.0	5.0
3-indolylmethyl glucosinolate *(Glucobrassicin)*	447.053	0.4	5.1
3-butenyl DS glucosinolate *(Desulfogluconapin)*	294.513	4.9	6.9
n-hexyl glucosinolate	402.089	0.6	8.3
1-methoxy-3-indolylmethyl glucosinolate *(Neoglucobrassicin)*	477.063	0.2	8.6

3.3 Separation and quantitative analysis of the products of glucosinolate hydrolysis – GC-MS

After learning the glucosinolate "fingerprint" of BSP extract it was necessary also to know what the hydrolysis products of the glucosinolates from BSP extract were. By using myrosinase (thioglucosidase from *Sinapis alba* - Sigma T4528) it was possible to enzymatically hydrolyse all of the glucosinolates present (see chromatograms at Appendix A, Figure A11-12). DCM as a solvent enabled good resolution and separation of compounds without having to use derivatisation. The most challenging aspect was identifying which compound was which as the alkyl glucosinolates only varied by the number of carbons in their alkane chain and/or the oxidation state (alkyl-

sulfinyl or alkyl-thiol). By using known pure compounds and available published spectral data, then allowing for retention time shifts for our system, it was possible to identify the main compounds of interest (Table 3.2).

Table 3.2. MS spectral signatures of identified end products of glucosinolate metabolism after incubation of broccoli seed extract with bacterial cultures or in acid medium for 24 h

Name	retention time (min)	Mass	MS spectral data m/z (% relative abundance)
Iberverin nitrile *4-(Methylthio)butanenitrile*	6.7	115.20	115 (40), 68 (12), 62, (12), 61, (100), 48(17), 45 (48), 41 (36)
Erucin nitrile *5-(Methylthio)pentanenitrile*	8.4	129.22	129 (72), 114 (11), 82 (68), 61 (100)
Iberverin *3-(Methylthio)propyl isothiocyanate*	10.4	147.26	147(11), 101(100), 72(38), 61(43), 45(26), 41(38)
Erucin *4-(Methylthio)butyl isothiocyanate*	11.5	161.28	161 (18), 115 (66), 85 (20), 72 (57), 61 (100)
Iberin nitrile *4-(Methylsulfinyl)butanenitrile*	11.3	131.19	131(21), 115(3), 87(2), 68(39), 64(68), 41(100)
Sulforaphane nitrile *5-(Methylsulfinyl)pentanenitrile*	12.8	145.22	145 (19), 129 (9), 82 (42), 64 (54), 55(100)
Iberin *3-(Methylsulfinyl)propyl isothiocyanate*	13.7	163.26	116 (20), 100 (15), 72 (100), 63 (34), 61 (18), 41 (98), 39 (56)
Sulforaphane *4-(Methylsulfinyl)butyl isothiocyanate*	15.3	177.28	160 (47), 72 (100), 64 (16), 55 (45)

3.4 Triple solvent extraction to determine partitioning efficiency

One of the systemic errors that occur in GC relates to the affinity the compound of interest has for the solvent. In order to detect the compounds of interest, first they have to be transferred from aqueous by partitioning into a volatile solvent, in this case DCM. Sulforaphane is insoluble in water but soluble in DCM. By using an extraction ratio of 1:3 (aqueous/non polar), fat soluble, and non-polar molecules including the nitriles or isothiocyanate products are partitioned out of the aqueous fraction and into DCM. Any nitriles or isothiocyanates with oxygen as part of a sulfinyl group increase the polarity and therefore decrease the degree of partitioning into DCM. As the products being assessed were mixtures of sulfinyl compounds as well as thiols, it was necessary to determine the extraction efficiency. This was done by triple extraction which confirmed an efficiency of >90% for all except sulforaphane at ~81% (Figure 3.4).

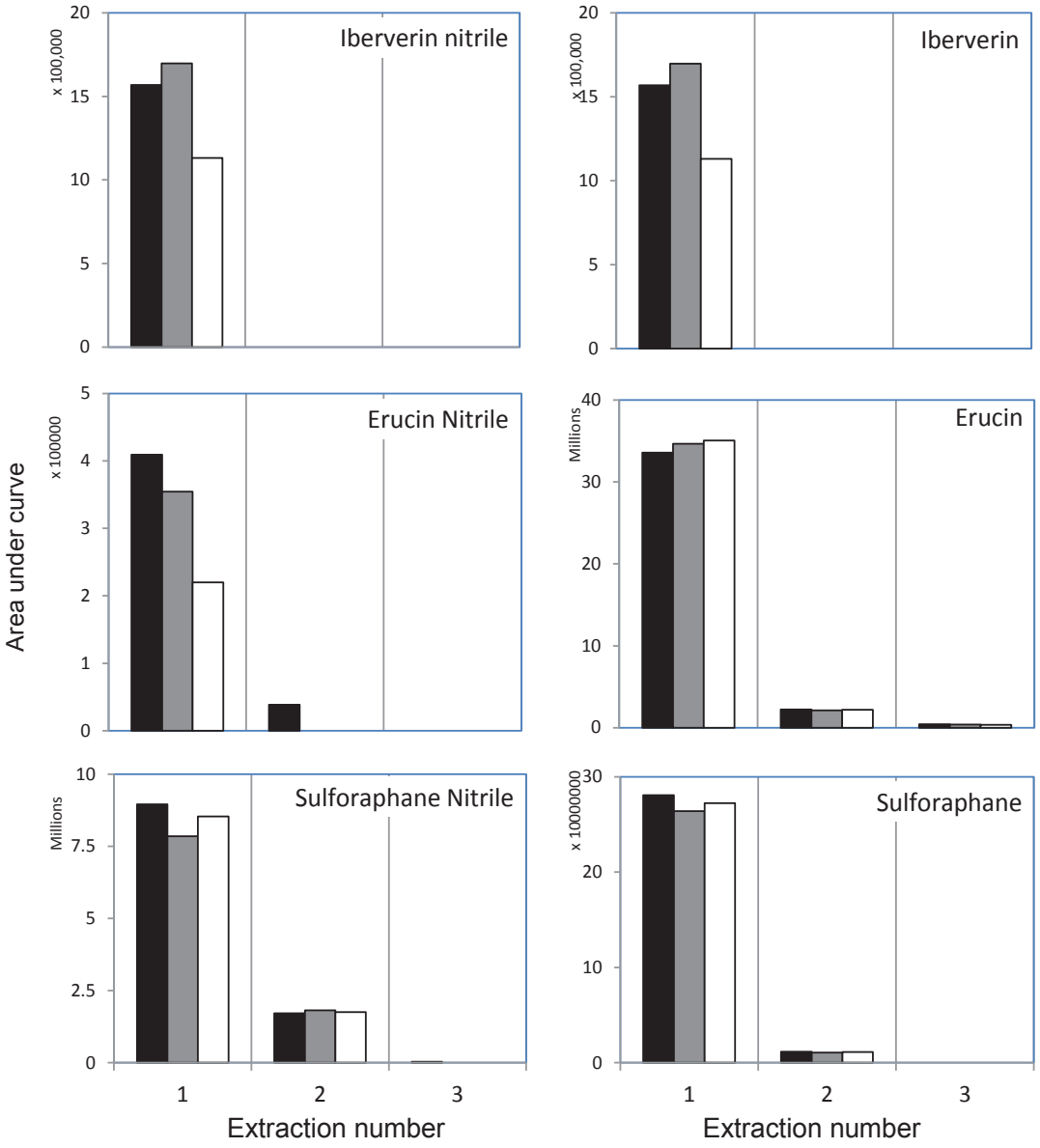
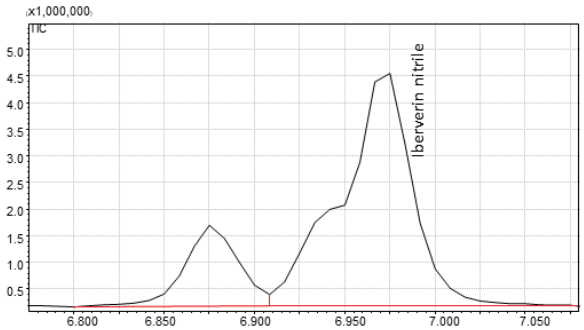


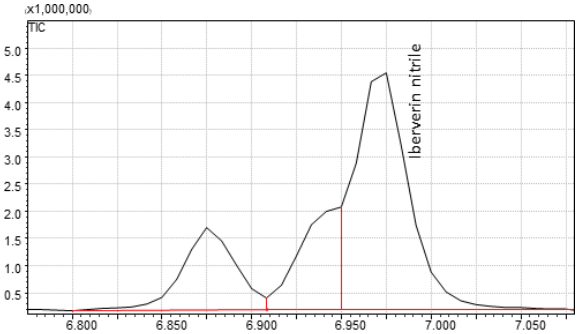
Figure 3.4. The extraction efficiency of various isothiocyanates and nitriles shows similar partitioning: Iberverin nitrile >90%, erucin nitrile >93%, sulforaphane nitrile >81%, sulforaphane >96% and iberin >90%. In general, the nitriles partitioned less efficiently than isothiocyanates, with sulforaphane nitrile lowest at 81%. Samples were in triplicate shown as black, grey and white bars.

3.4.1 Manual integration of peaks

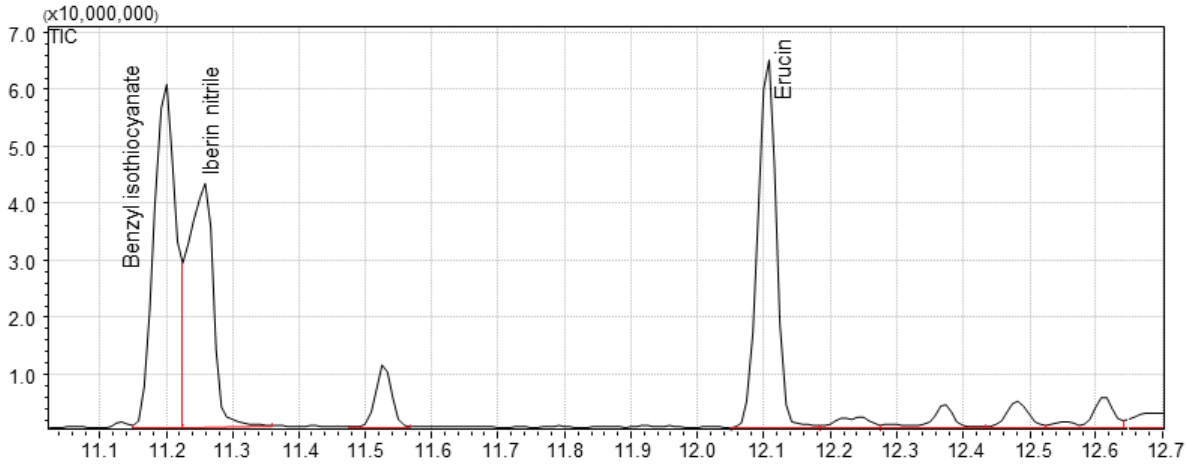
Automatic integration of peaks was performed by the software GCMS solution version 2.70 (Shimadzu Corporation) but manual integration was necessary for iberverin nitrile and iberin nitrile which did not separate from closely eluting compounds (Figure 3.5). Also manually integrated was BITC and iberin nitrile (11.2 versus 11.3 min) (Figure 3.5 and Appendix A, Figure A13). Reducing benzyl ITC concentration to <1mM improved peak resolution.



A. Iberverin nitrile automatic integration



B. Iberverin nitrile manual integration of peak



C. Benzyl isothiocyanate and iberin nitrile

Figure 3.5. (A) Iberverin double peak was separated by manual integration resulting in two peaks (B). (C) Iberin nitrile had to be manually integrated from the double peak with benzyl isothiocyanate. Ions are shown at Appendix A, Figure A13

3.5 Summary

The advantage of HPLC over spectrophotometry was that the use of columns and anion exchange allowed for separation of the analyte of interest from background interference and separated a number of compounds from a mixture. A disadvantage was that HPLC took a longer time to set up and calibrate. Spectrophotometry would have been the preferred method as it was available in the lab, easy to use, was able to process multiple samples in short time and did not require too much expertise to run. However, the samples did not lend themselves to assessment by spectrophotometry due to interference by other compounds. The next stage was identification of compounds.

In HPLC the only way to be certain that the correct compounds have been identified is by having that pure compound to compare against and the only analytical pure glucosinolate available commercially was allyl glucosinolate. Alternatively, the individual glucosinolates in the BSP extract could be identified with HPLC equipped with mass spectrometry. This was only available at Christchurch and getting samples analysed from here, then trying to extrapolate the data was technically difficult. Fortunately, LCQTOF-HRMS became available after only a few months of working with the other systems.

The next issue to overcome was in the identification and quantitation of the metabolites from glucosinolate hydrolysis. As small volatile compounds they lend themselves well to analysis by GC-MS. However, even with the availability of mass spectrometry, the only way to be certain of identification was to match spectra with pure compounds and the only commercially available analytical standard was sulforaphane (S6317), BITC (Aldrich 242494) and butanenitrile (Fluka 08436).

For example, the first GC-MS chromatogram using the NIST12 and NIST62 database libraries identified every nitrile as butanenitrile. As butanenitrile was not on the list of possible products and could not be giving multiple peaks across the chromatogram, butanenitrile (Fluka 08436) was purchased and compared with the multiple butanenitrile peaks from my chromatogram results. This confirmed that none of the peaks were butanenitrile because the retention time for the real butanenitrile is ~5 minutes and the butanenitrile peaks in our chromatograms were at 6.8, 8.6 and 12.3 min. In fact,

what the software had annotated by automatic integration of the peaks as butanenitrile, were actually iberverin nitrile, erucin nitrile and sulforaphane nitrile.

To achieve the identification of the compounds, the following approach was undertaken. GC-MS solutions software (Shimadzu) processed the samples as batch files which detected and integrated the peaks against NIST12 and NIST62 commercial libraries. Next the author's nitrile/ITC library was included and the samples reprocessed. Next manual integration was performed to separate any double peaks or rename incorrectly identified peaks and a manual visual scan was done over the whole chromatogram for any unidentified or interesting peaks.

The nitrile/ITC library was built from the commercially available analytical standards sulforaphane, butanenitrile and BITC (as described previously) and from others' published mass spectral data on all of the compounds in Table 3.2. The published spectral signatures of compounds were not identical to the same compounds identified in BSP extract because of differences in the GC-MS equipment. For example, the slight variations between columns meant that ion fragments were produced at different abundances. Also, as the column ages, the retention time shifts. What did not change however, were the mass values for the ions so sulforaphane for example, had a 160 m/z ion, a 72 m/z ion, a 64 m/z ion and a 55 m/z ion although the retention times and ion abundances did vary between columns and also between batch runs. The nitriles, erucin nitrile and sulforaphane nitrile share some of the same fragmentation ions because they are the same compound except for their oxidation state. As shown in chapter 1 (Figure 1.6), erucin nitrile and iberverin nitrile differ only by one carbon on their alkane chain as do iberin nitrile and sulforaphane nitrile. Identification for nitriles required matching both the ions then checking the retention times. The spectra of the identified compounds are given at the Appendix A, Figure A14 – A15).

While there is an advantage of having pure standards for comparison enabling identification, a drawback is that this can be limiting to finding only the products that match the standards. By not having the benefit of these standards, every peak was monitored by checking every chromatogram manually.

LCQTOF-HRMS enabled the profiling of individual glucosinolates and GC-MS allowed the fate of these glucosinolates to be monitored. Now experiments combining the bacteria and glucosinolates could proceed and if the glucosinolate composition of BSP extract was changing in any way, these analysis methods should capture these events.

3.6 References

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FOUR

4.0 Selection of bacteria

Plant myrosinase (thioglucosidase EC 3.2.3.1) is a member of glycoside hydrolase family 1 (GH1) (www.cazy.org/), but while GH1 family enzymes with diverse substrate specificities are commonly found in bacteria, none have been functionally characterised as having thioglucosidase activity. Bacterial genes encoding similar GH1 family glycoside hydrolases were sought from bacterial protein data (NCBI BlastP) with the criteria for bacterial selection being that the bacteria should be beneficial food grade organisms and be able to survive transit through the gut or at least remain metabolically active during transit.

By cloning the putative myrosinase-encoding genes from bacteria it may be possible to discover a novel bacterial myrosinase. Also from finding this, it may be possible to narrow down the number of bacteria that are screened for being the best at metabolising glucosinolates (GSLs).

The aim of this study was to identify myrosinase-producing bacteria and the first approach was to employ database mining to identify putative myrosinase-encoding bacterial genes and from this, select potential candidates, clone these genes and express them in recombinant *Escherichia coli.* It was hoped that pure recombinant protein could then be collected and assessed for myrosinase activity. In the event that this protein possessed myrosinase activity, this would be the first bacterial myrosinase identified since 1974[1,2] and the first to be cloned.

Another way to find GSL metabolising bacteria is to test bacteria and to this end, some preliminary assays for myrosinase activity were also undertaken.

4.1 Materials and methods for bacterial selection

4.1.1 BLAST identification of GH1 family genes

'BlastP' searches for proteins similar to the myrosinase protein from *Brassica oleracea* yielded a number of potential candidates. Included for alignment was the beta-glucosidase (1CBG) from *Trifolium repens* or white

clover (cyanogenic beta-glucosidase) (http://www.ncbi.nlm.nih.gov/protein/1CBG_A). While not a myrosinase, the crystal structure for 1CBG has been resolved and is reported to be similar to the myrosinases[3]. By collating the information on the characterised myrosinases, through information in the public domain, NCBI protein BLAST and alignment by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), a summary of the characteristics of myrosinases is given in Table 4.1.

Table 4.1. Comparison of myrosinases between plants, bacteria and fungi

Myrosinase	***Plant***	***1CBG***	***Enterobacter***	***B.animalis* subsp *lactis***	***Lactic acid bacteria***	***Fungi***
Reference	[3]	Fig A1**	[2]	Fig. A1**	Fig. A2**	[4]
5 glucose recognition sites	QHNYE	QHNYE	QHNYS	QHNYE	QHNYS	Not studied
Zinc ligand	HD 11-13 aa	HD	absent	HD	Absent except for 2 *L. rhamnosus* proteins	
Acid/Base proton donor	E	E	E	E	E	
Catalytic nucleophile	E	E	E	E	E	
Aglycon recognition	S or T, R, I, R, F or V, F	Absent	Absent	Absent	Absent	
Molecular mass kDa	120-150	56 (est*)	61 (est*)	53-56.5 (est*)	50-60 (est*)	90-120
Number of aa in protein	500-550	490	462	460, 490	450-500	
Inhibited by	Cu, Hg,		Cu, Hg, Ascorbic acid	Should be same as plant		
Activated by	Ascorbic acid					
Mercaptoethanol			Increased activity			
Salt			LiCl, SnCl, MnCl and NaCL did not affect activity			
pH stability max pH activity Heat stability			5-7 6.5 37			

*est – estimated from protein sequence data , **Appendix A Figures A16-A17

The KEGG database was used to find a beta glucosidase from Bifidobacteria (BIF_02125) for *B. animalis* subsp. *lactis* (http://www.genome.jp/kegg/kegg1.html) and this was also aligned with myrosinase from *Brassica oleracea* (Appendix A, Figure A16).

Some lactic acid bacteria had already been characterised as having myrosinase activity although the genes encoding this function had not been identified[5-7]. BlastP searches for similar proteins to the myrosinase from *Brassica oleracea* identified some putative myrosinases from the genera *Lactobacillus* and *Bifidobacterium* and from *Enterobacter cloacae*.

4.1.2 Expression of bacterial candidate genes encoding myrosinase activity

Lb. plantarum KW30 has recently been shown to produce a glycopeptide bacteriocin in which sugars are S-linked to a cysteine[8], and because of this we hypothesised that KW30 would be a candidate as a potential thioglucosidase producer. Analysis of the draft genome sequence of *Lb. plantarum* KW30[8] highlighted a region encoding GH1 family enzymes, together with beta-glucoside-specific PTS transporters (Figure 4.1). The genes for two GH1 family enzymes (GH1#1 and GH1#5) with best BLAST matches to 6-phospho-beta-glucosidase (YP_003064398.1) and beta-glucosidase (ZP_07078860.1) were selected as likely candidates. Figure A17 shows a ClustalW alignment for these.

Prior to cloning, all of the DNA sequences were checked for codon bias[9] because the genes to be expressed were from Gram positive high-GC organisms and the expression system was in Gram negative *E. coli*. Few unusual codons were noticed (see Appendix A, Figure A18) and as optimisation of the DNA sequences encoding these genes for *E.coli* expression required commerical synthesis of the genes, it was decided to continue without codon optimisation and if necessary, switch to the *E.coli* strain Rosetta-gami (DE3) which carries a plasmid encoding the rare *E. coli* codons AGG, AGA, AUA, CUA, CCC, and GGA. Constructs pETJAM1, 2 and 3 were designed to amplify and express the GH1 encoding genes from *B. animalis* subsp. *lactis* and *Lb. plantarum* KW30.

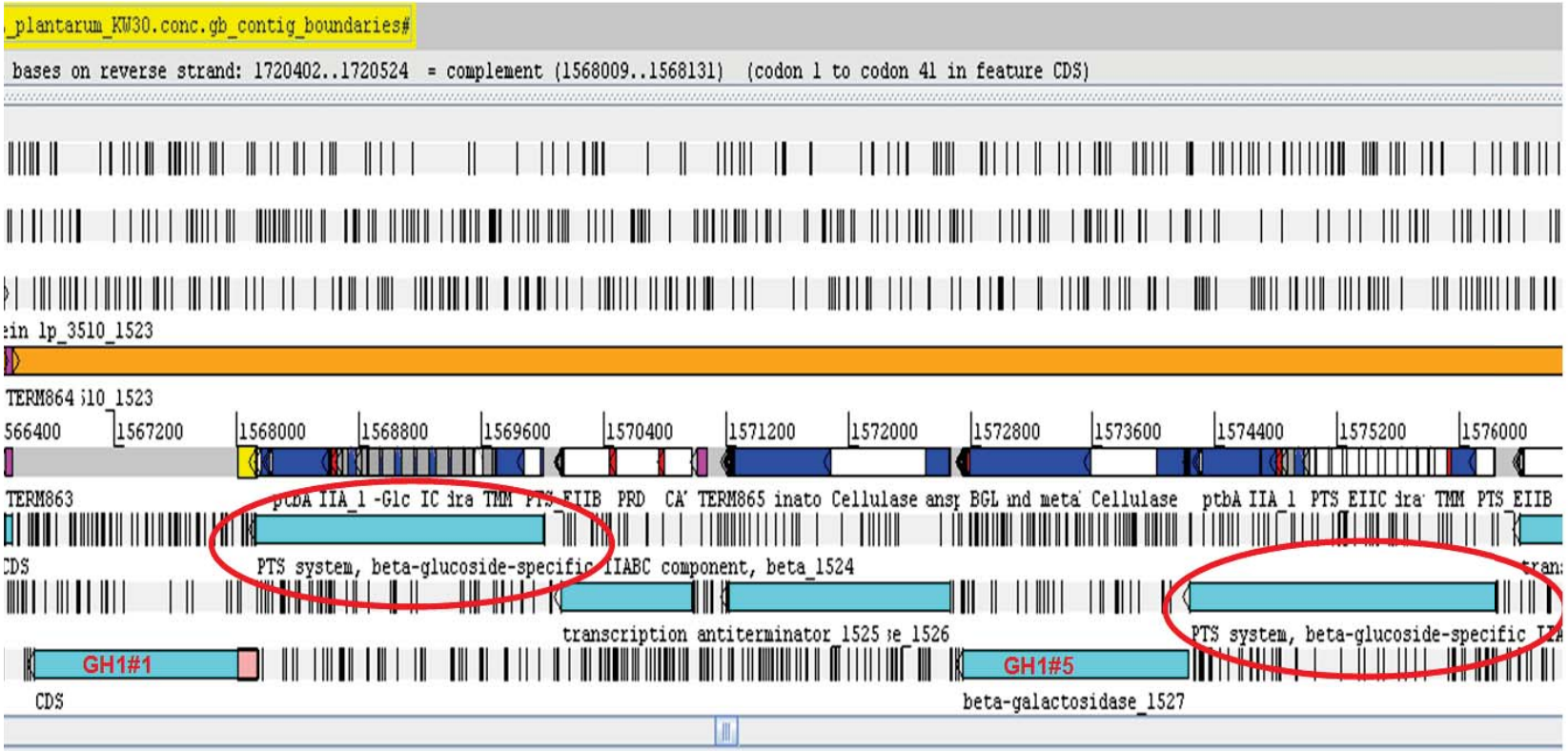

Figure 4.1. Annotated partial genomic sequence from L. Plantarum KW30. The genes identified and labelled in red GH1#1 and GH1#5 were chosen because both were identified as putative GH1 family genes and both were immediately downstream of PTS system components (circled in red)

4.2 Molecular manipulation and cloning

4.2.1 Cloning Strategy

The cloning strategy, primer design and all cloning steps were carried out by the author. Details of the cloning strains used are given in Table 4.2 Appropriate restriction sites were incorporated into the primers (Table 4.3) designed for the gene constructs to enable cloning into expression vector pET16b and the plasmids used are described in Table 4.4. All oligonucleotide primers were synthesised by Life Technologies and the bacterial strains that were tested listed in Tables 4.5 and 4.6.

Table 4.2. Characteristics of the cloning strains

Cloning Strain		**Strain and description**	**Source or reference**
Escherichia coli	XL1-Blue:	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *relA*1 *lac* *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	Stratagene New England Biolabs (NEB)
	BL21(DE3)	F^- ;*omp*T *hsdS*$_B$(r_B^- m_B^-) *gal* *dcm*(DE3);pLysS(Cam^r)	Novagen/Merck

Table 4.3. The primers used in this study.

Oligonucleotides	Restriction site underlined	Sequence from 5' to 3'
GH1#1 fwd	*Nco*I	CACCATGGATGCACCATCACCATCATCACTATAAACCAACTTATCCTAAAACGTTCC
GH1#1 rev	*Bam*HI	CCGGATCCTCAAAGCAAATTTCTGAAATAAGCTAGATCTTCACCATTAGATTTAATG
GH1#5 fwd	*Nde*I	CAGCATATGTATTCAAAAACAATGCCAACTGGCTTTCCAAAG
GH1#5 rev	*Bam*HI	TATGGATCCCTATCTCAAATCTTCCCCATTCGAAGCAATTAC
GH1 HN019 fwd**	*Nde*I	CACATATGACGATGACGTTCCCGAAGGGCTTCCAG
GH1 HN019 rev**	*Bam*HI	GTCGGATCCCGCTACTTGGCGGAGTGCTCG

****The HN019 primers failed to produce a PCR product.**

Table 4.4. The plasmids used in this study

Plasmid	Characteristics	Reference
pLUG-Multi TA	Cloning vector Ap^r : ColE1: blue-white selection	iNtRON
pET16b	Ap^r :T7 promoter with N-terminal hisx10 sequence	EMD4 Biosciences
pETJAM 2	pET16b containing *Nco*I to *Bam*HI DNA fragment encoding GH1#1 from KW30	This study
pETJAM 3	pET16b containing *Nde*I to *Bam*HI DNA fragment encoding GH1#5 from KW30	This study

Table 4.5. Lactic acid bacteria and whether the gene encoding GH#1 or GH#5 was present (✓)

Lactic Acid Bacteria	Strain	YP_003064398.1 (GH1#1)	ZP_07078860.1 (GH1#5)	Reference
Lactobacillus plantarum	KW30	✓	✓	[10]
Lactobacillus plantarum	WCFS1	✓	✓	BAA-793
Lactobacillus plantarum	ATCC8014		✓	ATCC8014
Lactobacillus plantarum	ATCC14917	✓	✓	ATCC14917
Lactobacillus plantarum	NC8	✓	✓	[11]
Lactobacillus brevis	LMG 11437		✓	ATCC367
Lactobacillus sakei	LB790	✓	✓	[12]
Lactococcus lactis subsp. *lactis*	KF147		✓	[13]
Lactococcus lactis subsp. *lactis*	KF152		✓	[13]
Lactococcus lactis subsp. *lactis*	KF282		✓	[13]

Table 4.6. Genomic DNA was extracted from Bifidobacterial species but did not produce PCR products using HN019 primers

Bifidobacteria species	**Reference**
B. adolescentis	NCTC11814
B. catenulatum	NTCC3933
B. catenulatum	NTCC3930
B animalis subsp lactis	HN001/DR10
B longum	NTCC3937
B. bifidum	VIII-210
B. breve	NTCC3932

The construct pETJAM1 was not continued with after several attempts to generate a PCR product from the template DNA failed. As the primers were designed from published genomic data on this strain, it was presumed that this was not the problem. It is possible that our strain did not have the same DNA sequence as that of the published data and although another strain was obtained (AgResearch), a PCR product could not be obtained (data not shown).

The DNA fragment encoding the genes “GH#1” and “GH#5” were generated by PCR with the primers listed in Table 2.5 and with genomic DNA from selected bacteria as a template (Table 4.5). The genes were amplified as per manufacturer’s instructions with the ‘pLUG-Multi TA-cloning Vector Kit’ (INtRON, Ngaio Diagnostics Nelson, NZ).The PCR products generated from genomic DNA were sequenced to confirm identity by the Massey Genome Service (Palmerston North, NZ). Next the fragments were a-tailed (as described at 2.1.7) and ligated into expression vector pET16b and these constructed plasmids named pETJAM2 and pETJAM3 respectively (Figure 4.2 and Table 4.4).

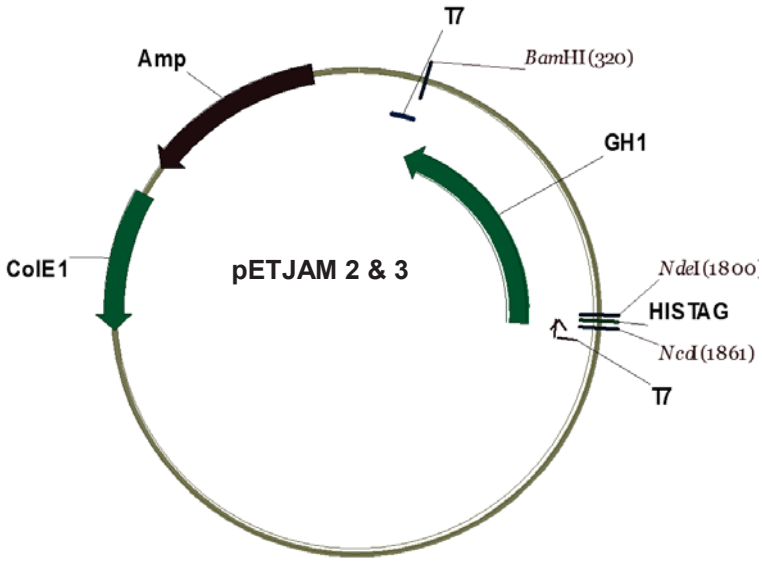

Figure 4.2. Vector map showing the basic design of the pETJAM 2 and pETJAM 3 expression vectors. pETJAM 2 had fragment GH1#1 cloned at *Nco*I-*Bam*HI sites of pET16b while pETJAM 3 had fragment GH1#5 inserted at *Nde*I-*Bam*HI sites

4.2.2 Myrosinase assay to discover bacteria capable of the hydrolysis of glucosinolates

Myrosinase activity assays have been characterised[14,15] and offered an option for screening whole cell extracts for myrosinase activity. An advantage of using this method (described in section 2.1.3) over measuring GSLs directly at UV ~230 nm is that special UV plates are not required and the 340 nm absorbance is more likely to be available on general lab spectrophotometers. A drawback of this assay was that purified enzymes work best, and in this case whole cell extracts were being used. As it was not known which if any glycoside hydrolases were involved, to avoid inhibition of any enzymes, protease inhibitors were not added to the whole cell extract which meant that extracts had to be stored at -80°C, thawed on ice and used immediately to avoid protease degradation.

The reagents used in the myrosinase assay[15] adapted for this thesis are given in chapter 2, Table 2.7.

A 96 microplate method of analysis was employed with a Spectromax Plus 190 plate reader (Molecular Devices, Biostrategy, Auckland, NZ) equipped with SoftMaxPro 4 software. The total volume in each well was 200 µL. Reactions were prepared in duplicate or triplicate with the assay reagents added first, followed by the bacterial lysate or protein. The reagents were mixed by reverse pipetting to avoid air bubbles. The plate was prepared on ice to maintain 4°C and the plate reader temperature was set for 30°C. When the temperature had stabilised in the plate reader, the microplate was loaded and incubated until the temperature stabilised to 30°C again. At this point the substrate (allyl glucosinolate) was added by reverse pipetting using an eight channel electronic autopipettor (Eppendorf, Raylab Ltd, Auckland). The microplate was returned to the plate reader and read immediately using the kinetic program recording the absorbance (340 nm) every 15 seconds for at least 15 minutes.

4.2.3 Cultivation conditions for glucosinolate tolerance

Bacteria were cultured according to the methods given in section 2. For GSL tolerance experiments the appropriate growth media (either RCM or MRS) were supplemented with 1% sugar (0.5% galactose and 0.5%

glucose). The bacterial growth was assessed by optical density (OD_{600}) and most probable number method (MPN). The GSL concentration used was based on previous studies using pure allyl glucosinolate at concentrations of between 0.3 - 0.4%[2,7] and concentrations of 1 mg (0.1%), 2.5 mg (0.25%) and 5 mg (0.5%) were selected for these experiments.

4.3 Results

4.3.1 Bacterial genes encoding YP_003064398.1 and ZP_07078860.1

The primers (Table 4.3) were tested against extracted genomic DNA from a number of Bifidobacteria, lactobacilli and lactococci (Table 4.5 – 4.6). Attempts to generate a PCR product from the HN019 primers in order to construct pETJAM1 were unsuccessful so further study on Bifidobacteria was abandoned.

The primers for GH1#1 produced products for all *Lb. plantarums* except strain ATCC8014 while the primers for GH1#5 yielded products from all *Lb. plantarums* except the unknown strain (Figure 4.3). All three *Lc. lactis* strains generated products for GH1#5 and *Lb sakei* LB790 produced products for both GH#1 and GH#5 and is summarised in Table 4.5.

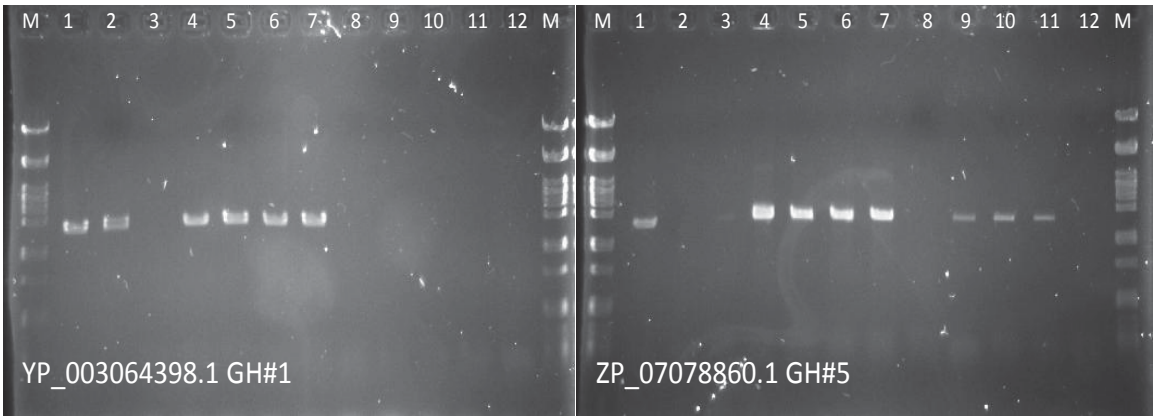

Figure 4.3. PCR primers were used to generate products from genomic template DNA as shown. Lane 1: *Lb. plantarum* KW30, 2: unknown *Lb. plantarum*, 3: *Lb. plantarum* 8014, 4: *Lb. plantarum* 14917, 5: *Lb. plantarum* WCFS1, 6: *Lb. sakei* LB790, 7: *Lb. plantarum* NC8, 8: *Lb. plantarum* 8014, 9: *Lc. lactis* KF147, 10: *Lc. lactis* KF152, 11: *Lc. lactis* KF282, 12: *Lb. brevis*

4.3.2 Production of recombinant protein

Expression of these genes from the pETJAM2 and 3 vectors in *E .coli* BL21 (DE3) resulted over-production of a protein ~50-55 kDa for both (Figure 4.4).

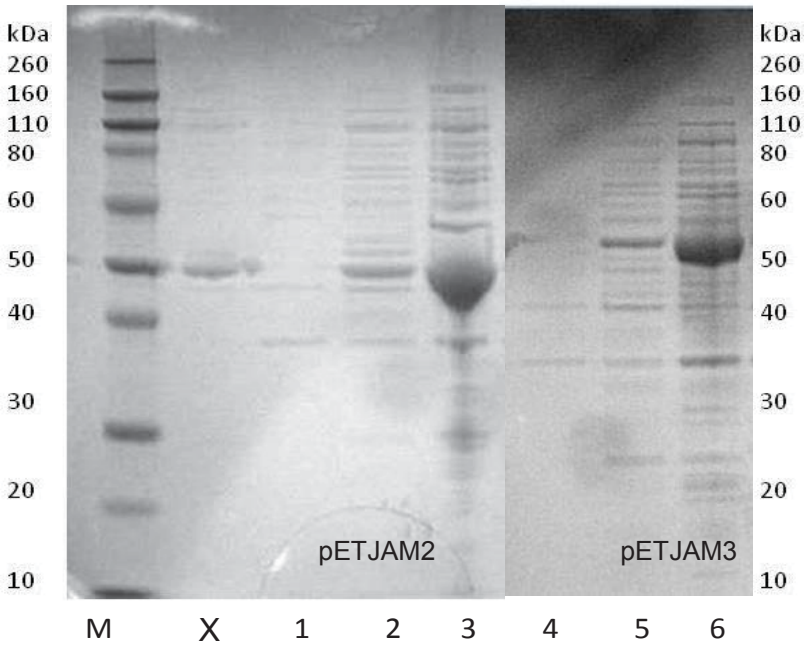

Figure 4.4. SDS-PAGE shows that pETJAM 2 and pETJAM 3 constructs produced proteins of the predicted size seen in this whole cell extract. M: marker, X: not used, 1: uninduced, 2: 4h post induction, 3: 20h post induction, 4: uninduced, 5: 4h post induction, 6: 20h post induction

Table 4.6 shows that both proteins were identified by the peptide fingerprinting method of MALDI-TOF (section 2.24).

Table 4.7. MALDI-TOF of the identified peptides from proteins produced

Protein	Identified peptides
GH1#1	R83-K104, F108-R114, V135-K162, A170-K184, Q242-K251, M259-K316, Y321-R410, R453-R471, S486-R495
GH1#5	G2-R21, N34-K55, E56-K96, G99-K119, I130-R200, Y241-R314, S375-R397, I421-R431, Y470-R487, D491-R508

Whole cell extract was fractionated as previously described (section 2.1.1) and the soluble-insoluble fractions assessed. Most of the protein was

found to be insoluble so different cultivation conditions were tried from temperature (37°C down to 28°C) and decreased induction time (2 – 4 h from 6 h) but both constructs produced insoluble protein each time (Figure 4.5). Several attempts were made to purify the small fraction that appeared to be in the soluble fraction but were unsuccessful.

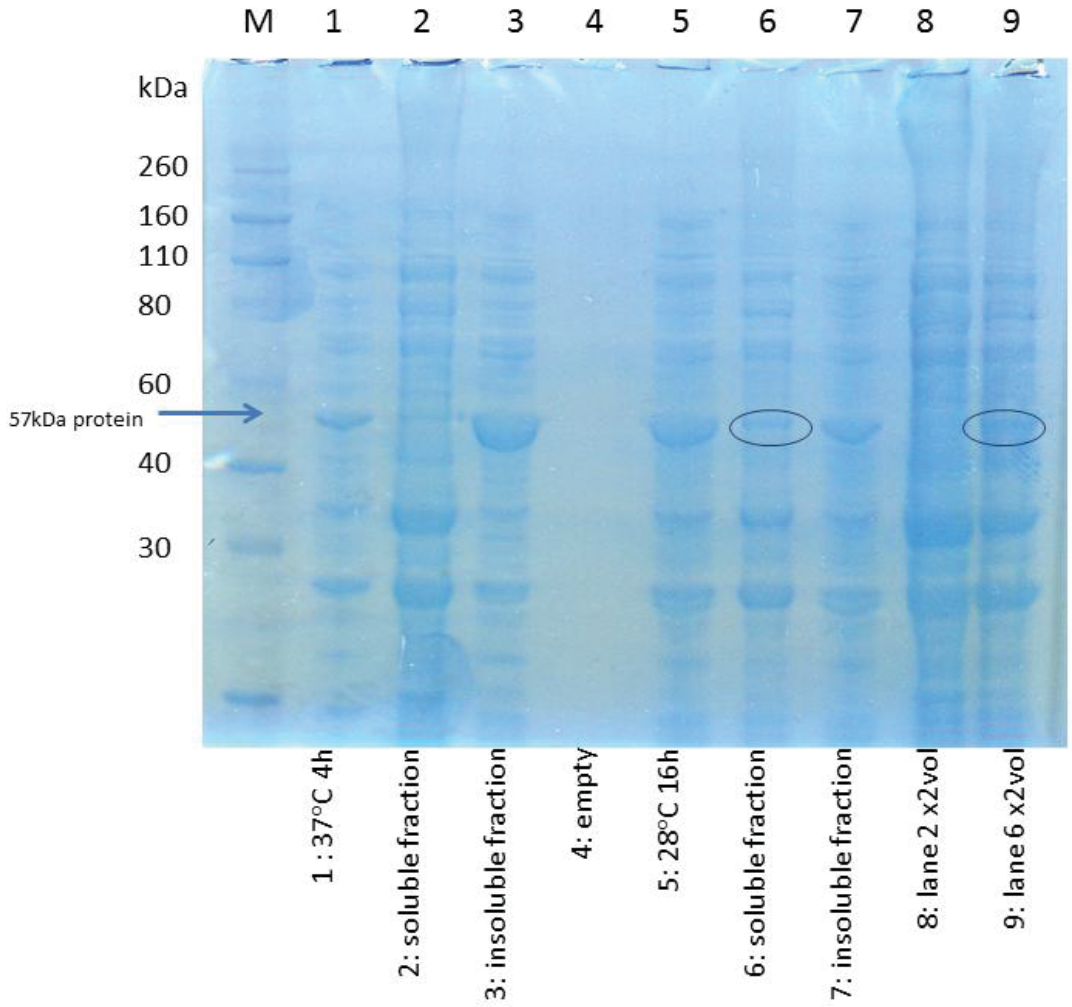

Figure 4.5. Insoluble protein resulted from expression of both GH1#1 and GH1#5 (only GH#5 is shown)

Unfortunately many attempts to produce soluble protein were unsuccessful and without soluble active protein myrosinase activity of the possible GH1 enzymes could not be determined. Attention was given to measuring myrosinase activity using a whole cell extract instead.

4.3.3 Myrosinase assay to identify myrosinase-producing bacteria

When plant myrosinase from *Sinapus alba* (Sigma T4528) was incubated with allyl glucosinolate, a change in absorbance was recorded at 340 nm. Shown in Figure 4.6 are duplicate samples of allyl glucosinolate incubated with myrosinase and control allyl glucosinolate samples incubated with the protein bovine serum albumin (BSA). The myrosinase change in aborbance data points are shown as triangles and diamonds while BSA data points are represented by squares and circles. Data was generated using SoftmaxPro Version 5 on a SpectraMax Plus 190 Microplate Reader (Molecular Devices, Biostrategy, NZ).

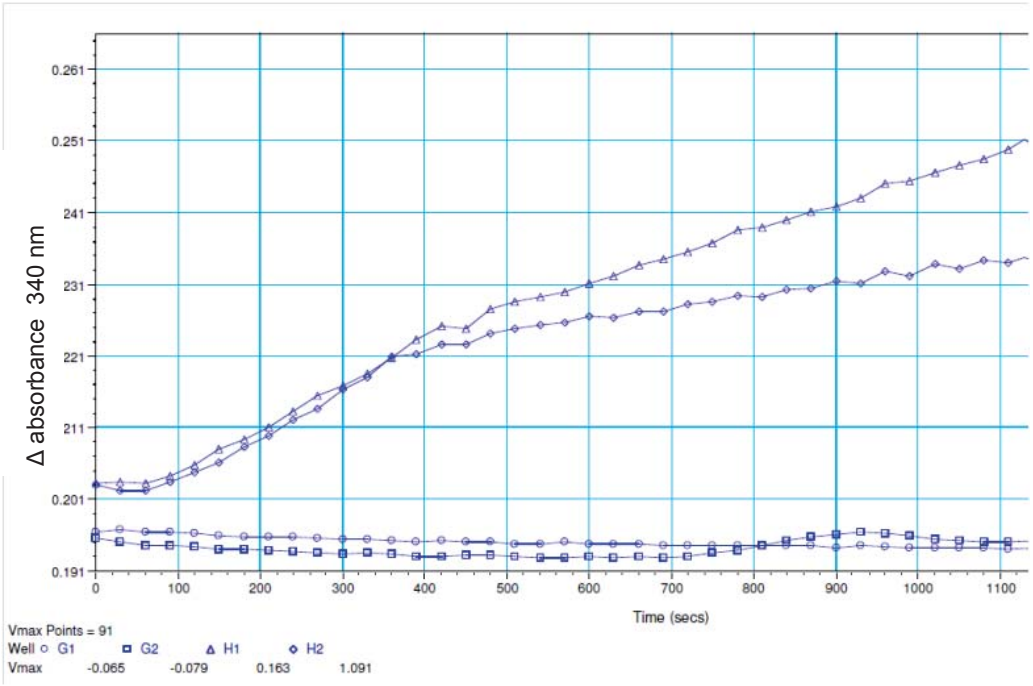

Figure 4.6. Myrosinase assay recorded the change in absorbance at 340 nm for duplicate samples of allyl glucosinolate incubated with myrosinase (diamond/triangle) while allyl glucosinolate incubated with BSA (square/circle) showed no change at all. Controls lacking the substrate allyl glucosinolate did not change in absorbance (data not shown)

Next, bacterial lysates were incubated under the same conditions again with allyl glucosinolate as substrate and the change in absorbance (340 nm) recorded. All of the bacterial lysates demonstrated myrosinase activity (Figure 4.7 – 4.9) but only *E. cloacae* showed a possible adaptive

response to GSLs defined by whether the substrate turnover increased if the bacteria had been pre-cultured in GSLs (0.3% (w/v)) (Figure 4.7).

The coupled assay was abandoned after these preliminary experiments. As the LC-QTOF-HRMS system had become available, it was decided to continue investigating the bacterial metabolism of GSLs in experiments with this because the biggest advantage of this system was that individual GSL consumption could be measured rather than just the total. It also allowed for hundreds of samples to be processed directly from supernatants at once maintaining consistency, and without the need for the enzyme reagents used in the coupled assay.

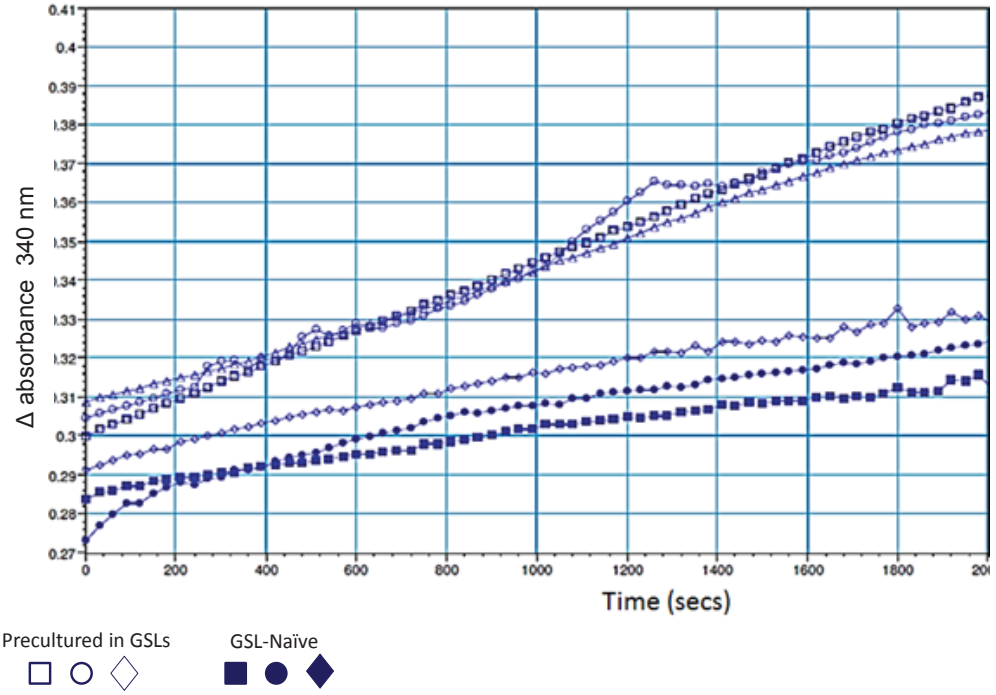

Figure 4.7. Myrosinase activity demonstrated by *E. cloacae* lysate incubated with allyl glucosinolate. An adaptive response is evident also because the change in absorbance of lysate from cells pre-cultured in GSLs was greater than the GSL-naive lysates

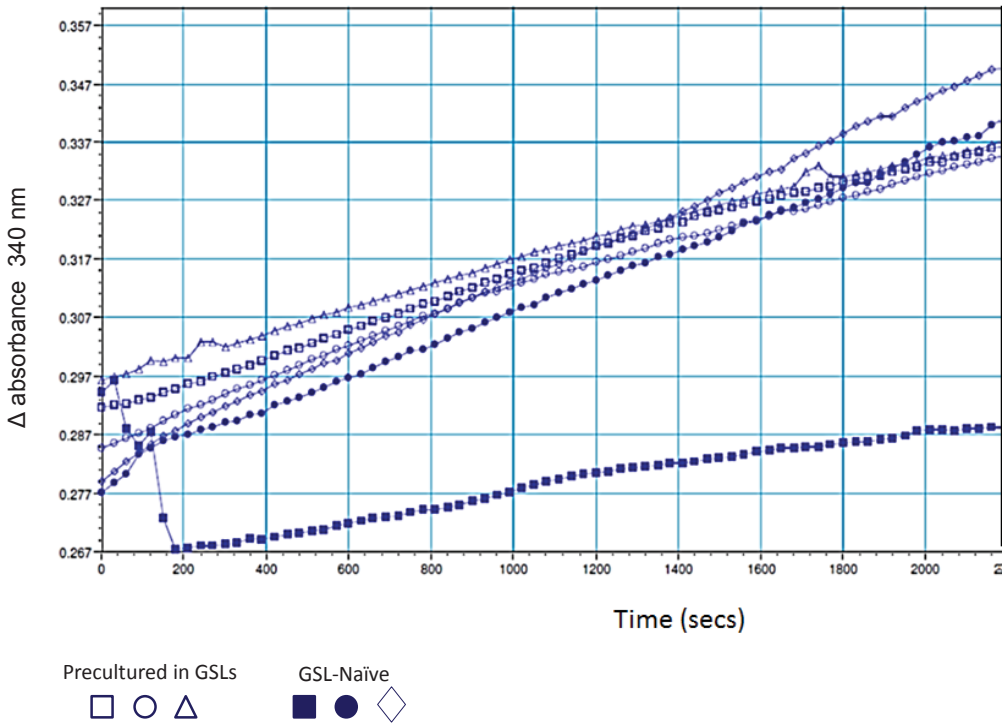

Figure 4.8. Myrosinase activity demonstrated by *E. coli* Nissle but not an adaptive response as pre-cultured and GSL naive lysates were similar

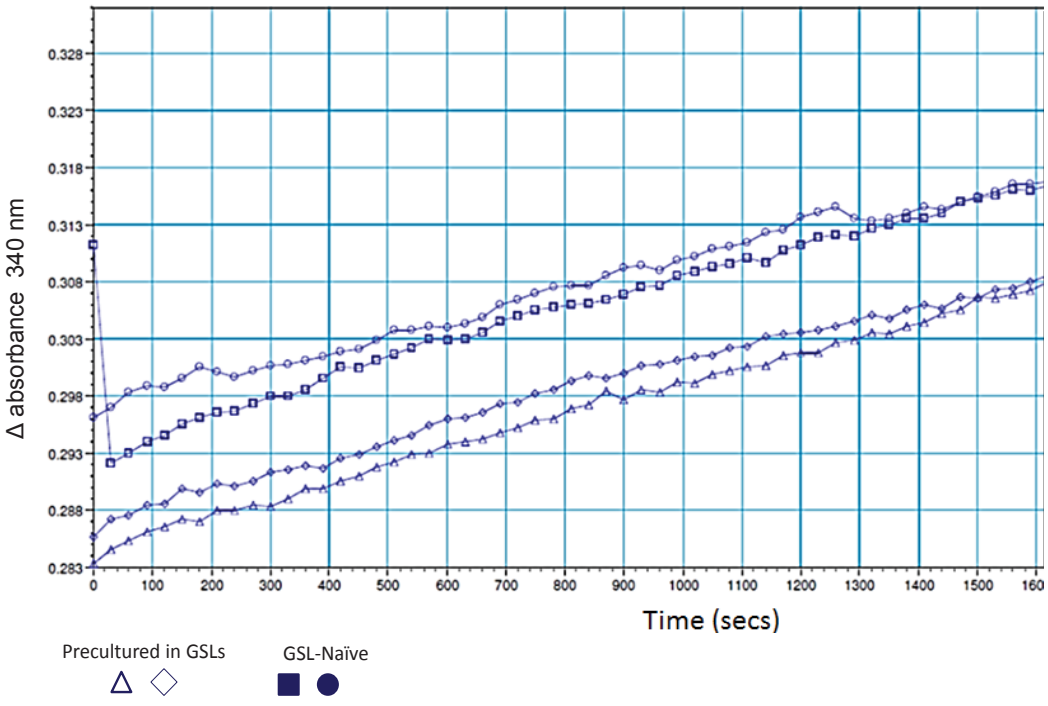

Figure 4.9. Myrosinase activity demonstrated by KW30 lysate but no adaptive response as like E. coli Nissle , the changes in absorbance between pre-cultured and GSL-naive were the same

4.3.4 Glucosinolate tolerance by bacteria

Next, experiments for GSL tolerance were carried out to be certain that the presence of GSLs did not inhibit bacterial growth. Lactococci, lactobacilli, bifidobacteriaceae, *E. coli* Nissle and *E. cloacae* were cultivated in the presence of GSLs extracted from broccoli seed powder (BSP). Bifidobacteriaceae were discontinued after this point for reasons given in the previous chapter.

None of the bacteria were inhibited in their growth by the presence of GSLs although some bifidobacterial species failed to grow well even without GSLs in the media (data not shown). MPN method indicated that all were viable after five days (data not shown). There were no differences between *Lc. lactis* strains KF147, KF152 and KF282 (Figure 4.10). There were also no differences in GSL tolerances between *Lb. plantarum* strains WCFS1, NC8, KW30, ATCC14917, and ATCC8014 (data not shown) and the *Lb. plantarum* strain shown in Figure 4.11 is WCFS1.

Enterobactericaeae *E. coli* Nissle and *E. cloacae* were not inhibited by GSLs (Figure 4.12 and 1.13). Although bifidobacteriaceae were not continued with, *B. animalis* subsp. *lactis* showed tolerance to GSLs especially over 48 hours (Figure 4.14). Previous work by others[1] had identified *Enterobacter cloacae* #506 (*E. cloacae*) as a myrosinase producing organism and the genome sequence for *E. cloacae* ATCC 13047 contains genes for GH1 family enzymes (GenBank accession numbers CP001918, CP001919, and CP001920)[12]. Therefore, this strain was included for future experiments as a putative myrosinase-positive organism. However, while *E. cloacae* is commensal, it has been known to exhibit pathogenicity[16,17] so where the possibility of pathogenicity was an issue, only *E. coli* Nissle was used as it is a non-pathogenic beneficial bacterium which can be safely consumed as part of a diet.

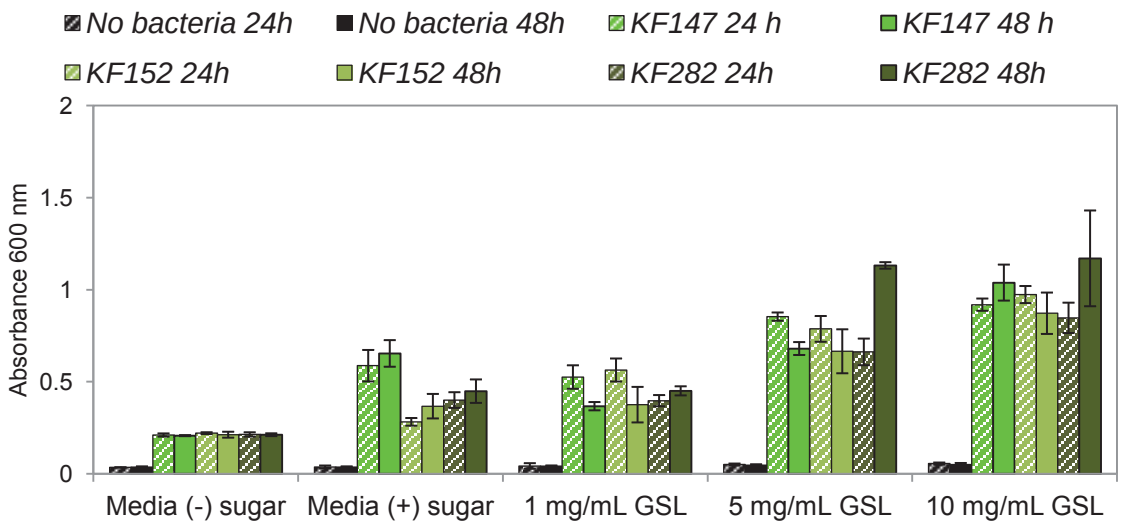

Figure 4.10. *Lactococcus* strains KF147, KF152 and KF282 did not find the GSLs in the media inhibitory reaching optical densities higher than for the media (+) sugar control at 10 mg/mL GSL

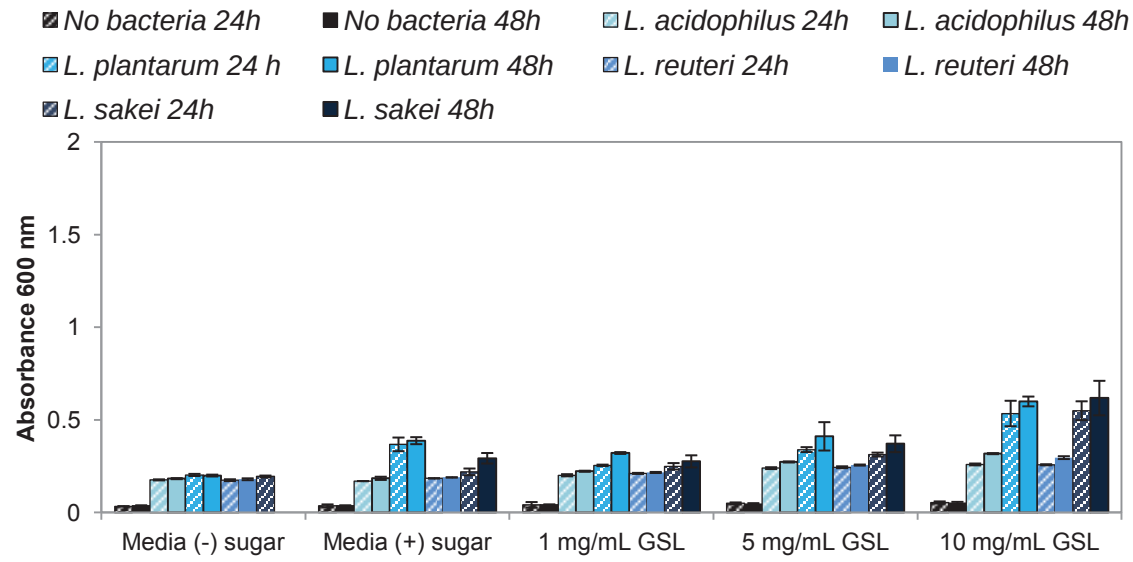

Figure 4.11. Lactobacilli were not inhibited by GSLs and at 5 mg/mL GSL, reached similar densities to the control media (+) sugar

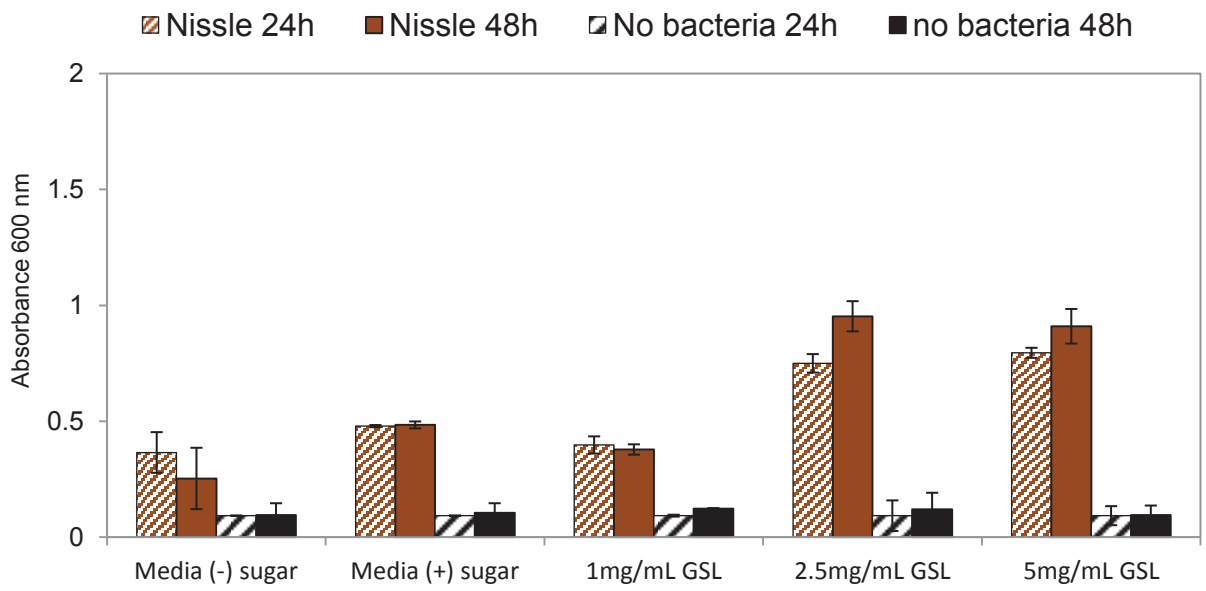

Figure 4.12. *E. coli* Nissle was not inhibited by GSLs and in 2.5-5 mg/mL GSLs continued to increase in optical density at 48h above the media (+) sugar control suggesting that it was able to utilise the GSLs

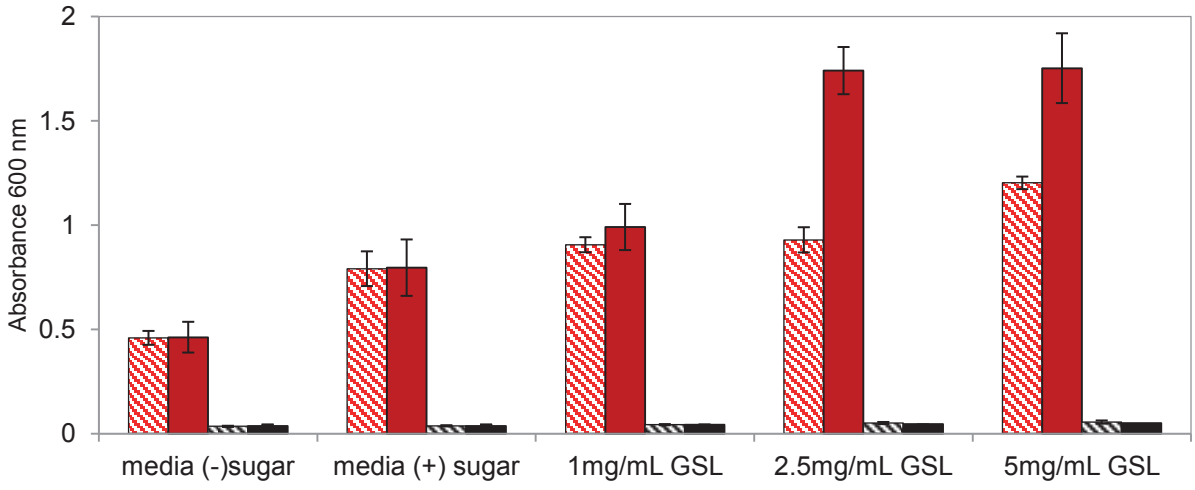

Figure 4.13. *E. cloacae* achieved the highest optical density after 48h of all bacteria and continued to increae in optical density in the 2.5-5 mg/mL GSL media greater than in the media (+) sugar control suggesting that it also could utilise the GSLs

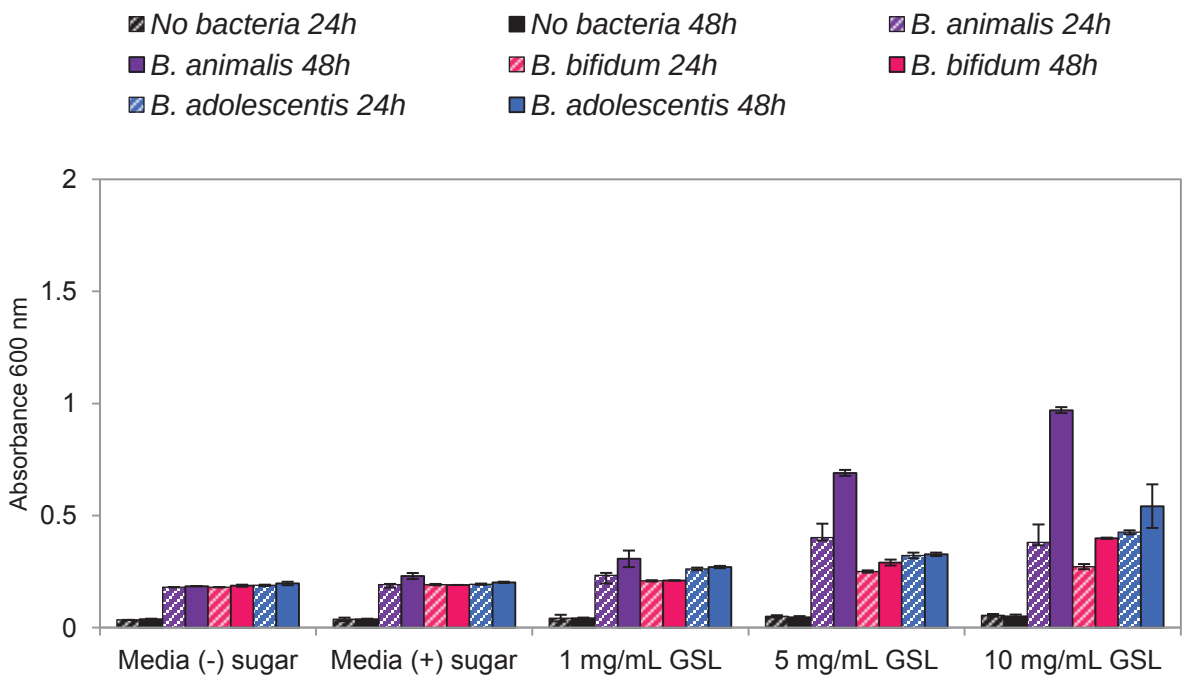

Figure 4.14. Bifidobacterial strains shown were not inhibited by GSLs .*B. longum, B. breve* and *B. catenulatum* grew poorly even without GSLs and are not shown

4.4 Discussion

Codon bias can cause problems in cloning and in this case there were some rare *E. coli* tRNAs, however, this did not impact on the host's ability to produce the recombinant protein as confirmed by MALDI-TOF. There were a number of possible reasons as to why the recombinant protein was insoluble. One was that the protein may have misfolded during synthesis due to the strong expression system (T7). Decreasing the inducer (IPTG) concentration from 1 mM down to 0.5 mM did not remedy this nor did decreasing the temperature from 37°C to 28°C to slow the rate of growth. Protein prediction software (http://www.predictprotein.org/ viewed August 2010) predicted a soluble protein but clearly the results showed that it was not.

While there are a number of work-around solutions including chaperone proteins to assist folding, host expression systems for lactobacilli, or cell free expression systems, it was decided not to pursue this line of research for a bacterial myrosinase-encoding gene and to focus instead on

studying the effects of GSL consumption by the strains that had showed promise as GSL metabolisers.

4.5 Summary

The purpose of cloning the putative myrosinase-encoding genes from bacteria was to discover a novel bacterial myrosinase. By identifying and cloning potential gene targets it was hoped that the recombinant protein produced by *E. coli* could be purified and used in enzymatic activity assays.

Even though the protein was insoluble and inactive, by cloning the putative GH1 encoding genes from KW30, it was possible to continue with choosing strains to testing for GSL consumption and metabolism. Genes encoding GH1#1 and GH1#5 were present in six of the *Lactobacillus* strains tested while the *Lactococcus* strains had GH1#5 only. Despite being unsuccessful at obtaining the pure recombinant enzyme, it was still possible to continue screening for myrosinase-producing bacteria by looking for this activity in whole cell extracts.

When whole cell extract was studied with the myrosinase assay, all demonstrated activity. Lysate from *E. cloacae* cells was the only one to exhibit GSL-adaptation as lysate from pre-cultured bacteria produced a more rapid change in absorbance compared to GSL-naive (Figure 4.7).

These results are in contrast to previous work which found activity only when whole cells were used[7]. Their method of obtaining cell lysate was by sonication to break up the cells whereas for this thesis protein extraction was performed using a commercial bacterial protein extraction product (section 2.1). The only other difference was in the buffer used which was MES buffer for the previous study and potassium phosphate for this.

It is possible that in this past study the whole cell extract obtained had lost its myrosinase activity. They made no mention of the use of protease inhibitors and for this study none were used for the reasons given in at 4.2.2. These results were interesting if for no other reason than that they throw doubt on the results of an earlier study. It was not possible to directly compare myrosinase activity between the pure plant enzyme and bacterial lysate because the lysate contained the total protein from the bacterial cell

and the bacterial glycoside hydrolase responsible for the myrosinase effect was of unknown identity and unknown concentration.

However myrosinase assays were only one way to find myrosinase-producing bacteria and as part of finding whether bacteria tolerated GSLs, LC-QTOF-HRMS allowed for both to be assessed.

It showed that addition of GSLs in the BSP extract did not impact negatively on the growth of the bacteria tested.

From these results a GSL-adaptive beneficial bacterium might be identified by screening for GSL consumption and metabolism. Once found, this beneficial microbe could be added to a diet in combination with GSLs and it may even be possible that by doing so, health benefits are increased if the combination of beneficial bacteria with GSLs increases the production of bioactives and thereby host exposure to them.

From the results of the GSL tolerance experiments, two lactic acid bacteria were selected which were *Lactococcus lactis* KF147 (KF147) and *Lactobacillus plantarum* KW30 (KW30). The Enterobacteriaceae were also included for comparison with the lactic acid bacteria. These were *Escherichia coli* 1917 Nissle and *Enterobacter cloacae* ATCC13047 (*E. cloacae*).

4.6 References

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FIVE

5.0 Bacterial metabolism of glucosinolates

Bacteria are known to be capable of glucosinolate (GSL) metabolism and gut bacteria are naturally exposed to dietary GSLs. What is not known is whether regular consumption of dietary GSLs has an induction effect on the bacteria in that they become better GSL metabolisers after exposure to GSLs, or whether some GSLs are preferred over others. When GSLs are transformed to ITCs or nitriles, they become bioactive and it is the side chain or R group and the active group whether isothiocyanate or nitrile that is known to impact on the bioactivity[1-3]. What is not known is whether bacteria that are part of the gut microbiota also recognise these groups and respond to them.

Past studies investigating the products of GSL metabolism by bacteria have some common themes. For example, often pure glucoraphanin or allyl glucosinolate has been tested rather than a combination of GSLs that would be found found in food such as broccoli[4-11]. Another common element is cooked versus raw vegetables and how this impacts on bioavailability of the active components. It has been confirmed that cooking destroys myrosinase activity and in its absence, the gut microbiota can, and do, hydrolyse GSLs[12-16]. Another common element in these studies is that not all of the GSLs are ever accounted for.

5.0.1 The aim of this study

There were several parts to this study. The first was to find the best GSL metabolisers from the chosen bacteria and of those, to find which were GSL-adaptive (better at metabolising GSLs after exposure). Next was to find whether these bacteria preferred some GSLs over others and then to find what the products of the GSL metabolism were.

The last part was to find the mechanism responsible for the transport of GSLs in and out of bacterial cells.

Chapter four outlined the strategy used to help find the best GSL metaboliser. The gene search approach was undertaken with the hope that a gene with myrosinase function would be found but as described, while a few

candidate genes were located, recombinant expression of these genes resulted in either no PCR product at all or products that could be cloned into expression systems resulted in production of insoluble protein without activity.

The other approach taken was to test by simply screening lysates then growing bacteria with GSLs and measuring the fate of the GSLs over 24 hours. This method proved to be the most successful and from these results, two lactic acid bacteria were selected which were *Lactococcus lactis* KF147 (KF147) and *Lactobacillus plantarum* KW30 (KW30). The Enterobacteriace included for further study were *Escherichia coli* 1917 Nissle (*E. coli* Nissle) and *Enterobacter cloacae* ATCC13047 (*E. cloacae*).

5.1 Materials and Methods

5.1.1 Glucosinolate consumption experiments

Bacteria were cultured according to the methods given in section 2.0.3.

5.1.2 Cultivation method for co-cultures

It was decided to combine one lactic acid bacterium with one Enterobacteriaceae for this experiment and *E. coli* Nissle and KW30 were chosen because they are able to grow at body temperature (37°C). For this experiment bacteria were pre-cultured overnight in their appropriate media (either RCM for *E. coli* Nissle or MRS for KW30) with GSLs (0.3% (w/v)). 1 mL of this overnight culture was pelleted, washed twice by re-suspending the pellet in sterile 50 mM potassium phosphate buffer then centrifuging at 10,000 *x g* for 10 min and the cells re-suspended in 1 mL sterile potassium phosphate buffer (pH 7.2). A 2% inoculum of each bacterial culture was added to the media prepared as follows:

MRS media and RCM media were combined at a 1:1 ratio. GSLs (0.3% (w/v)) were added and 10 mL of this medium was added to a 15 mL sterile culture tube. After addition of the bacterial cultures, the tube was incubated anaerobically for 24 hours at 37°C.

5.1.3 Cultivation method for glucosinolate uptake coupled to sugar transporter

For the individual sugar experiments bacteria were cultured overnight in the appropriate culture medium (either RCM or MRS). 2 mL of this overnight

culture was pelleted, washed twice by re-suspending the pellet in sterile 50 mM potassium phosphate buffer then centrifuging at 10,000 *x g* for 10 min and the cells re-suspended in 2 mL sterile potassium phosphate buffer (pH 7.2). 2% inocula were added to minimal media (see section 2.0.5.3) that contained 0.3% (w/v) of either: glucose, galactose, fructose, arabinose, sucrose, trehalose or lactose. Incubation was at 37°C for 24 hours. *E. coli* Nissle was not included for these experiments due to the experiment being scaled down to cope with the number of samples required for LC-QTOF-HRMS.

5.1.4 Preparation for LC-QTOF and GC-MS

After cultures were incubated for the appropriate time, the spent medium was clarified by centrifugation at 10,000 *x g* for 10 minutes, the supernatant removed, filtered and the products of the GSL consumption extracted and assessed by GC-MS and LC-QTOF-HRMS (see method section 2.3.6 and 2.3.7).

5.2 Results

5.2.1 Glucosinolate consumption by bacteria

The preliminary experiments with HPLC (Appendix, Figures A6 – A9) showed that there were changes in the GSL peaks and that these differed between bacteria. Despite efforts to extrapolate the known peaks from the analysed sample of known broccoli GSLs (Appendix, Figure A3) against these peaks, it was not possible to identify with certainty anything other than glucoraphanin. As LC-QTOF-HRMS was now available this was used to identify the peaks in the chromatograms.

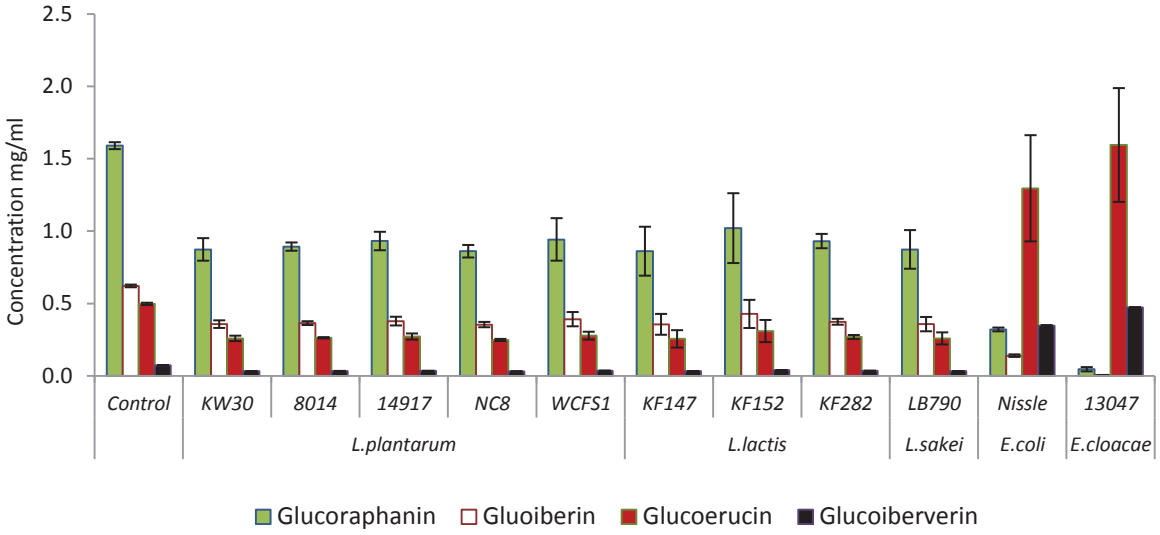

Figure 5.1. The consumption of individual glucosinolates between lactic acid bacteria and Enterobacteriaceae was different. The Enterobacteriaceae were consuming all of the glucoraphanin and glucoiberin while appearing to produce glucoerucin and glucoiberverin. Error bars = standard deviation (*n*=3)

LC-QTOF-HRMS enabled monitoring of individual GSLs and consumption of GSLs was investigated. Several lactobacilli and lactococci were compared against one another and with *E. coli* Nissle and *E. cloacae* (Figure 5.1). Here it can be seen that all the lactic acid bacteria have similar GSL consumption profiles consuming between 38 – 51% of glucoraphanin and glucoiberin and 32 – 43% of glucoerucin and glucoiberverin. In contrast, while the Enterobacteriaceae consumed nearly all of the alkylsulfinyl GSLs glucoraphanin and glucoiberin (81 - 97%), a concomitant increase was observed for alkylthiol GSLs glucoerucin and glucoiberverin. Glucoerucin increased 2.6 – 3.2-fold and glucoiberverin increased by 4.7 – 6.4-fold for *E. coli* Nissle and *E. cloacae* respectively. This result was unexpected and warranted further investigation.

As the consumption of GSLs was similar between the lactobacilli and lactococci, KF147 and KW30 were selected as the representative lactic acid bacteria. KF147 possesses one putative GH1 encoding gene (GH#5) while KW30 has GH1#1 and 5 (see section 4.13).

5.3 Glucosinolate metabolism by *Lc. lactis* KF147, *Lb. Plantarum* KW30, *E. coli* Nissle and *E. cloacae*

As shown in Figure 5.2 the major GSLs in the broccoli seed powder (BSP) extract were glucoraphanin and glucoiberin with minor GSLs glucoerucin, glucoiberverin progoitrin and allyl glucosinolate. Six were monitored chosen on the basis of abundance and or their properties (see chapter 3, section 3.2). These were the alkylsulfinyl GSL glucoraphanin, its alkylthiol redox pair glucoerucin, the alkylsulfinyl GSL glucoiberin and its alkylthiol redox pair glucoiberverin. Allyl glucosinolate and progoitrin were also monitored because in contrast to the other four, these were not able to shift to different state of redox or at least did not interconvert into other GSLs.

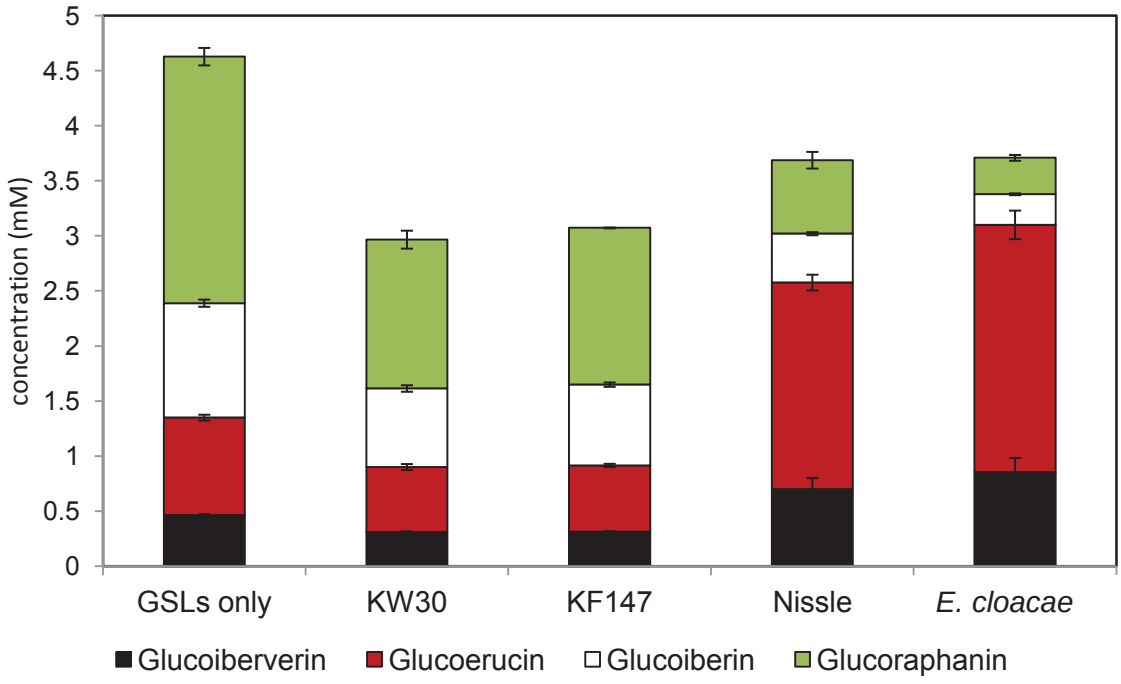

Figure 5.2. Glucosinolate profile of culture medium as determined by LC-QTOF-HRMS after 24 h anaerobic incubation of named bacteria with broccoli seed powder (BSP) extract at 37°C. Control samples had BSP extract added to uninoculated culture medium. The Enterobacteriaceae interconversion of GSLs can be seen as glucoraphanin and glucoiberin decrease while their reduced forms glucoerucin and glucoiberverin increase. Error bars = standard deviation (*n*=3)

Figure 5.2 illustrates the differences in GSL consumption between the lactic acid bacteria and the Enterobacteriaceae where the alkylthiol GSLs (glucoerucin and glucoiberverin) increased in concentration while the alkylsulfinyl GSLs (glucoraphanin and glucoiberin) disappeared.

When progoitrin and allyl glucosinolate were considered however, there were no differences in GSL metabolism between bacteria (Figure 5.3).

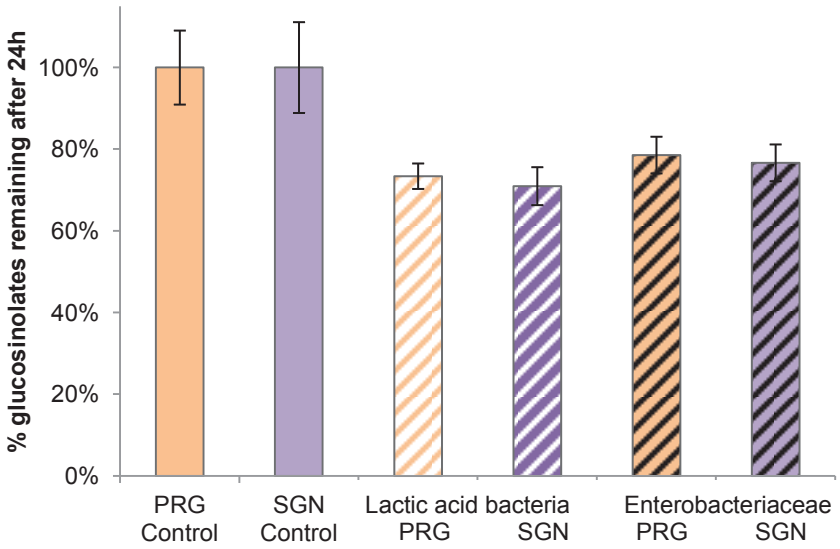

Figure 5.3. Progoitrin (PRG) and allyl glucosinolate (SGN) were consumed similarly by the lactic acid bacteria and the Enterobacteriaceae which removed about 25-30%. Error bars = standard deviation (*n*=3)

As previously described (Figure 1.5), glucoraphanin and glucoerucin are the same molecules in different redox states and so are glucoiberin and glucoiberverin.

These redox pairs of GSLs are able to reversibly shift between states. The data shows the GSLs are increasing in concentration, evidence that the alkylsulfinyl GSLs are being reduced to their alkylthiols by the Enterobacteriaceae.

5.3.1 Bacterial consumption of glucosinolates generated nitriles

As well as converting glucoiberin and glucoraphanin to their redox equivalents, the Enterobacteriaceae also metabolised the GSLs differently to the lactic acid bacteria. The products generated from metabolism of the GSLs

by the lactic acid bacteria were iberverin nitrile, erucin nitrile and sulforaphane nitrile whereas the only products detected for the Enterobacteriaceae were erucin nitrile and iberverin nitrile (Figure 5.4).

The missing hydrolysis products of lactic acid bacterial metabolism could have been produced at earlier time points than 24 hours (then degraded) Four-hourly sampling however, failed to detect any metabolites until after 16 hours when the emergence of the first nitriles was observed (data not shown).

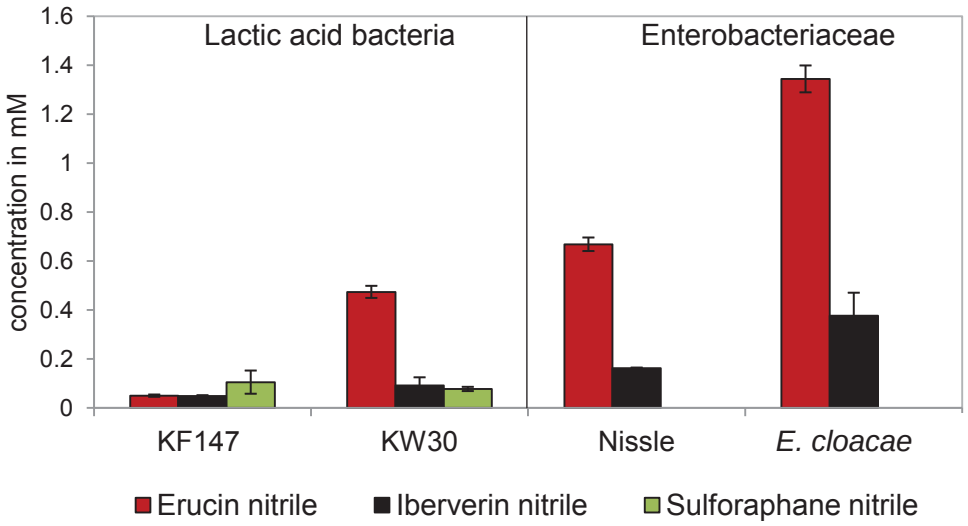

Figure 5.4. The products of GSL metabolism by lactic acid bacteria and Enterobacteriaceae show that the lactic acid bacteria produced the alkylsulfinyl nitrile sulforaphane nitrile in contrast to the Enterobacteriaceae which only produced the reduced nitriles, iberverin nitrile and erucin nitrile. Error bars = standard deviation (*n*=3)

5.3.2 Enterobacteriaceae reduce both alkylsulfinyl glucosinolates and alkylsulfinyl nitriles

Enterobacteriaceae *E. coli* Nissle and *E. cloacae* were incubated for 24 hours in RCM media containing 0.3% GSLs (w/v) extracted from BSP and the nitriles sulforaphane nitrile, iberin nitrile, erucin nitrile and iberverin nitrile (see Figure 5.7). To determine whether oxygen played a role in the reduction of GSLs, bacteria were also cultivated anaerobically and under aerobic conditions (as described at section 2.01). Growth of both was similar and both grew

equally well in anaerobic and aerobic conditions as assessed by optical density (data not shown).

LC-QTOF-HRMS analysis confirmed that *E. coli* Nissle was unaffected by oxygen and reduced the alkylsulfinyl GSLs (see Figure 5.5). and both *E. coli* Nissle and *E. cloacae* consumed the alkylsulfinyl GSLs under aerobic or anaerobic conditions (Figure 5.5 - 5.6). In general the alkylthiol species either increased or were similar to starting concentrations for both (iberverin nitrile was the exception for *E. coli* Nissle). This suggests that the reduction of the sulfinyl GSLs was not due to the anaerobic or aerobic conditions.

GC-MS analysis showed that the Enterobacteriaceae were reducing the alkylsulfinyl nitriles sulforaphane nitrile and iberin nitrile to erucin nitrile and iberverin nitrile because the concentration of these were decreasing while concomitantly alkylthiol nitriles erucin nitrile and iberverin nitrile were increasing in concentration. This was due to the bacteria because in the control media without bacteria, there was no change (Figure 5.7).

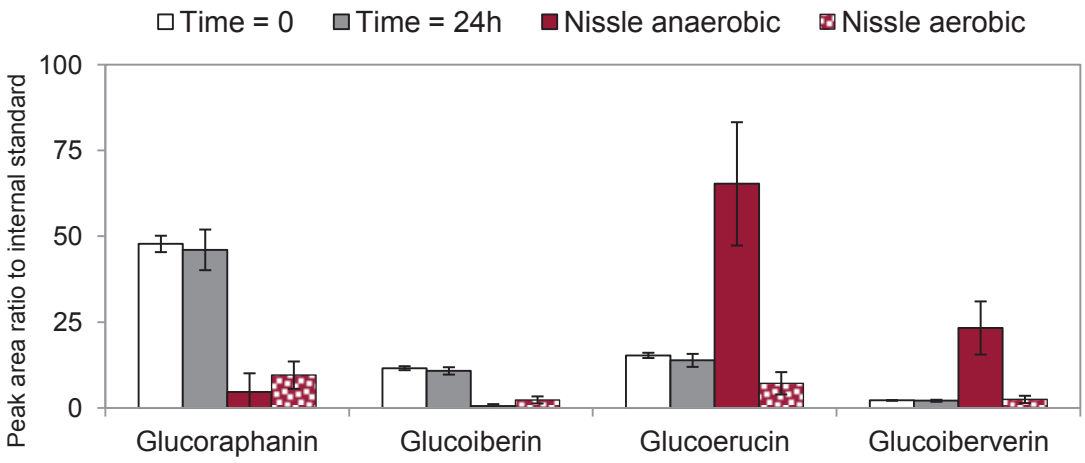

Figure 5.5. *E. coli* Nissle consumed GSLs under anaerobic and aerobic conditions but transformed more alkylsulfinyl GSLs to alkylthiol GSLs under anaerobic conditions. Nissle aerobic and anaerobic are shown at 24 h while the no bacteria media only controls are shown as white for time=0 and grey for time=24h. Error bars = standard deviation (*n*=5).

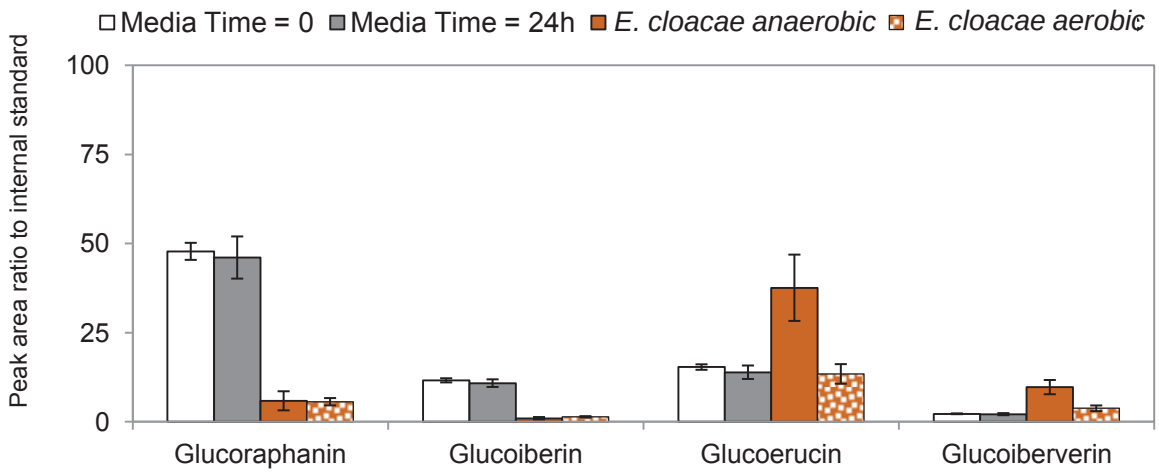

Figure 5.6. *E. cloacae* was similar to *E. coli* Nissle and also consumed GSLs under anaerobic and aerobic conditions, while transforming more alkylsulfinyl GSLs to alkylthiol GSLs under anaerobic conditions*E. cloacae* aerobic and anaerobic are shown at 24 h while the no bacteria media controls are shown as white for time=0 and grey for time=24h. Error bars = standard deviation (*n*=5).

The GC-MS analysis of the hydrolysis products also showed that *E. coli* Nissle and *E. cloacae* were different when grown under aerobic conditions. *E. coli* Nissle produced iberverin nitrile and erucin nitrile under both anaerobic and aerobic conditions (Figure 5.7). In contrast, *E. cloacae* only produced iberverin nitrile and erucin nitrile under anaerobic conditions and under aerobic conditions produced no nitriles (or other GSL hydrolysis products) at all (Figure 5.8). This was unexpected especially as *E. cloacae* were thought to be myrosinase producing organisms from aerobic cultivations[17].

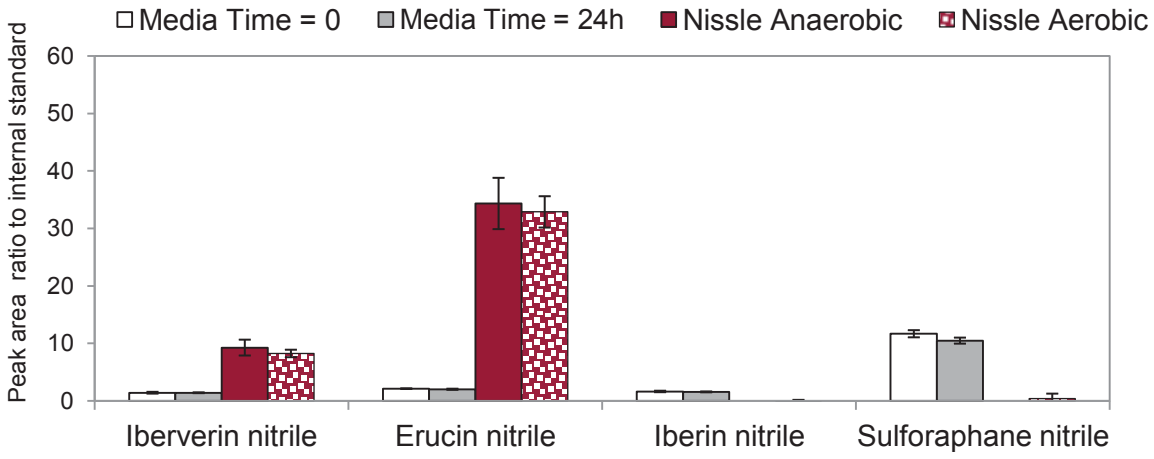

Figure 5.7. No matter whether conditions were anaerobic or aerobic, iberin nitrile and sulforaphane nitrile decreased in the presence of *E. coli* Nissle with iberverin nitrile and erucin nitrile the only products produced. Nissle aerobic and anaerobic are shown at 24 h while the no bacteria media only controls are shown as white for time=0 and grey for time=24h. Error bars = standard deviation (*n*=5).

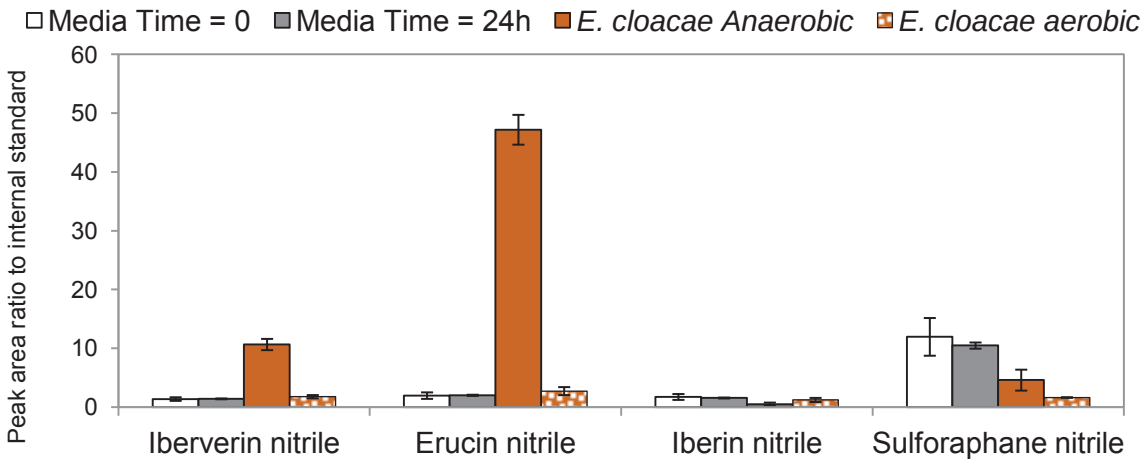

Figure 5.8. Under anaerobic conditions, *E. cloacae* produced iberverin nitrile and erucin nitrile but under aerobic conditions, it produced no nitriles at all. *E. cloacae* aerobic and anaerobic are shown at 24 h while the no bacteria media controls are shown as white for time=0 and grey for time=24h. Error bars = standard deviation (*n*=5)

5.3.3 A glucosinolate-adaptive response was seen for KW30

When the total nitriles were combined for each group and compared with plant myrosinase enzymatic hydrolysis, only *E. cloacae* produced hydrolysis products at concentrations approaching myrosinase (~1 mM) followed by *E. coli* Nissle at ~0.65 mM. Both lactic acid bacteria that were GSL-naive produced less than 0.25 mM of nitriles and only KW30 showed a GSL-adaptive response by increasing its nitrile output from 0.15 mM for GSL-naive up to 0.6 mM for the GSL-adapted (Figure 5.9). The Enterobacteriaceae produced around ten times the concentration of nitriles compared to the lactic acid bacteria and at levels similar to plant myrosinase (Figure 5.9).

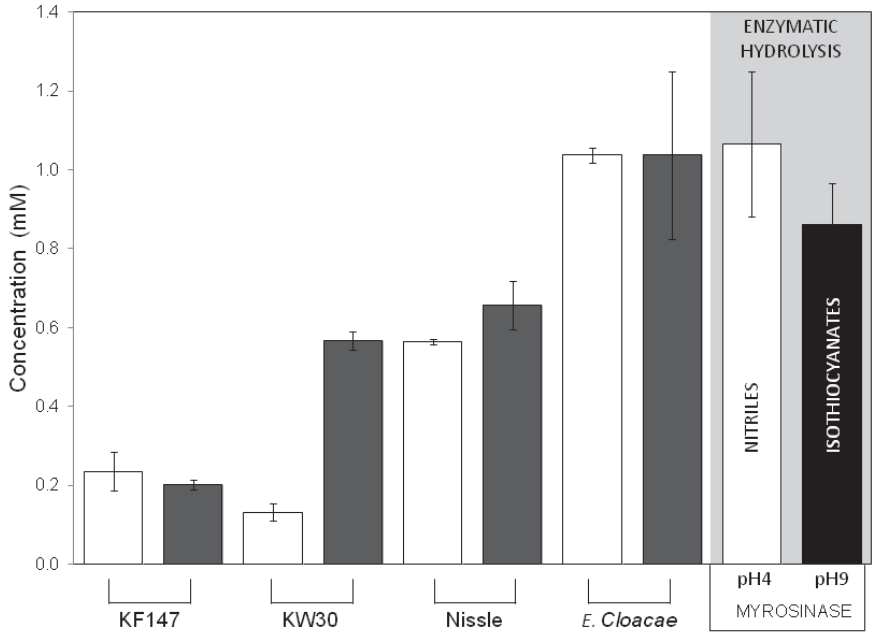

Figure 5.9. Total combined nitriles generated by bacteria compared with plant myrosinase showed *E. cloacae* produced similar concentrations of products to myrosinase but only KW30 showed a glucosinolate-adaptive response. The white bars for bacteria represent GSL-naive and grey GSL-pre-cultured. 1 unit of myrosinase was used for the enzymatic hydrolysis at pH4 and 9. Error bars = standard deviation (*n*=3)

5.3.4 Co-culturing KW30 with *E. coli* Nissle

Oxygen rich or anaerobic environments did not appear to be a factor causing the redox changes between the alkylsulfinyl GSLs and alkylthiol GSLs. Another possibility was that because lactic acid bacteria lower the pH, this

affects the ability of GSLs to shift to the reduced redox state. If so, it would be expected that co-cultures of lactic acid bacteria with Enterobacteriaceae would produce more alkylsulfinyl products than alkylthiol since lactic acid bacteria lower the pH within hours and samples were analysed after 24 hours.

When GSL-adapted KW30 and *E. coli* Nissle were co-cultured in 0.3% (w/v) GSL supplemented media, the GSLs that remained in the media were in similar ratios to the starting ratios and the consumption profile was similar to previous results obtained for KW30 monocultures as approximately 40% of the available GSLs were consumed (Figure 5.10). Even though buffered media (pH 7.4) was used, the pH after 24 hours was less than 4.

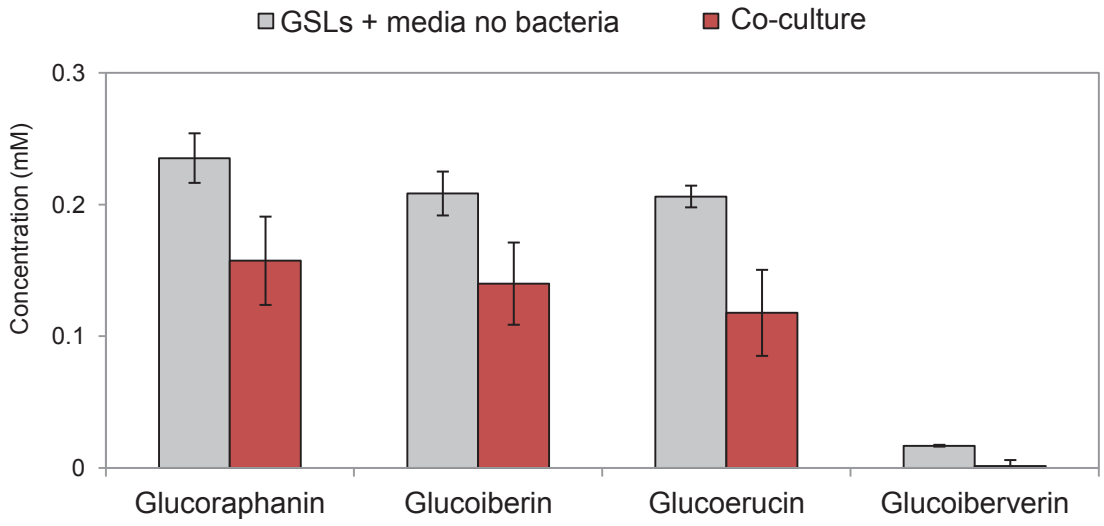

Figure 5.10. 24 hours incubation in GSL supplemented media by co-culturing KW30 and *E. coli* Nissle shows approximately half of the available individual GSLs were consumed and the alkylthiol GSLs did not increase as they did with *E. coli* Nissle monocultures. Error bars = standard deviation (*n*=3)

When the hydrolysis products were assessed by GC-MS, the KW30 and *E. coli* Nissle co-cultures produced iberverin nitrile, iberin nitrile, erucin nitrile and sulforaphane nitrile and the ratios were different than for monocultures (Figure 5.11). Although co-cultured *E. coli* Nissle no longer produced as much erucin nitrile it was still the major product and represented 58% of the total nitriles versus 76% as a monoculture. KW30 in comparison, produced 31%

erucin nitrile. Iberin nitrile was absent in *E. coli* Nissle but represented <2% of the total nitriles in co-cultures while in KW30 it represented ~0.3%.

It appeared as *E. coli* Nissle was affected more than KW30 and both interfered with one another to some degree because while sulforaphane nitrile was produced similarly when co-cultured or KW30 was alone, iberverin nitrile and erucin nitrile were produced at around one third of the concentration achieved by either as mono-cultures.

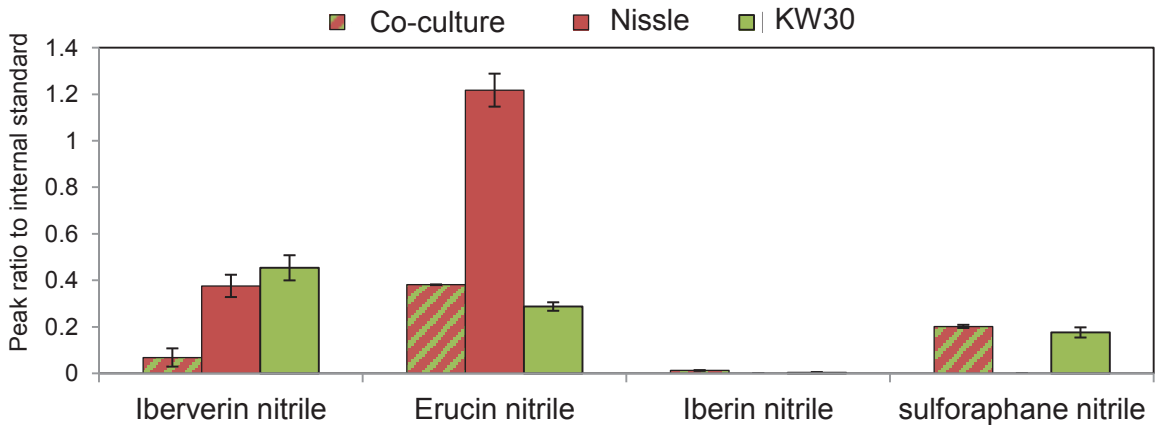

Figure 5.11. Comparison of co-cultured *E. coli* Nissle and KW30 shows that coculturing produced different ratios of products. Error bars = standard deviation (*n*=3)

5.3.5 Glucosinolate utilisation as an alternative carbon source

Bacteria were inoculated into minimal media supplemented only with 0.3% (w/v) GSLs extracted from BSP. Optical density measurements taken at 24 hours showed that growth had stalled. After three days, the most probable number method was used on these cultures and they were all viable indicating that while the GSLs were available as a carbon source, they were not used as such nor were they toxic (data not shown). When grown in minimal medium supplemented with GSLs (0.3% (w/v)), and sucrose (0.3% (w/v)), there were no differences in GSL consumption between lactic acid bacteria but *E. cloacae* consumed more glucoraphanin and glucoiberin when sugar was absent (Figure 5.12). Due to limitations on the number of samples that could be processed, *E. coli* Nissle was not included in these experiments.

5.4 Glucosinolates uptake coupled to a sugar transporter system

Evidence of a transporter system that may be involved in the taking up and metabolism of GSLs was investigated. Table 5.1 gives the sugars that were tested.

Table 5.1. Sugars used to compare glucosinolate metabolism

Sugar	Common name
β-D-glucose	Glucose
β -D-Galactose	Galactose
Arabinose (aldopentose)	Arabinose
α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside	Sucrose
α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside	Trehalose
β-D-galactopyranosyl-(1→4)-D-glucose	Lactose

Bacteria were again cultivated in minimal media containing 0.3% (w/v) GSLs extracted from BSP with individual sugars as listed in Table 5.1. None of the sugars tested were implicated in uptake of GSLs which can be seen in Figure 5.12 which shows KW30 and *E. cloacae* versus consumption of glucoraphanin and glucoerucin for simplicity. *E. cloacae* reduced glucoraphanin and glucoiberin to glucoerucin and glucoiberverin (data not shown for glucoiberverin) while KW30 and KF147 (data not shown) did not and none of the sugars appeared to affect the rate of GSL consumption. *E. cloacae* consumed more glucoraphanin and glucoiberin with sugar than without but this did not equate to producing more glucoerucin and glucoiberverin so this reduction reaction apparently occurs whether GSLs are metabolised or not.

GSLs were not available as a carbon source when bacteria were cultivated in RCM and MRS media in the absence of other sugars (see Figure 5.13). It was observed that the Enterobacteriaceae consumed more GSLs with sugar than without (in minimal media without sugar bacteria did not grow). However, MRS and RCM media are rich media and would have provided the bacteria with alternative carbon sources to sugar such as soluble starch (RCM) for Enterobacteriaceae and sodium acetate (MRS) for lactic acid bacteria.

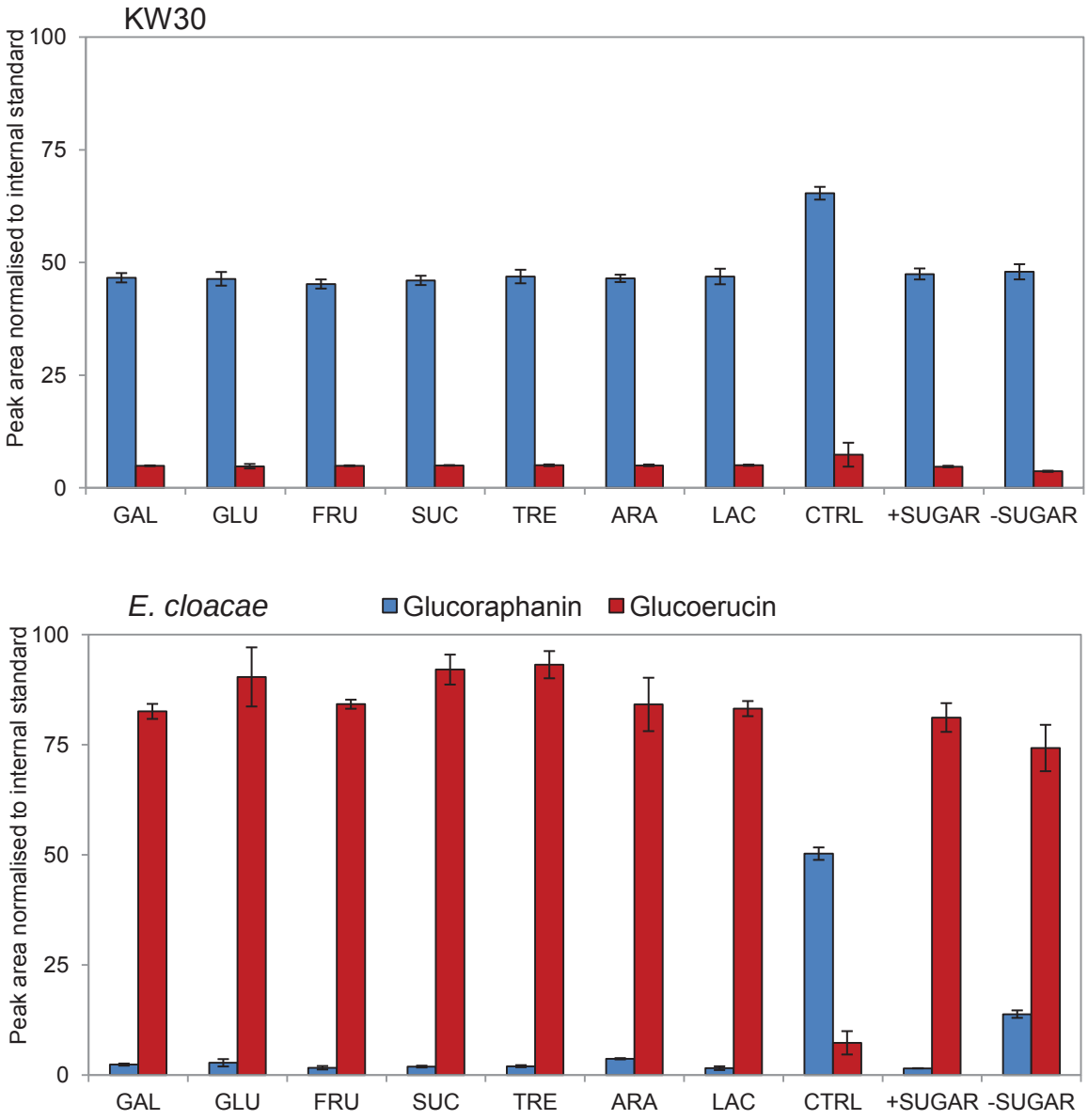

Figure 5.12. The consumption of glucoraphanin (GR) and glucoerucin (GE) by KW30 (top) and *E. cloacae* (bottom).

Key: CTRL = no bacteria, GSLs in media only.

GLU = glucose,GAL = galactose, FRU = fructose, SUC = sucrose, TRE = trehalose ARA = arabinose, LAC = lactose, SUGAR = 0.5% glucose and 0.5% galactose. Standard deviation (n=3)

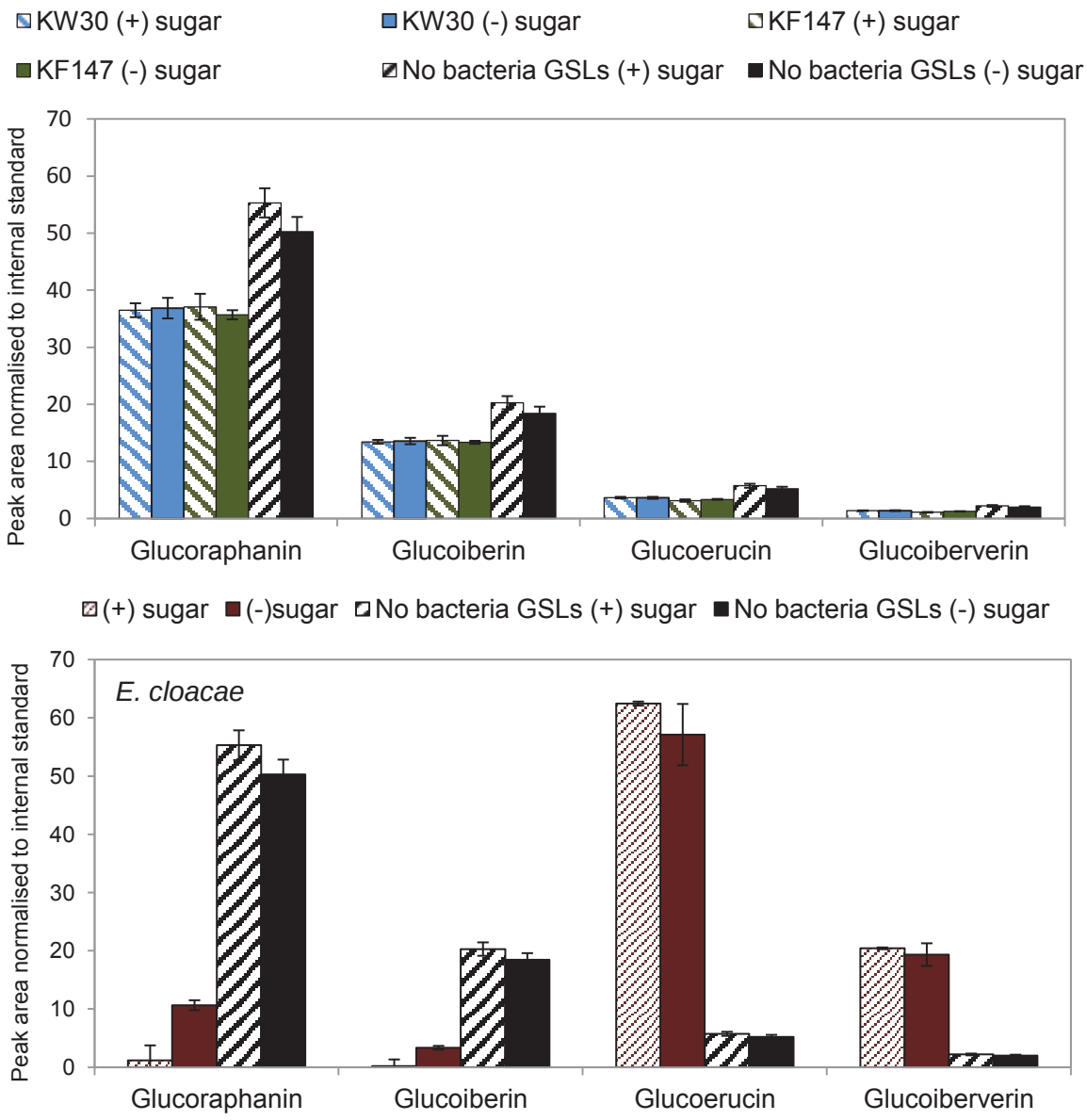

Figure 5.13. Glucoraphanin and glucoiberin profiles show a similar pattern of consumption for lactic acid bacteria while *E. cloacae* reduced the alkyl-sulfinyl glucosinolates and was better at GSL consumption with sugar addded to the RCM media. Error bars are the standard deviation (*n*=3)

5.5 Summary

While unstable metabolites such as isothiocyanates (ITCs) are thought to be too labile to remain intact, their absence while nitriles were present instead is not thought to be due to rapid ITC degradation, but more likely because the beneficial bacteria in these experiments have demonstrated a preference for production of nitriles over ITCs (Figure 5.9). The reason for this may be because ITCs are known to be inhibitory to bacteria (section 1.1.2).

In vitro, myrosinase activity was exhibited by both lactic acid bacteria and Enterobacteriaceae as assessed first by the myrosinase assay. Next HPLC showed that glucosinolate peaks were changing but it was only possible to determine the identities of one of these peaks and that was glucoraphanin. LC-QTOF-HRMS enabled identification of all the peaks and showed that the lactic acid bacteria metabolised GSLs differently to Enterobacteriaceae. It was also apparent that after pre-culturing in GSLs, KW30 was GSL-adapted, generating hydrolysis products four-fold the concentration of GSL-naive KW30. The products of GSL hydrolysis by KF147 and KW30 after incubation with BSP extract were iberverin nitrile, erucin nitrile and sulforaphane nitrile.

In contrast, the Enterobacteriaceae did not demonstrate GSL-adaptation and reduced the alkylsulfinyl GSLs into alkylthiol GSLs at the same rate whether pre-cultured with GSLs or not. For GSL metabolism *E. coli* Nissle produced the same products for aerobic or anaerobic conditions while *E. cloacae* only produced nitriles under anaerobic conditions. Both *E. coli* Nissle and *E. cloacae* reduced the alkylsulfinyl nitriles to alkylthiol nitriles when these were present in the cultivation media.

In the co-cultures it was found that neither performed as well together as when cultivated as monocultures and the hydrolysis products were a combination of what *E. coli* Nissle and KW30 would usually produce as monocultures with both sulforaphane nitrile and erucin nitrile the major products. The pH did not appear to be a major determinant of the redox state because for the major products of hydrolysis by the co-cultures were still erucin nitrile, followed by sulforaphane nitrile with minor amounts of iberverin nitrile and trace amounts of iberin nitrile and it is possible that KW30 and *E. coli* Nissle simply interfered with one another's metabolism of GSLs.

A co-culture does not represent a complete gut microbiota and the GSL metabolism experiments with a microbiota could provide answers as to whether the observed redox shift is real and due to facultative anaerobic activity by Enterobacteriaceae.

None of the individual sugars tested appeared to share the same transport system required for the uptake of GSLs even though GSLs have a sulfur-linked glucose side chain. *E. cloacae* consumed more glucoraphanin and glucoiberin when sugar was provided in the media but did not produce

equivalently more glucoerucin and glucoiberverin. This suggests that the redox shifting is not related to GSL metabolism. Because the results did not implicate the involvement of the sugar transporter systems being tested, GC-MS analysis was not performed.

5.6 References

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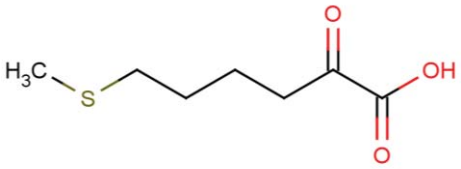
SIX

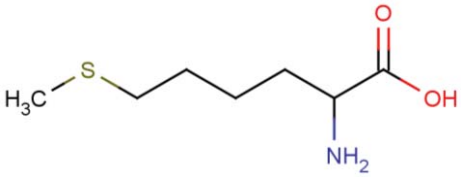
6.0 Metabolism of selenoglucosinolates by *Lb. plantarum* KW30 and *E. coli* Nissle

6.0.1 Plant biosynthesis of glucosinolates

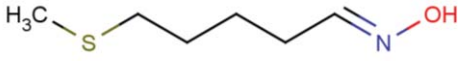
All glucosinolates (GSLs) originate from an alpha-amino acid. They all share three common features which are a beta-thioglucose, a sulfonated oxime and a variable aglycone side chain. Classification of GSLs is determined by the variable side chain or R group according to their chemical structures which may be aliphatic or aromatic[1]. The biosynthesis of GSLs by plants begins with an amino acid precursor. Aliphatic GSLs are made from methionine, alanine, leucine, isoleucine or valine, while benzenic GSLs are synthesised from phenylalanine or tyrosine, and the indole GSLs from tryptophan[2]. Biosynthesis is a staged process with each stage occurring independently. Glucoerucin (4-methylthiobutyl GSL) for example is synthesised from methionine, undergoing chain elongation to 2-oxo-6-methylthiohexanoic acid[3]. This is modified to dihomomethionine, then methyl thiopentanaldoxime. Next, glucose is added to form methylthiobutyl desulfo-GSL and the sulfate group added to complete the synthesis (Figure 6.1)[4].



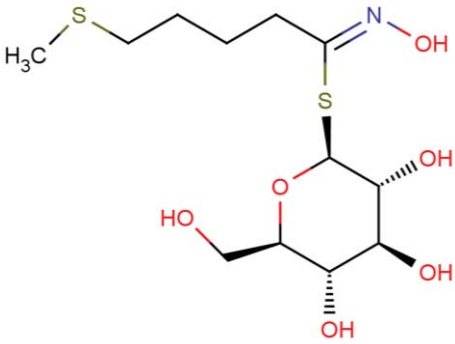
1: 2-oxo-6-methylthiohexanoic acid



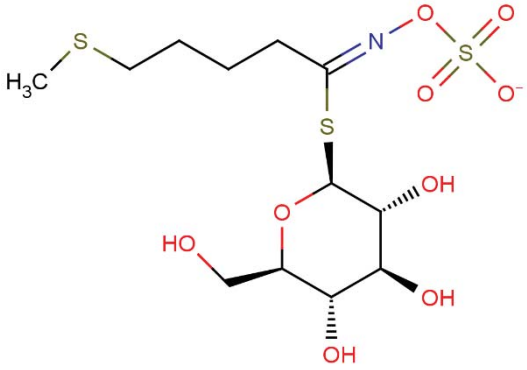
2: dihomomethionine



3: methylthiopentanaldoxime



4: Methylthiobutyl-desulfoglucosinolate



5: Methylthiobutyl glucosinolate (glucoerucin)

Figure 6.1. The biosynthesis of glucoerucin proceeds in stages starting with methionine undergoing chain elongation to 2-oxo-6-methylthiohexanoic acid (1). From this forms, dihomomethionine (2), next, an aldoxime by addition of nitrogen (3), followed by the addition of the sulfur-linked glucose (4) and finally addition of the sulfate group (5)

6.0.2 Selenium analogues of glucosinolates

Fertilisation of soil with selenium, in the form of sodium selenate, during the growing of broccoli results in the broccoli plant taking up selenium and incorporating it into tissue. This led to the discovery that during GSL biosynthesis selenium could become incorporated into GSLs in place of sulfur, and that broccoli grown in selenium fertilised soil had greater phase II induction capabilities than non-selenium fertilised broccoli even though selenium fertilisation also caused a decrease in the total GSLs present[5,6]. Isoselenocyanates were chemically synthesised and these were also found to have greater phase II induction capabilities than ITCs which had sulfur at the active site[7,8]. However, as described in section 1.5, it was confirmed recently that the position of the selenium in the manufactured isoselenocyanates was not the same position selenium would occupy in the corresponding selenoisothiocyanates resulting from hydrolysis of plant-produced selenoglucosinolates (SeGSLs)

The broccoli grown in selenium fertilised soil incorporated selenium in some of the methionine derived GSLs confirming that chain elongation of selenomethionine rather than methionine was occuring[9]. No oxidised SeGSLs were found suggesting that selenium is not capable of forming a stable bond with oxygen the way sulfur does in sulfinyl GSLs as the only SeGSLs found were selenoglucoiberverin (methylselenopropyl GSL), selenoglucoerucin (methylselenobutyl GSL) and selenoglucoberteroin (methylselenopentyl GSL)[9].

6.0.3 Bacterial metabolism of glucosinolates

From the previous chapter it was apparent that there were differences in the way the lactic acid bacteria and the Enterobacteriaceae metabolised GSLs. The Enterobacteriaceae reduced the sulfinyl GSLs glucoraphanin and glucoiberin under anaerobic conditions into glucoerucin and glucoiberverin respectively. Sulfinyl GSLs have a sulfur atom at the terminal methyl group, which in turn bonds with oxygen creating an electrophilic reactive site. The redox shift only occurs in the presence of bacteria and it was hypothesised that this reaction was due to bacterial reductases. These are produced for the detoxification of electrophilic and reactive compounds in the same way

that eukaryotic phase II reductases protect from reactive oxygen species (ROS).

SeGSLs are not oxidised and their hydrolysis products are selenoisothiocyanates and selenonitriles; it is not known whether these are as reactive as the chemically synthesised isoselenocyanates. It is not known whether bacteria can transform SeGSLs but it may be possible to determine this by feeding SeGSLs to bacteria.

6.1 Aim of this study

The aim of this study was to see whether bacteria could transform SeGSLs and if so, what the products would be. If bacteria are unable to transform or metabolise SeGSLs, it may provide clues about how bacteria metabolise and transform GSLs and whether the oxidation state plays an important role. If gut bacteria preferentially alter the redox state of GSLs and their products to a reduced form, what do they do to SeGSLs that are modified and cannot be oxidised, and what effect might this have?

6.2 Materials and methods

Bacteria were cultivated under the same conditions as for the GSL consumption experiments in chapter 5 (section 5.1.1). Broccoli GSLs were extracted as described in chapter 2 (section 2.2.3). Analysis was by LC-QTOF-HRMS and GC-MS as described previously in chapter 2 (section 2.3.6 and 2.3.7). Compounds were identified using published data for SeGSLs, selenoisothiocyanates and selenonitriles[9]

6.3 Results

6.3.1 Standard broccoli compared with high-selenium broccoli

The GSLs glucoraphanin, glucoiberin, glucoerucin and glucoiberverin were monitored in standard broccoli (STD) and high-selenium broccoli (HighSe)[1] which was also monitored for the SeGSLs, selenoiberverin and selenoerucin. Figure 6.2 shows that after allowing for some redox shifting

[1] Author acknowledges with grateful thanks the samples of high selenium broccoli heads and standard broccoli donated by Marian McKenzie (Plant & Food Postharvest Fresh Foods)

between glucoraphanin and glucoerucin, glucoiberin and glucoiberverin, the total yield of GSLs in high-selenium was only 18% of that in standard.

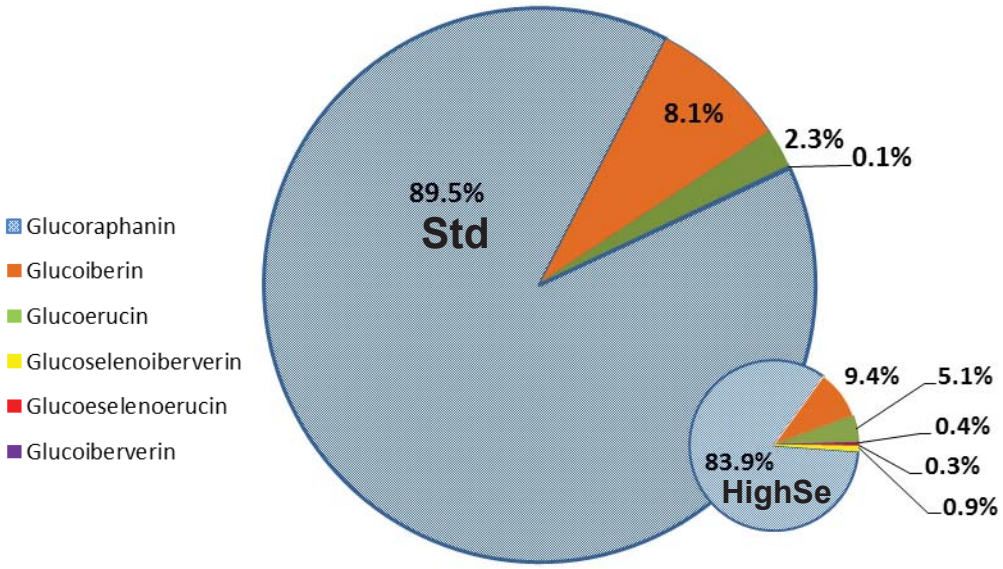


Figure 6.2. Pie graph depicting the relative ratios of glucosinolates for the broccoli extract taken from broccoli grown with selenium fertilization (HighSe) or without (Std)

Selenoglucoberteroin (5-methylthiolpentyl-GSL), selenoglucoiberverin and selenoglucoerucin have been characterised for mass and spectra[9]. Selenoglucoiberverin and selenoglucoerucin were chosen for this experiment because they are selenium analogues of glucoiberverin and glucoerucin which were monitored for bacterial metabolism with glucoiberin and glucoraphanin in chapter five.

6.3.2 Glucosinolate and selenoglucosinolate consumption

The selenium fertilized broccoli (+Se) had a similar ratio of sulfur glucosinolates but at lower concentration than controls as shown in Figure 2. This was expected as selenium fertilization is known to affect several plant biosynthesis pathways including phenolics and GSLs leading to a reduction in GSL biosynthesis which is dose dependent (Robbins, *et al.*, 2005). Also, only one plant was used to extract the GSLs from and it may have had a GSL profile that was atypical. As expected, selenoiberverin and selenoerucin were

minor components of the high-selenium broccoli GSLs and represented 0.3 and 0.8% of total GSLs. Both high-selenium and standard extracts were adjusted to a GSL concentration of 0.3% (w/v) for incubation with the bacteria with the high-selenium extract results shown in Figure 3. Glucoraphanin consumption was similar between extracts with 100% for *E. coli* Nissle and 71% for *Lb. plantarum* KW30 in the standard group (data not shown) and 92% for *E. coli* Nissle and 82% for *Lb. plantarum* KW30 in the high selenium glucosinolate extract.

When high-selenium GSLs were incubated with bacteria for 24 hours, it was observed that while much of the sulfur GSLs were consumed (Figure 6.3 (A)), selenoglucoerucin was still present in both bacterial supernatants and while selenoglucoiberverin was apparently absent in *Lb. plantarum* KW30 supernatant, it was still present in *E. coli* Nissle's (Figure 6.3 (B)).

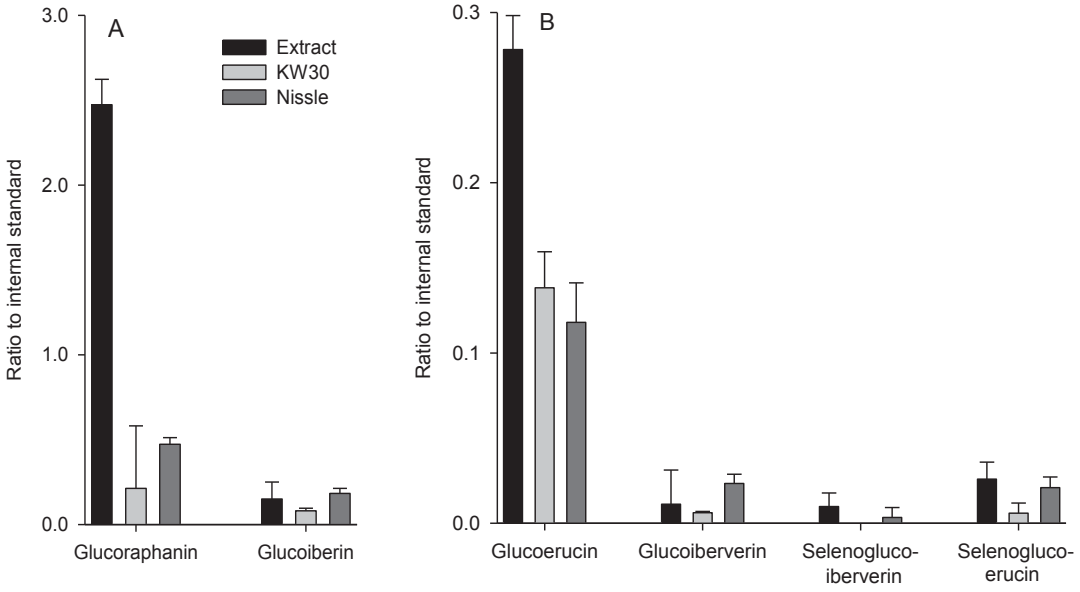


Figure 6.3. (A) Sulfinyl glucosinolates glucoraphanin and glucoiberin (B) thiol glucosinolates glucoiberverin and glucoberucin with selenoglucoiberverin and selenoglucoerucin. Black represents the extract ratios, light grey KW30 and dark grey *E. coli* Nissle. Note that the scale for (B) is 10-fold lower than (A). The error bars are standard deviations of the mean (*n*=3)

6.3.3 The hydrolysis products

When bacterial GSL metabolism was assessed, erucin nitrile was the only metabolite produced by *E. coli* Nissle while *Lb. plantarum* KW30 produced erucin nitrile and also some sulforaphane nitrile (Figure 6.4). Additionally, even though most of the glucoraphanin was consumed, It was not responsible for the concomitant increase in the corresponding nitriles or ITCs. We were unable to detect any metabolites of selenoglucosinolates such as selenoisothiocyanates or selenonitriles.

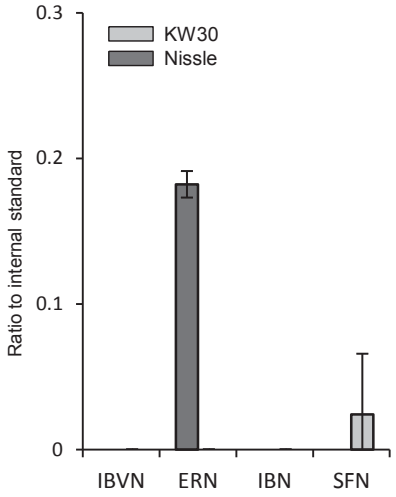


Figure 6.4. GC-MS identified the products of glucosinolate hydrolysis by *E. coli* Nissle which was erucin nitrile (ERN) and by KW30 which was sulforaphane nitrile (SFN). No selenoisothiocyanates or selenonitriles were detected. IBVN=Iberverin nitrile and IBN=iberin nitrile. Error bars are the standard deviations of the mean (*n*=3)

While the selenoglucoerucin and selenoglucoiberverin concentration decreased over 24 hours, the starting concentration was less than 1% of the total GSLs and so the decrease in concentration was also minor. The variation between samples was also relatively large and nothing was found in volatile products with GC-MS that could be identified as seleno-ITCs or seleno-nitriles. It is possible that *Lb. plantarum* KW30 consumed selenoglucoiberverin while avoiding selenoglucoerucin and that the hydrolysis products were further metabolised into undetected products

however, it is more likely that neither were consumed and selenoglucoiberverin was not detected because the system was not sensitive enough to determine concentrations below a certain limit. The error bars for the control SeGSLs were relatively large as well suggesting that there could be as little as none to some present in the sample (see Figure 6.3B). Both bacteria consumed the sulfur GSLs which could be easily determined as they were present in concentrations far in excess of the SeGSLs.

6.4 Discussion

Both *E. coli* Nissle and *Lb. plantarum* KW30 were able to consume GSLs which was evident by their disappearance (Figure 6.3) but the only hydrolysis products detected after incubation were iberverin nitrile, erucin nitrile and sulforaphane nitrile. Standard broccoli's dominant GSL was glucoraphanin (>89%) as shown in Figure 6.2 and after incubation with *E. coli* Nissle and *Lb. plantarum* KW30, only erucin nitrile was detected (Figure 6.4). The high-selenium broccoli had only about 18% of the glucoraphanin found in standard broccoli but did produce SeGSLs including selenoglucoiberverin and selenoglucoerucin (Figure 6.2). Erucin nitrile was the only end product detected for *E. coli* Nissle, and sulforaphane nitrile the only end product detected for *Lb. plantarum* KW30 (Figure 6.4).

Although the high-selenium broccoli contained SeGSLs, these were at very low levels and were there at similar concentrations at the end of the incubation for *E. coli* Nissle suggesting they were not consumed by these bacteria. For *Lb. plantarum* KW30, they were present but below the threshold of the limits of detection (LOD). As the error bars for the control were greater than the differences, it suggests that they were probably not consumed either. Their presence after incubation while their sulfur analogues that were present in much greater concentrations were consumed suggests that the selenium analogues of glucoerucin and glucoiberverin are not metabolised by *E. coli* Nissle or *Lb. plantarum* KW30 in the same way that glucoerucin or glucoiberverin are. It is curious that the bacteria in this experiment did not remove the sulfur-linked glucose from selenoglucoerucin or selenoglucoiberverin when presumably it is their bacterial glycoside hydrolase which performs this task as the glucose is at the other end of the

molecule where selenium should be distant enough not to be able to interfere with this process. There was no environmental pressure for bacteria to scavenge the glucose as the media contained 1% glucose and all nutrients necessary including sulfur but as earlier *in vitro* experiments have shown, bacteria were better at transforming GSLs when sugar was available rather than without (Figure 5.13). For although GSLs were not a carbon source as such and this was found from earlier GSL tolerance and consumption experiments that form chapter 4 and 5 of this thesis, in the presence of a carbon source the Enterobacteriaceae did hydrolyse more GSLs. This would translate to extra glucose available for the bacteria and it was observed in the earlier GSL tolerance experiments (Figures 4.9 – 4.12) that bacteria achieved greater optical density in media containing GSLs at 5 – 10 mg/mL, than media alone.

The as yet, unidentified uptake mechanism employed by bacteria in the metabolism of GSLs may be more specific than just being able to recognise the glycoside group or another possibility is that GSLs do not use a transport mechanism and are able to be passively taken up due to their high solubility; selenium analogues are however, apparently not able to be taken up as easily and this must be due to the selenium; either selenium's physical properties prevent it from being transported across the cell membrane or there is a mechanism that transports GSLs and it is not flexible enough to accommodate SeGSLs. *E. coli* Nissle and *Lb. plantarum* KW30 had different responses from one another towards the SeGSLs for reasons as yet unknown.

While others have shown that selenium fertilised broccoli has more potent phase II inducing potential than standard broccoli, these results support earlier studies that show fertilisation with selenium causes the total GSL content in the broccoli to be substantially reduced[6].

If the SeGSLs present are not able to be transformed by bacteria, then active myrosinase is required to generate selenoisothiocyanates or selenonitriles because without hydrolysis, SeGSLs will follow the fate of the other excess GSLs and be excreted in urine intact. Feeding trials would be required to see whether eating this broccoli will generate hydrolysis products and if so what these are. From this it may be possible to identify whether it is

the selenoisothiocyanates responsible for the observed increased phase II effect (compared to ordinary ITCs), or whether selenium on its own is the inducer. While some selenium does become incorporated into SeGSLs, selenium is also taken up in other tissues as selenomethionine, selenocysteine, inorganic selenium (selenite or selenate) and can become incorporated in proteins that use selenomethionine or selenocysteine as happens in the biosynthesis of SeGSLs[9]. Selenoproteins have been found to be phase II inducers[10] and while selenium is a potent antioxidant it can also be toxic depending on dosage and the form it takes.

6.5 Summary

The aim of this study was to see whether bacteria could transform SeGSLs and if so, what the products would be.

After twenty-four hours incubation with *Lb. plantarum* KW30, selenoglucoiberverin was absent while selenoglucoerucin was still detected in the spent culture media but no hydrolysis products were detected (data not shown). For *E. coli* Nissle, selenoglucoiberverin and selenoglucoerucin were detected in the spent culture media and no hydrolysis products could be detected (data not shown). Because the starting amounts were very small and the standard deviation relatively large, no conclusions can be drawn about the ability of either bacteria to metabolise these SeGSLs.

However, there was a trend and that was that the bacteria consumed the sulfur GSLs at a ratio of one hundred to one of SeGSLs. While the SeGSLs were in concentrations too small for these results to be considered conclusive and it is possible that these bacteria consumed SeGSLs, they did not metabolise them into hydrolysis products that could be detected.

It may be that bacteria take up, metabolise and transform GSLs because they recognise them and a selenium substitution at the methionine end affects their ability to do so.

Bacteria change the redox state of alkylsulfinyl GSLs and their products to a reduced form but because SeGSLs do not have this property and perhaps because the selenium substitution prevents uptake by bacteria, removal of the sulfur-linked glucose and the sulfate group cannot proceed so the selenoglucoerucin and selenoglucoiberverin remained in the media.

6.6 References

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SEVEN

7.0 Metabolism of dietary glucosinolates in an animal model

Dietary glucosinolates may reach the lower intestine if plant myrosinase is inactive either by processing or cooking of the vegetables[1,2]. They are thus available as a substrate for the colonic microflora and we have seen in chapter 5 that lactic acid bacteria and Enterobacteriaceae are capable glucosinolate transformers. Having this bacterial transformation occur in the intestine may be providing a protective effect on the colon, rectum, bladder, and prostrate. From *in vitro* experiments, it was apparent that glucosinolates did not affect bacterial growth and previous work by others has also shown that glucosinolate-rich material does not significantly perturb the gut microbiota[3]. Yet while other studies have examined the bacterial metabolic activity in the upper digestive tract[4,5], the effect of bacterial transformation of glucosinolates to isothiocyanates in the lower digestive tract has so far only been assessed *in vitro*[6,7].

Additionally, there are reported to be therapeutic benefits from the consumption of beneficial bacteria that include the production of bioactives from dietary components such as phytochemicals, polyunsaturated fatty acids (PUFAs) and prebiotics all of which contribute to our well-being[8,9]. Beneficial bacteria are also attributed with increased bioavailability of nutrients and the suppression of pro-carcinogenic enzymes[10,11] but to this author's knowledge there have been no studies of whether the health benefits of both could be augmented by combining consumption of beneficial bacteria with dietary glucosinolates from cruciferous vegetables. If GSLs and beneficial bacteria offer health benefits individually, then it was hypothesised that consuming the two together would have a synergistic effect increasing the health benefits to a greater degree than each alone.

7.1 The aim of this study

The aim of this study was to combine beneficial bacteria with dietary glucosinolates from broccoli and determine whether consumption of these together or alone can increase or induce phase II enzymes in the host which may be interpreted as leading to a health benefit.

An *in vivo* experiment to see whether phase II enzymes are upregulated in response to dietary glucosinolates was conceived and an animal feeding trial designed in order to answer this.

7.2 Materials and methods

7.2.1 Rationale for using animals

The complexity of the mammalian gut system with the interrelations between the food, the microbial gut ecosystem, and the host cannot be reproduced outside a living organism. There are also currently no *in vitro* models for the gastrointestinal tract and its complex microbial community for determining the effects of food and the gut ecosystem.

While human trials are to be preferred over animal for nutritional studies that relate to human health, the invasive sampling of tissue required for assessment of phase II enzyme activity prevented participation by human volunteers.

Although cell culture work has its place, in the case of phase II enzyme activity, previous publications have shown that cell culture work results differ from animal work[12].

The rat has been shown to be a valid model for the mammalian digestive tract and particularly the lower bowel[13,14]. They are readily available, easily handled and require small quantities of food. The Sprague Dawley rat strain has been routinely used in many research facilities.

7.2.2 Animals and housing

Male Sprague Dawley rats (6-7 week old,~180 g live weight), were sourced from Food Evaluation Unit, Plant & Food Research. Rats were placed in hanging wire cages with trays beneath and after acclimatization for 1 week, were randomly allocated into their eight (A-H) experimental groups (n = 10 per group).

7.2.3 Diets

The composition of the diet is given next in Table 7.1.

Table 7.1. Broccoli seed nutritional analysis and AIN-76A diet composition. 0.3% broccoli powder was added to the AIN-76A-BSP diet and the ingredients of the AIN-76A adjusted to balance ingredients where appropriate (total sugars and sodium).

Freeze dried broccoli seed analysis	
Ash Unit: % m/m	13.4
AOAC 923.03/942.05	
Fat SBR*	8.8
Unit: %m/m	
AOAC	
Fructose*	0.2
Unit: % w/w	
AOAC 980.13	
Glucose*	1.5
Unit: % w/w	
AOAC 980.13	
Lactose*	<0.1
Unit: % w/w	
AOAC 980.13	
Maltose*	<0.1
Unit: % w/w	
AOAC 980.13	
Moisture	2.4
Unit: %m/m	
AOAC (2005)	
Protein, Foods, (Nx6.25)*	18.4
Unit: %m/m	
AOAC 992.15	
Saturated Fat	2.4
Unit: g/100g	
Product basis AOAC	
Sodium*	680
Unit: mg/100g	
AOAC 984.27	
Sucrose*	26.0
Unit: % w/w	
AOAC 980.13	
Total Carbohydrate*	57.0
Unit: % m/m	
Food Standards Code	
Total Sugars*	27.9
Unit: %w/w	
AOAC (1995) 980.13	80.0
Diet supplies	
Fibre 5%, Protein 18.7%	
Fat 5%, Carbohydrate	
Digestible Energy,	

AIN-76A	
INGREDIENTS	mg/g
Casein	200
DL-methionine	3.0
Sucrose	500
Corn starch	150
Arbocel	50
Corn Oil	50
Choline Bitartrate	2.0
AIN-76 Mineral Mix	35
AIN-76A Vitamin Mix	10
Ethoxyquin	0.1

AIN-76 Mineral Mix	mg/g
Calcium Phosphate Dibasic	500
Sodium chloride	74
Potassium citrate monohydrate	220
Potassium sulphate	52
Magnesium oxide	24
Manganous carbonate (43-48%	3.5
Ferric citrate (16-17% Fe)	6.0
Zinc carbonate (70% Zn)	1.6
Cupric carbonate (53-55% Cu)	0.3
Potassium iodate	0.01
Sodium selenite	0.01
Chromium potassium sulphate	0.55
Sucrose	118.03

AIN-76A Vitamin Mix	mg/g
Thiamine Hydrochloride	0.60
Riboflavin	0.60
Pyridoxine hydrochloride	0.70
Nicotinic acid	3.00
Calcium pantothenate	1.60
Folic acid	0.20
Biotin	0.02
Cyanocobalamin CFR premix	0.10
Vitamin A (Retinol acetate)	0.14
Vitamin E (alpha-tocopherol) 5,000	9.98
Vitamin D3 (Cholecalciferol)	0.30
Vitamin K (Menadione)	0.05
Sucrose	982.72

7.2.4 Experimental design

7.2.4.1 Power Analysis

Power analysis* with respect to the endpoint outcome of phase II enzyme activity was performed by using published data (Table 7.2)[15] with particular attention on the difference between the Broccoli HP and Broccoli GS treatments.

Table 7.2. The power analysis for the animal trial

Variable	Colon	Liver
Standard deviation (SD)	78	18
Minimum true difference of interest	150	17
Resulting sample size (*n*) per group one-tailed test	*n*=4	*n*=14
For *n*=10 power =	99%	70%

Based on this power analysis, (*n*=10), and with the choice of combinations of glucosinolates and bacteria (*Lc. lactis* KF147, *Lb. plantarum* KW30 and *E. coli* Nissle), eight groups (A-H) were required.

7.2.5 Choice of beneficial bacteria

Although included in previous *in vitro* work, *Enterobacter cloacae* ATCC13047 was not included in these *in vivo* experiments because while it is commensal, there are recorded instances of it being pathogenic[16,17]. As it shared similarities with *E. coli* Nissle in that both were members of the Enterobacteriaceae and both had shown similar glucosinolate metabolism, and because *E. coli* Nissle is non-pathogenic, it was decided to use only *E. coli* Nissle.

7.2.6 The diet intervention groups

Eighty rats were divided into eight groups of ten in each group. All groups received the diet (AIN-76A) which is a standard rat diet containing all of the energy requirements and nutrients necessary for maintaining a grown rat. Group A, the control group received this diet alone. Group B had added to their AIN-76A diet, broccoli sprout powder (BSP) at 0.32% (w/v). Groups C, D and E

* Author acknowledges Duncan Hedderley biometrician Plant & Food Research, Palmerston North who provided the power analysis

had the AIN-76A diet, but were given beneficial bacteria, either *Lactococcus lactis* subsp. *lactis* KF147, *Lactobacillus plantarum* KW30 or *Escherichia coli E. coli* Nissle. Groups E, F and G had BSP (0.32% (w/v)) added to their AIN-76A diet the same as group B but were also given beneficial bacteria as described for groups C, D and E. The beneficial bacteria were delivered in 50 µL milk at a concentration of 10^{13} cells per mL and fed to the rats while they were held, offering the drink through a pipette tip. A test run was done prior to the trial using milk only and confirmed that the rats would readily drink milk in this manner. During the trial, groups A and B which were not receiving beneficial bacteria, were offered the same volume of milk. The food (AIN-76A +/- BSP 0.32% (w/v)) and water were available ad libertum throughout the trial.

7.2.7 Housing and conditions

The animals were housed from weaning in family groups in familiar cages and were transferred one week prior to the start of the experiment to hanging cages. During the experiment, the rats were housed individually in these hanging cages which contained pressed wood chips and a plastic tube for environmental enrichment. The room was temperature controlled (22 – 24°C, humidity of 60 ± 5%) with 12 h light/dark cycle. a plastic tube for environmental enrichment. General health was monitored daily on a scale of 1 - 5, with 5 being the healthiest score. Animals were weighed weekly and monitored for food and water intake as well as the condition of their coat, their behaviour and excretion daily.

For the first 7 days, all animals received AIN-76A diet and in the mornings were fed 50 µL of either milk from reconstituted skim milk powder or milk that had the bacteria added. The broccoli diet provides 80 mg per day of extract to 25 g diet and the broccoli powder extract provides ~12% GSLs w/v, or 9.6 mg GSL per 25 g of diet a day.

7.2.8 Collection of tissue

After 21 days of feeding, rats were euthanized by CO_2 asphyxiation. Entire caeca were collected under CO_2 gas to maintain anaerobic conditions and transferred to 50 mL plastic tubes pre-filled with CO_2 being stored on ice briefly before being used for GSL degradation experiments. Livers were harvested and transferred to 50 mL plastic tubes then snap frozen in liquid

nitrogen and stored at −80°C for use in quinone reductase (QR) assays. Bladders were collected, transferred to 2 mL screw top vials and also frozen immediately in liquid nitrogen then stored at −80°C for QR assays. Urine was collected by syringe from the rats' cages on day 20 and stored at −80°C for analysis of metabolites. Blood was collected from a cardiac puncture post-mortem, stored on ice and processed for analysis of metabolites the same day.

7.2.9 Protein extraction

For liver, approximately 1 g was added to a 15 mL falcon tube and 5 mL of ice cold 0.3M sucrose in PBS buffer (pH 7.2) added. The sample was homogenized using a (Omni THQ digital tissue homogenizer) while on ice, then 2 mL was removed and centrifuged at 9,000 *x g* at 4°C for 10 minutes. The supernatant (S9 fraction) was collected for analysis of QR and the protein concentration determined as follows: Solubilised protein content in the obtained S9 fractions were measured with a Bradford assay procedure. A protein standard, bovine serum albumin (BSA), ranging from 0.05 mg to 0.4 mg/mL was used to generate a standard curve. These dilutions were incubated with Bradford's solution (Biorad, Auckland NZ) at room temperature for 5 min and were then the absorbance was measured at 595 nm (SpectraMax190, Molecular Devices, Biostrategy, Auckland, NZ). Comparison of the absorbance of the extract of rat liver S9 fraction with the BSA standard curve provided a relative measurement of protein concentration. S9 fractions were diluted accordingly so that all contained approximately the same concentration (40 mg/mL). For bladder tissue, the bladders were ground with a mortar and pestle in liquid nitrogen until they were powdered. The powdered bladder tissue was then added to ice cold 0.5 mL 0.3M sucrose in PBS buffer (pH 7.2), vortexed to mix and then centrifuged at 13,000 *x g* at 4°C for 15 minutes. The supernatant was carefully removed and the protein estimated as for the liver S9 fractions adjusting to 35 mg/mL.

7.2.10 Quinone reductase assay

This was adapted[18] as follows: 100 mL of MTT buffer contained 5 mL 0.5M Tris-HCl (pH7.4), 5 mL 10% bovine serum albumin, 670 µL 1.5% Tween 20, 67 µL 7,5 mM flavin adenine dinucleotide (FAD), 670 µL 150 mM glucose-6-phosphate, 60 µL 50 mM nicotinamide adenine dinucleotide phosphate (NADP),

173 µL 2U/mL glucose-6-phosphate dehydrogenase (G-6PH), 30 mg 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 100 µL 25 mM menadione dissolved in acetonitrile. 200 µL of MTT buffer was added to wells containing either 2.5 µL of soluble cell extract or BSA controls. Duplicate plates had 50 µL 0.3M dicoumarol added prior to the addition of the MTT buffer as a QR enzyme inhibition control. Incubation was for 5 minutes at room temperature then the reaction was stopped in the test plate with the addition 50 µL 0.3M dicoumarol and the plates read immediately at 570 nm (Spectra Max Pro, Molecular Devices, Biostrategy Ltd, Auckland NZ. Samples were analysed in duplicate with five biological replicates per group.

7.2.11 Glucosinolates

Glucosinolates in the diet were provided from broccoli seed powder (BSP) (previously described in chapter 2). The composition of this was determined prior to the feeding trial and is shown in Table 7.1. Two batches of BSP powder were analysed and the average given. Individual glucosinolates where they represent <1% of the extract are not shown.

Table 7.3. Glucosinolate composition of extract used.

Glucosinolate	% of total
Glucoraphanin	52.0
Glucoiberin	15.6
Progoitrin	7.1
Glucoerucin	11.9
Glucoiberverin	2.8
Sinigrin	2.7
Gluconapin	2.2

7.2.11.1 LC-QTOF-HRMS

The method for LC-QTOF-HRMS has previously been described in chapter 2. However urine and blood were to be analysed and these were prepared as follows:

7.2.11.1.1 Urine

Samples were prepared for analysis by making a 1:3 dilution of the urine with methanol/ H_20 (20:80), then the sample was centrifuged to precipitate any particulates and 100 µL of supernatant transferred to a vial.

7.2.11.1.2 Blood

Samples were prepared by treatment with 100 mM acetic acid[19]: Briefly, 0.5 mL of plasma was treated with 100 mM acetic acid in methanol (1 mL), left on ice for 30min, and centrifuged at 2000 *x g*, for 5 minutes at 4 °C. 100 µL of supernatant was added to a vial containing 100 µL of 100 mM acetic acid in methanol.

7.2.11.2 GC-MS

The method for GC–MS has previously been described in chapter 2. However urine and blood were to be analysed and these were prepared as follows:

To verify retention times known broccoli hydrolysis products or pure standards were added to urine or blood. The areas under the peaks identified were normalised to the internal standard and the partitioning efficiency validated empirically using triple solvent extraction. Based on the efficiency and where possible, sulforaphane was used to generate a standard curve for estimating nitrile concentration. The limit of detection was determined for each experiment at three times the signal to noise ratio (6.0×10^3 for urine analysis and 5.0×10^3 for blood).

7.2.11.3 Statistical analyses

Statistical analyses were conducted using analysis of variance (ANOVA) and results were expressed as means of five biological replicate determinations ± standard deviation (SD).

7.3 Results

7.3.1 Food intake and weight

All animals were monitored for food and water intake as well as the condition of their coat, their behaviour and excretion (Table 4), scoring the maximum health score of 5 each day throughout the trial.

Table 7.4. Several indicators of health were used to monitor the animals

Frequency of monitoring	
Daily	Water intake
	Food intake
	Posture
	Gait/movement
	Coat condition
	Faecal consistency
Weekly	Weight

By the end of the fourth week, the animals' weights averaged 358.8 grams and they were consuming on average, 23.5 g of food per day. There were no differences in weights between groups at week 1 (p=0.23), week 2 (p=0.38), week 3 (p=0.12) and week 4 (p=0.34). The groups that had BSP (0.32% w/v) supplemented diets consumed between 9.6 - 11.5 mg of GSLs made up of glucoraphanin (4.9 – 6 mg), glucoiberin (1.5 – 1.8 mg), glucoerucin (1.1 – 1.4 mg) and glucoiberverin (0.3 mg). Plotting food intake versus weight showed a spread with no particular pattern of clustering within or between groups (Figure 7.1).

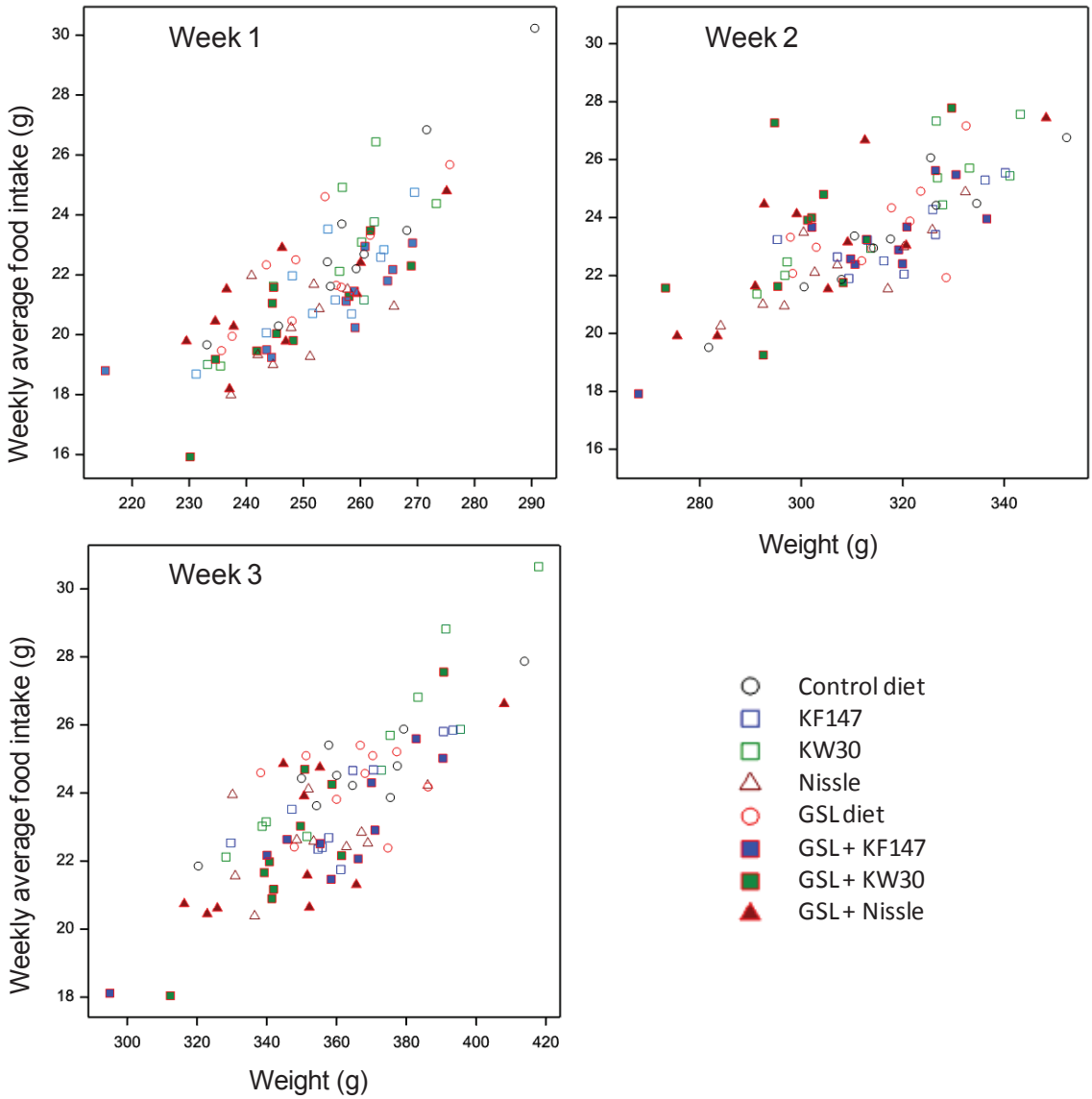


Figure 7.1. Scatter plot of food intake versus weights (*g*) of all 80 animals during the trial from week 1 to week 3, and across all treatment groups. Groups (n=10) are colored as follows: Blue, KF147; green, KW30; brown, *E. coli* Nissle; GSLs, red; control diet, black. A red border around solid colored markers show GSLs in combination with the beneficial bacteria.

7.3.2 Phase II induction

QR was chosen for measuring phase II induction because it is a phase II enzyme known to be produced by all cells, a biomarker for health having being used extensively in studies of GSLs and their hydrolysis products and existing protocols for its measurement are well established[20]. Liver tissue QR activity in the dietary intervention groups was assessed by measuring the change in absorbance (570 nm) using the control diet group as an indicator of baseline

(background) levels. QR activity was greatest in the control diet group, the KW30 diet-supplemented group, the GSL group and the GSL plus *E. coli* Nissle diet-supplemented group (Figure 7.2). Groups with significantly less QR values were the KF147 supplemented groups with or without GSLs, *E. coli* Nissle and KW30 with GSLs. These results were confusing and it was concluded that the most likely reason for these findings was that the liver was not reflecting any 'accumulation' effects in a trial such as this where food intake was uncontrolled. The liver, while responding to the bioactive products of GSL hydrolysis, does so rapidly; it is perhaps not the best tissue to record QR elevation over time and where the food intake time points may vary between groups and in this 'snapshot' the current liver activity was being observed which may not have been related to GSLs or beneficial bacteria consumed in the diets.

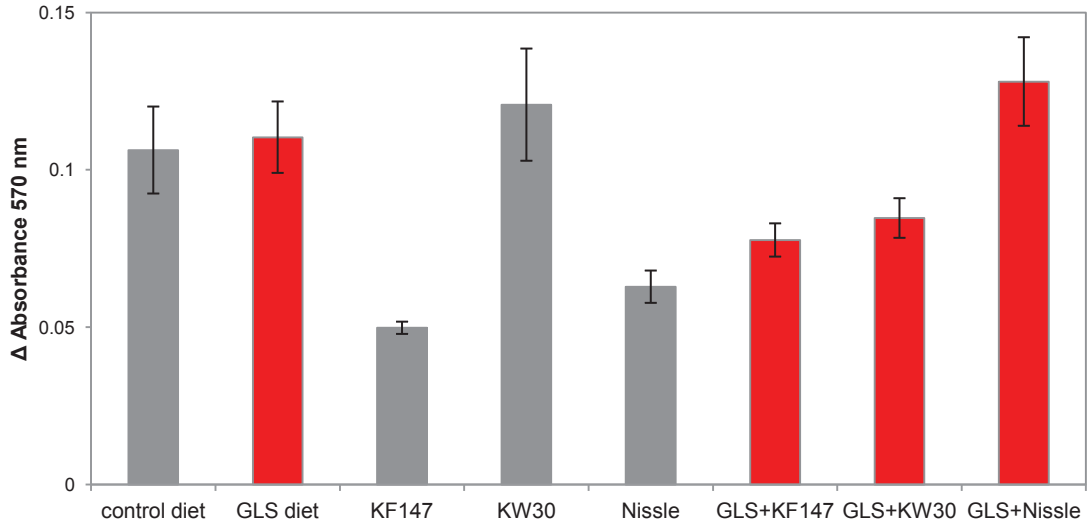


Figure 7.2. Quinone reductase (QR) assay of liver showed no particular pattern of induction except that KF147 (group C) appeared to be particularly low compared to the rest. Diets containing BSP are solid red bars. All samples were normalised for protein concentration SEM shown (*n*=5)

7.3.3 Quinone reductase effects were observed for bladder

Figure 7.3 shows the QR results from the bladder where a significant increase in QR activity was observed between the control diet (no GSLs and no beneficial bacteria) compared to all the other groups ($p<0.001$). Significant differences were found between the groups that had no added beneficial bacteria (control diet and GSL diet) and the other groups that had combinations

of the two (p<0.001). There was even a significant difference in QR activity for all groups that received added beneficial bacteria with or without GSLs (*p*<0.001). However, when grouped by diet (GSLs or not), there were no significant differences (*p*=0.28) nor were there significant differences between the type of bacteria (lactic acid or *E. coli* Nissle, *p*=0.54) (Figure 7.3). It appeared that the addition of beneficial bacteria increased the QR activity in the bladder as much if not more than GSLs without additional bacteria and there was no statistical significant difference between groups (p=0.44). However there was a trend that the groups receiving beneficial bacteria and GSLs had higher QR values compared to the GSLs-only and beneficial bacteria alone.

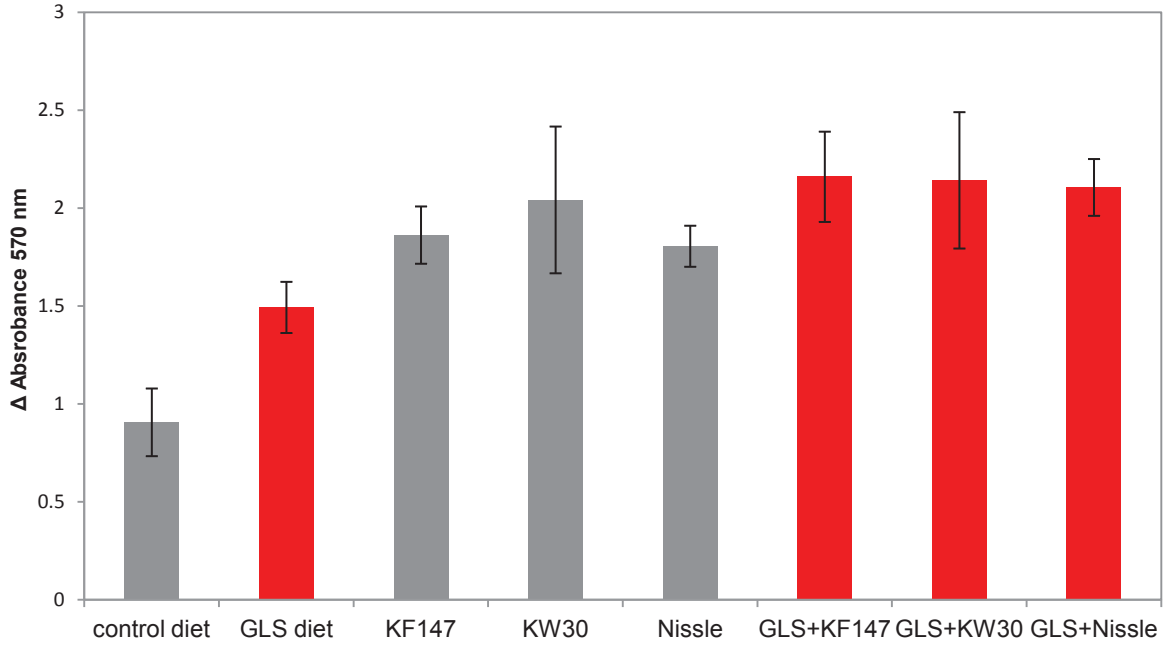


Figure 7.3. The QR assay of bladder tissue suggests an induction effect for all diets containing GSLs and/or beneficial bacteria. Diets containing GSLs are shown as solid red bars. All samples were normalised for protein concentration SEM shown (*n*=5)

7.3.4 Urine

LC-QTOF-HRMS analysis of urine for the rats fed the diets supplemented with BSP showed intact GSLs glucoraphanin, glucoiberin, glucoerucin and glucoiberverin while rats fed the standard AIN-76A diets had no detectable GSLs in their urine. While the GSLs fed to the rats was comprised of 55% of glucoraphanin, 14.8% glucoiberin, 9% glucoerucin and 5% glucoiberverin w/v, the urine profiles differed markedly with reduced GSLs

glucoerucin and glucoiberverin dominating over glucoraphanin and glucoiberin. This suggested that the GSLs glucoraphanin and glucoiberin had been interconverted to glucoerucin and glucoiberverin respectively (Figure 7.5) and supports results of earlier work (chapter 5 and chapter 6).

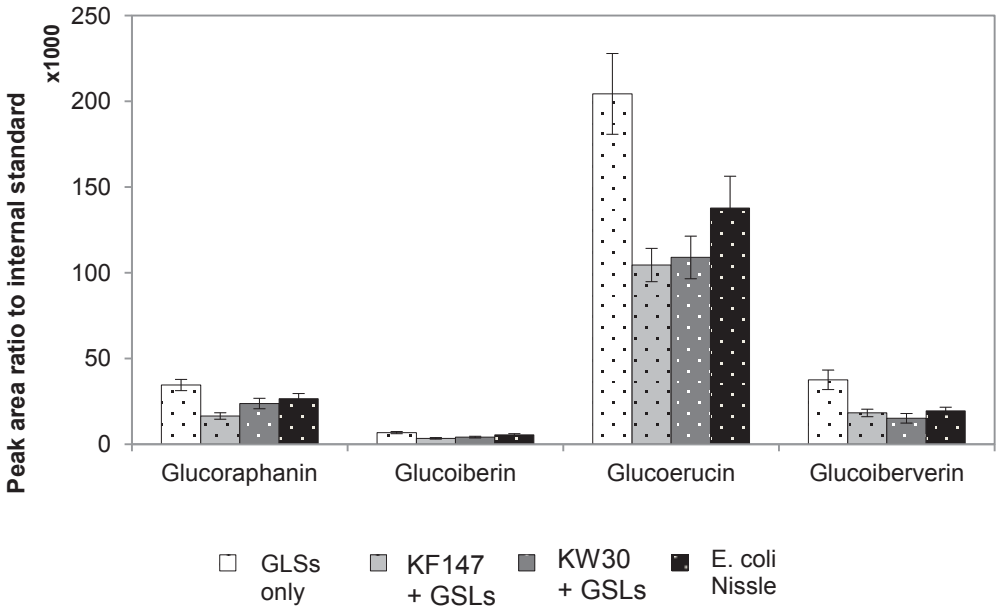


Figure 7.4. LC-QTOF-HRMS urine analysis identified the GSLs glucoraphanin, glucoiberin, glucoerucin and glucoiberverin in all groups. Group GSLs only urine contained significantly more GSLs than the other groups (p<0.001). The groups which had beneficial bacteria with their GSL diet were not significantly different. SEM shown . Error bars = SEM (n=10)

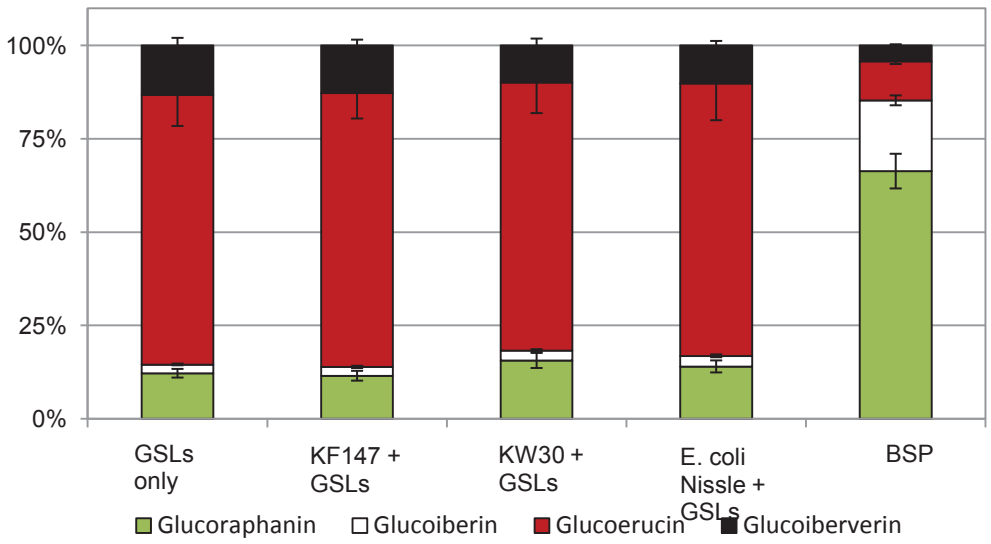
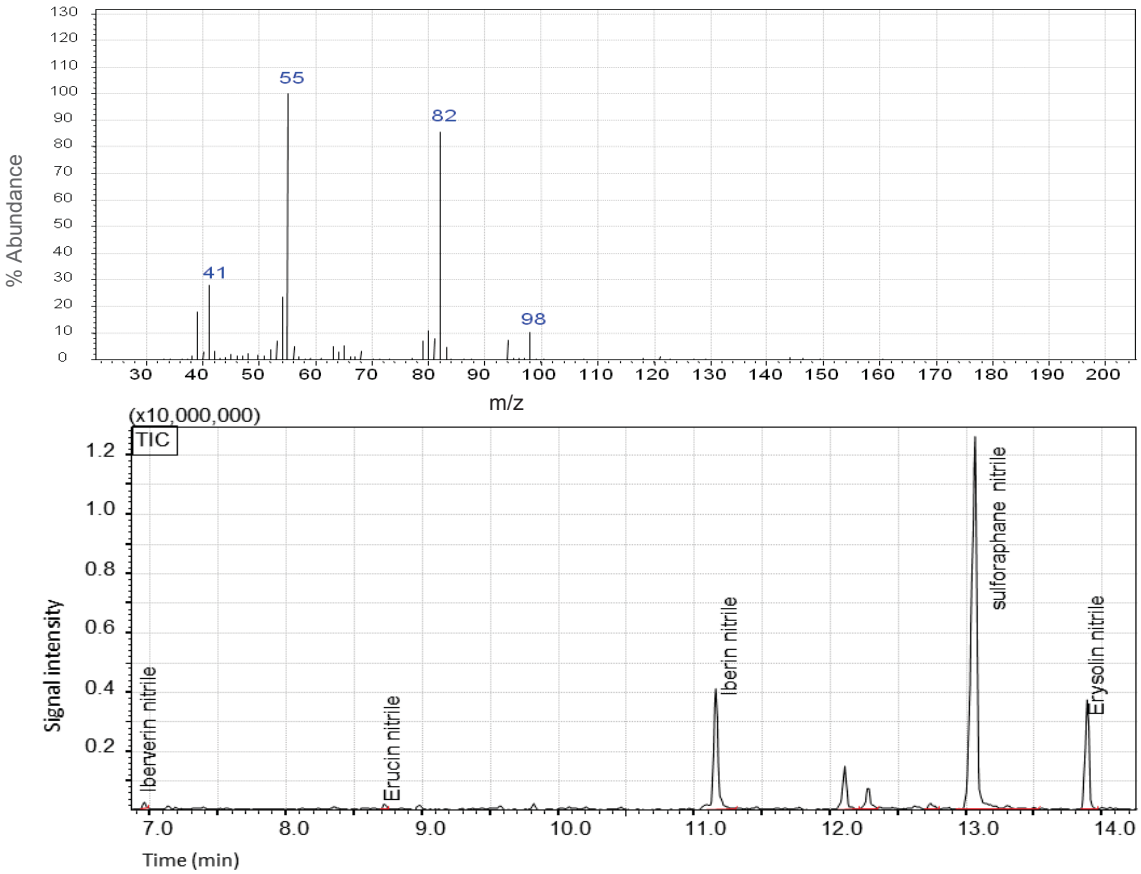


Figure 7.5. Stacked bar graph (100%) demonstrates a similar redox shift from sulfinyl to thiol across all GSL groups. 'None' had GSLs only added to their diet and 'BSP' gives the relative proportions of the four GSLs of interest that were fed in the diet. Error bars = SEM (n=10)

When the urine was analysed by GC-MS, one of the major peaks was a new product identified as erysolin nitrile (3-methylsulfonyl) butanenitrile which is the most oxidized form of sulforaphane nitrile. Erysolin nitrile was identified based on the published spectral data[21] who also gave the parent ion (m/z 161) at <1% although we did not find this ion. The chromatogram, spectral data and retention time is given in Figure 7. 6.



Name	Retention time (min)	Mass	MS spectral data m/z (% relative abundance)
Erysolin nitrile 5-(Methylsulfonyl)pentanenitrile	13.9	161.22	98 (10), 54 (25), 41 (30), 82 (88), 55 (100)

Figure 7.6. Top: LC chromatograph from urine: Center: MS spectral ion signature of the peak observed at 13.9 minutes. Bottom: The major peak, identified as erysolin nitrile, was seen in all of the urine and blood samples of the glucosinolate diet intervention groups. Error bars = SEM (n=10)

When urine was analysed for the hydrolysis products of GSL metabolism the major peaks detected were sulforaphane nitrile, erysolin nitrile and iberin

nitrile with minor peaks of iberverin nitrile, erucin nitrile and sulforaphane (Figure 7.7).

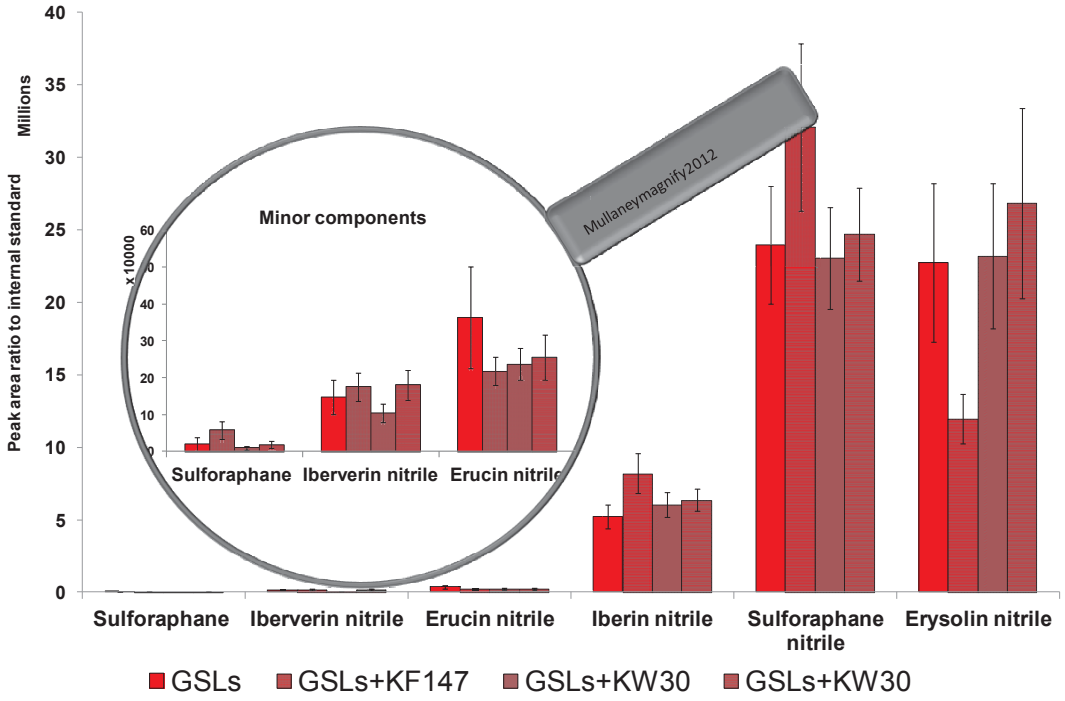


Figure 7.7. GC-MS analysis of rat urine showing the major metabolites of glucosinolate transformation. Magification of the minor components. Sulforaphane, iberverin nitrile and erucin nitrile is shown with sulforaphane just above the limits of detection (LOD) = 6 x 10^5. Error bars = SEM (n=10)

7.3.5 Blood

Analysis of blood showed similar products to the urine including erysolin nitrile which was the predominant product again (Figure 7.8). No GSLs were detected in the blood but this was expected because GSLs are known to be cleared rapidly from the blood and blood was taken from the animals post mortem who had been feeding *ad libitum*. Iberin nitrile values for group B and F were just at or just below the limits of detection (Figure 7.8). While ITCs were not detected in the blood, we were operating at the limits of detection and subsequent efforts to concentrate the sample by evaporation under nitrogen were unsuccessful. Erysolin nitrile can be produced via the oxidation of sulforaphane nitrile or erucin nitrile, or from hydrolysis of the GSL glucoerysolin, which has so far only been found in *Erysimum allionii* (Siberian wallflower) a Brassiaceae that is cultivated for the flowers and not eaten[21]. As there was no

glucoerysolin in the BSP providing the GSLs for the diet, and the only groups that accumulated erysolin nitrile in their urine and blood were those fed this diet, it was most probably formed through oxidation of erucin nitrile and or sulforaphane nitrile. Also erysolin nitrile was not found in any of the *in vitro* bacterial experiments so it was hypothesised that the oxidation of erucin nitrile and or sulforaphane nitrile was host initiated.

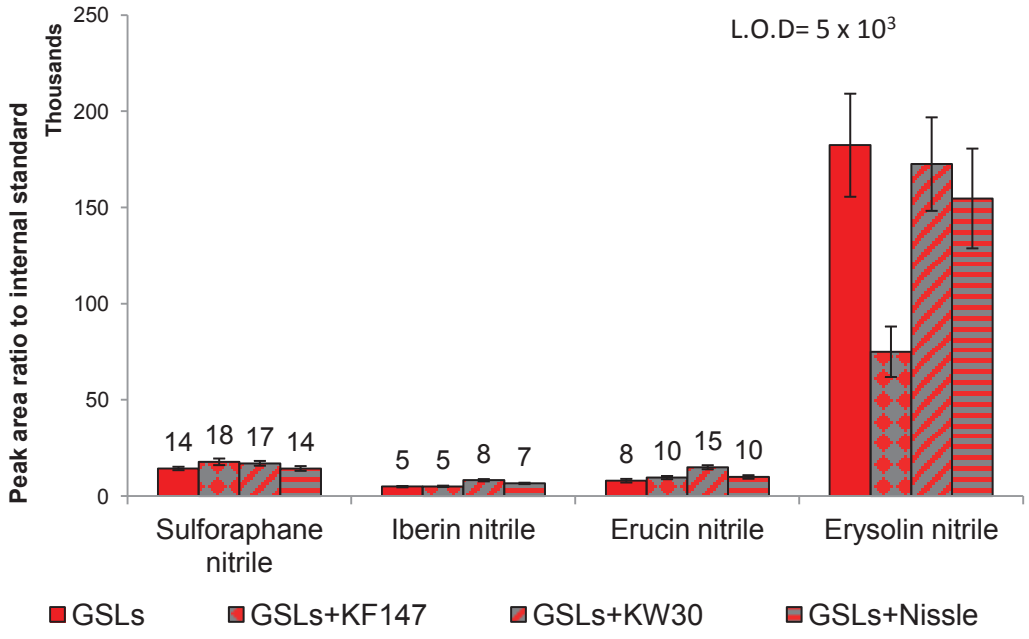


Figure 7.8. GC-MS of blood identified similar compounds as the urine. Sulforaphane nitrile, iberin nitrile, erucin nitrile and erysolin nitrile were the only products of GSL metabolism that could be identified above the limits of detection (LOD=5x10^3). Error bars = SEM (n=10)

7.4 Discussion

Both the consumption of dietary glucosinolates and beneficial bacteria, have been associated with increased health benefits. This study investigated whether a broccoli extract containing multiple glucosinolates could stimulate the phase II antioxidant pathways and also whether the addition of beneficial bacteria could augment this benefit. The aim of this study was to determine whether consumption of these together or alone can increase or induce phase II enzymes in the host which may be interpreted as leading to a health benefit. To answer these questions *L. lactis*, *Lb. plantarum* and *E. coli E. coli* Nissle were

chosen due to their ability to transform GSLs into ITCs and nitriles and also because they are food grade microorganisms and safe as part of a diet.

The host microbiota comprises a complex community representing a vast mix of different bacteria, many of which possess oxidative capabilities enabling them to oxidise reduced GSLs and their hydrolysis products, the nitriles and ITCs. Indeed, as chapter 5 and 6 showed, *in vitro,* the enterobacteriaceae reacted to sulfinyl GSLs and their hydrolysis products by reducing them into their equivalent thiol species while the lactic acid bacteria did not. *In vivo,* the GSLs glucoraphanin and glucoiberin were consumed while their reduced species, glucoerucin and glucoiberverin were found in urine in greater quantities than could be accounted for by diet, supporting the *in vitro* evidence in chapter 5 and 6 that a shift in redox from sulfinyl to thiol for glucoraphanin and glucoiberin was occurring.

However this was not the case for the GSL hydrolysis products. As shown in Figure 7.7 the major GSL hydrolysis products found in urine were the oxidised species and this time the major peaks were that of sulforaphane nitrile, iberin nitrile and erysolin nitrile. The minor peaks this time were sulforaphane, erucin nitrile and iberverin nitrile.

There are two possible reasons for the alkylthiol - alkylsulfinyl redox shift that was observed in the GSL hydrolysis products identified in the urine and blood. One is that the bacteria were responsible and the other is that the host was responsible. Supporting evidence that bacteria were not involved was seen in the *in vitro* bacterial experiments (chapter 5) where alkyl-GSLs and their products only shifted towards alkylthiols. In these experiments, after incubation, glucoraphanin was always consumed while glucoerucin increased in concentration. Erucin and erucin nitrile were the main hydrolysis products while sulforaphane and sulforaphane nitrile were either absent or as seen with sulforaphane nitrile that was present in the media at the start, decreased in concentration. It is possible that sulforaphane nitrile simply degraded into non-detectable products but media without bacteria showed no loss of nitriles suggesting it was stable and also when lactic acid bacteria were grown with this same GSL media (chapter 5), sulforaphane nitrile was found in its media after 24 hours.

The gut is an anaerobic environment where oxygen is toxic to the obligate anaerobes and while the enterobacteriaceae are facultative anaerobes, they showed no inclination in the previous *in vitro* experiments to oxidise the GSLs or their hydrolysis products and it is unlikely that they did so when part of the microbiota.

7.4.1 Discovery of a new compound, the sulfone erysolin nitrile

Erysolin nitrile has never been reported as a product of bacterial metabolism of glucoraphanin or glucoerucin before and was only detected in the urine and blood of the groups of animals which had GSLs added to their diet. It was also the dominant nitrile product found at similar concentrations to sulforaphane nitrile and at one hundred-fold concentration to erucin nitrile. The chemical structure of erysolin nitrile identifies it as the sulfone of sulforaphane nitrile, and erucin nitrile. Its presence can be explained by a single oxidation of the methyl sulphur group in sulforaphane nitrile (Figure 7.9). As shown, while the sulfur bond with oxygen (sulfinyl) is reversible, the sulfur double oxygen bond (sulfone) is not.

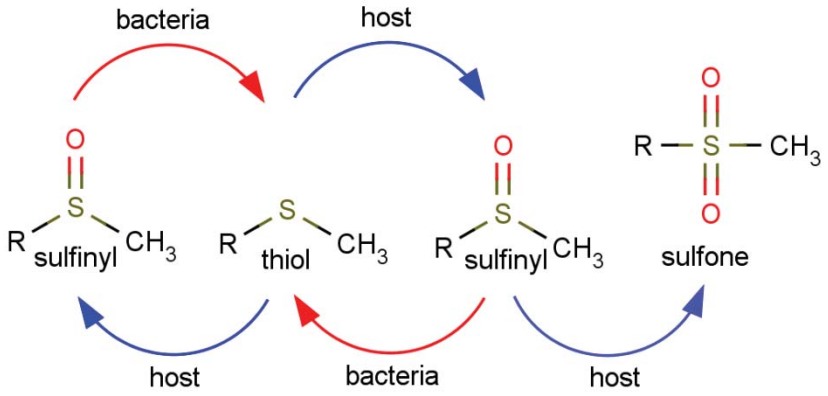


Figure 7.9. Sulfinyl to thiol redox reaction. Reduction is due to bacteria and oxidation is due to the host. Double oxidation to the sulfone is irreversible

7.4.2 Evidence that the oxidation of the thiol to sulfinyl was host-derived

Because there was no active myrosinase available to catalyse the transformation of the GSLs, the appearance of nitriles had to be due to microbiota. From this point, it appears that the microbiota reduced the GSLs glucoraphanin and glucoiberin but the major hydrolysis products were the oxidised, sulforaphane nitrile and iberin nitrile, even erysolin nitrile. The

hypothesis for bacterial reduction followed by host oxidation was supported by the result showing that in the urine, the major GSL present was not glucoraphanin, which accounted for 63% of the total of the four GSLs assessed. It was now glucoerucin at 72% and glucoiberverin now on 13% where in the broccoli seed powder they were 14% and 3% respectively (summarised in Table 7.5).

The change occurred after this point for the GSL hydrolysis products, the nitriles. In contrast to the *in vitro* experiments from chapter 5 where the GSL hydrolysis products were reduced as well as the GSLs, this time as Table 7.5 shows, the oxidised species of nitrile were found in excess of the reduced and these results more closely matched the proportions from the broccoli seed powder.

Table 7.5. The proportion of sulfinyl to thiol species changed during host metabolism and supports the hypothesis that host oxidation reversed the bacterial reduction of the glucosinolate hydrolysis products (refer to Table A1-A2 in Appendix for diagrams of the structures)

Glucosinolate	% in broccoli seed powder	% in urine	Corresponding nitrile	% in urine
Glucoraphanin (sulfinyl)	63	12	Sulforaphane	43
Glucoiberin (sulfinyl)	19	2	Iberin	12
Glucoerucin (thiol)	14	72	Erucin	<1
Glucoiberverin (thiol)	3	13	Iberverin	<1
Erysolin nitrile (sulfonyl)	Not added	Not a GSL	Erysolin	43

The question of why only the hydrolysis products should be oxidised by the host can be explained thus: GSLs are already very hydrophilic due to their β-thioglucoside and sulfate group and oxidation would have no effect on the solubility. Nitriles and ITCs on the other hand, lose those hydrophilic components becoming hydrophobic; oxidation would therefore increase solubility which in turn facilitates elimination.

Finally, erysolin nitrile was found for the first time and had not been seen in the previous *in vitro* experiments. Its presence in both blood and urine supported the hypothesis of host oxidation. It also suggested that if erysolin

nitrile had formed from the oxidation of sulforaphane nitrile then iberin nitrile might also be oxidised to cheirolin nitrile, however none was detected.

An explanation for this is that the host directed the oxidation of nitriles only if sufficiently hydrophobic in order to facilitate elimination. Evidence that supports this hypothesis is that the Cyp450 enzymes are part of the phase I detoxification system and these are able to oxidise reactive groups within molecules in order to inactivate them[20,22-24].

Perhaps the thiol group in some glucosinolates nitriles is available to react with Cyp450 enzymes becoming oxidised to the sulfinyl form which decreases the nitrile's hydrophobicity. Hydrophobicity may be a contributing factor of whether nitriles accumulate in tissue even though this is not the same for ITCs. For ITCs, uptake by cells is directly related to their conjugation with glutathione (GSH) a tripeptide of glutamate, glycine and cysteine (Figure 7.11). The cellular enzyme glutathione S transferase (GST) promotes ITC uptake by enhancing the conjugation reaction[24] As shown in Figure 7.11, sulforaphane is conjugated with glutathione then subsequently deconstructed by stepwise removal of glutamate, then glycine becoming the mercapturic acid as sulforaphane conjugated to N-acetyl cysteine for excretion.

But nitriles do not have an isothiocyanate group for glutathione conjugation (Figure 7.10), and past studies using mouse and rat hepatoma cells have confirmed that while the ITC sulforaphane induces glutathione *S*-transferase, sulforaphane nitrile does not[25].

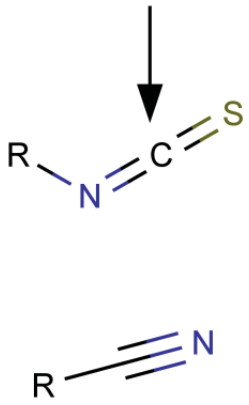
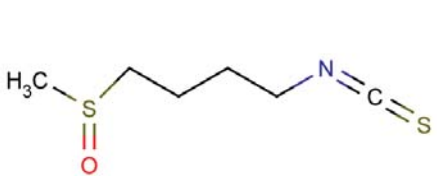


Figure 7.10. The isothiocyanate group (top) and nitrile group (bottom) demonstrate the difference between ITCs and nitriles with R representing the side chain which can be a number of different structures including alkyl, aromatic or branched. The arrow indicates the carbon that forms the conjugate with glutathione

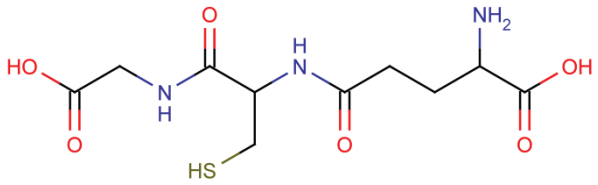
If host oxidation was responsible for the redox shift to sulfinyls then one would expect to find in urine and blood, sulforaphane nitrile in excess of erucin nitrile and iberin nitrile in excess of iberverin nitrile which is what was observed (Figure 7.8).

All of these results support the hypothesis that nitriles are oxidised by the host to facilitate elimination. However, whether nitrile accumulation increases with increased hydrophobicity or whether other factors contribute to accumulation is not known.

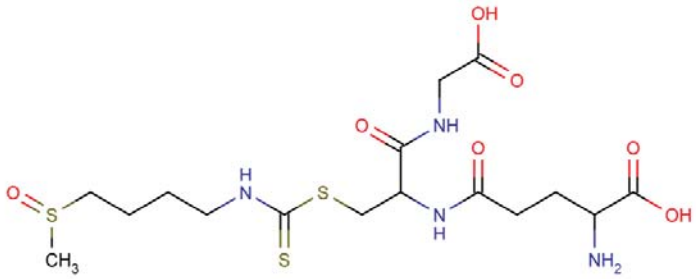
The only other reason to find erysolin nitrile in urine and blood would be if glucoerysolin was one of the GSLs in the mixture given from broccoli seed. Glucoerysolin has so far only been found in the Siberian Wallflower (*Erysimum allionii*)[21] and was not in the broccoli seed powder that contained the GSL mixture.



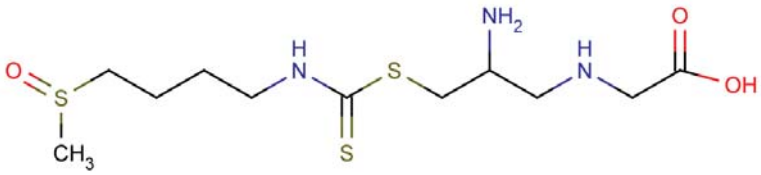
Sulforaphane



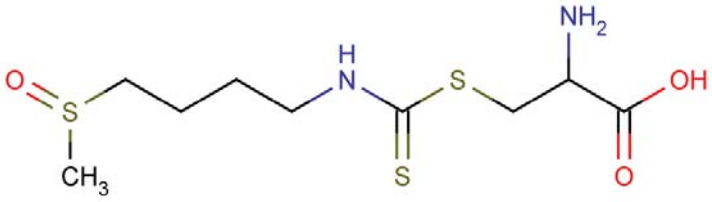
Glutathione



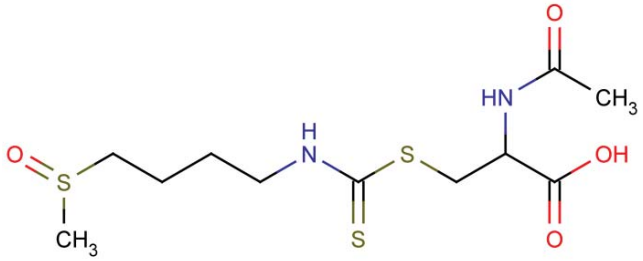
Sulforaphane-glutathione conjugate



Sulforaphane-glycine-cysteine conjugate



Sulforaphane-cysteine conjugate



Sulforaphane-N-acetyl cysteine conjugate

Figure 7.11. Sulforaphane undergoes stepwise deconstruction once conjugated with glutathione

7.5 Summary

The aim of this study was to combine beneficial bacteria with dietary glucosinolates from broccoli and determine whether consumption of these together or alone could increase or induce phase II enzymes in the host which may be interpreted as leading to a health benefit.

The animal trial was therefore designed to determine whether phase II enzymes were upregulated in response to dietary GSLs. The results showed no obvious induction effects in the liver tissue but did show an increase of QR activity in the bladder for some groups. Those groups were those receiving GSLs with or without additional beneficial bacteria at the concentration of 9.6 mg GSL per 25 g of diet a day and those receiving beneficial bacteria alone.

Analysis of the urine and blood showed that major glucosinolates found in urine were the reduced species whereas the major hydrolysis products found in the blood and urine were oxidised. Erysolin nitrile was identified which could only have been generated by oxidation of erucin nitrile and or sulforaphane nitrile. Oxidised species of nitriles had not been found in any previous *in vitro* bacterial glucosinolate metabolism experiments using broccoli seed powder but only two lactic acid bacteria and two enterobacteriaceae were studied and these are not representative of a gut microbiota.

The hypothesis formed was that the microbial metabolism of GSLs results in a redox shift of both GSLs and their hydrolysis products towards reduced while the host modifies the products' redox state to the oxidised.

An *ex vivo* experiment was designed using intact caeca from the animals that were used in this feeding trial with the intention of studying the products of *ex vivo* caecal metabolism of GSLs. If the redox shift was due to microbial activity by microbiota, this will be reflected in the products of GSL metabolism and give similar products as in the feeding trial. However, if the redox shift was due to host modification, the products of *ex vivo* GSL metabolism will be the reduced species, similar to the results of the *in vitro* bacterial experiments that formed chapters 5 and 6 of this thesis.

7.6 References

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EIGHT

8.0 The caecal metabolism of glucosinolates – *ex vivo*

After pre-exposure to glucosinolates (GSLs), the lactic acid bacterium *Lb. plantarum* KW30 demonstrated an adaptive response in that it became a more efficient GSL metaboliser (chapter 5). Previous experiments from this chapter indicated that Enterobacteriaceae reduce the sulfinyl GSLs glucoraphanin and glucoiberin to alkylthiol GSLs glucoerucin and glucoiberverin respectively.

When urine was analysed in chapter 7, the reduced species of GSLs dominated over the oxidised yet for the hydrolysis products (nitriles and ITCs) the reverse was true; oxidised were the dominant species and erysolin nitrile which had never been reported before was found at the greatest concentrations in both blood and urine. In order to determine what had caused this shift back to the oxidised, an *ex vivo* experiment was conceived that enabled the products of caecal metabolism of GSLs by host bacteria to be ascertained. A higher concentration of the hydrolysis products would be evidence that oxidation was occurring and was therefore a result of host metabolism.

A the same time it had been shown in chapter 5 that prior exposure to dietary GSLs primed KW30 making it a more efficient GSL metaboliser. It is not known, however, whether it is possible to prime endogenous host microbiota in a similar manner and if so, whether this has any effect, beneficial or otherwise.

8.1 Aim of this study

The aim of this study was to determine whether the reduction and oxidation of GSLs, ITCs and nitriles was of bacterial origin, host initiated or both. In chapter 7 it appeared that both reduction and oxidation were occurring but it wasn't possible to be certain whether the reduction and or oxidation of GSLs, ITCs and nitriles were due to bacterial metabolism, host metabolism, or a combination of both. In chapter 5 it was evident that KW30 was able to adapt to GSLs becoming more efficient at metabolising them after pre-exposure. This study therefore sought to determine whether host microbiota and host microbiota that has been manipulated by the addition of beneficial bacteria, can be primed by pre-exposure to GSLs.

8.2 Materials and methods

Post mortem, intact caeca were transferred under anaerobic conditions into 50 mL polypropylene tubes (previously described in chapter 7). Whilst in the tube, 10 mL of sterile 50 mM potassium phosphate buffer (pH 7.4) containing 0.3% (w/v). GSLs was gently added using a pipette through the caecal wall through a small incision made with a sterile scalpel blade. After gentle mixing by pipetting, 2 mL was removed immediately for GSL baseline quantification analyses. Caeca were incubated in airtight anaerobic chambers containing oxygen absorbing and CO_2–generating sachets (AnaeroPack™ System, Mitsubishi Gas Chemical Co. Ltd, Ngaio Diagnostics, Nelson, New Zealand) for 24 h at 37°C. Supernatants were removed for short chain fatty acid (SCFA) analysis and for LC-QTOF-HRMS/ GC-MS analysis. Mean weights of caeca are given in Figure 8.1. While there was variation within the groups, there were no significant differences between the groups (p=0.3).

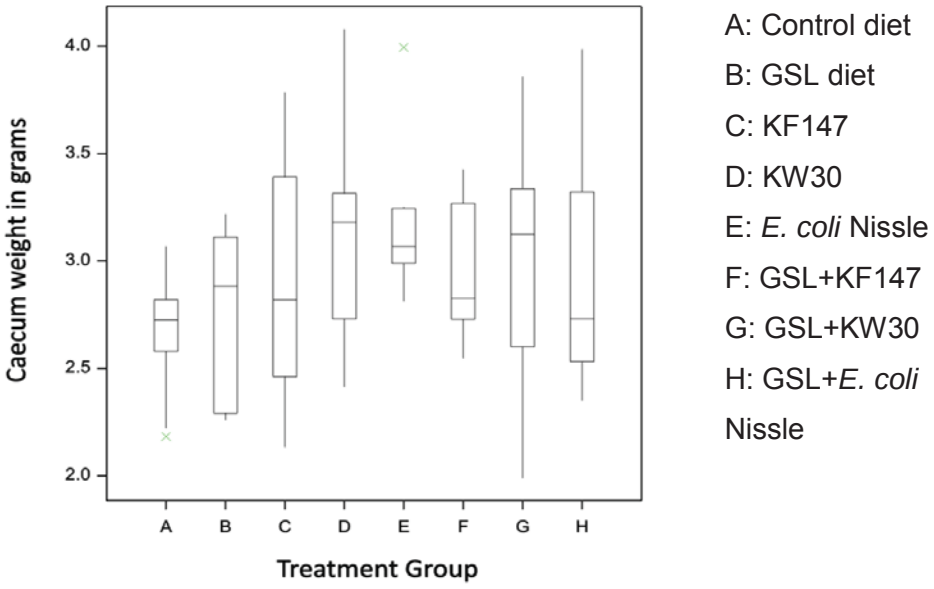


Figure 8.1. Mean weights of caeca from all treatment groups (n=10, p=0.3). Outliers are shown as a green X (group A and group E)

8.3.5 LC-QTOFHRMS

Caecal samples were prepared for analysis by centrifugation to precipitate solids, transferring 100 µl of the supernatant to a vial containing 900 µl of water that had added to it the internal standard epicatechin at a concentration of 5 µg/mL.

8.3.6 GC-MS

Caecal samples were prepared for analysis by mixing the sample by vortexing for 10 seconds, then transferring one volume of sample to a tube containing 2 volumes DCM (Sigma-Aldrich 650463). The tube was tightly capped, shaken and mixed by inversion for 15 min at room temperature. The samples were centrifuged for 5 min at 2000 x *g* to separate the phases and the organic phase (bottom layer) carefully transferred to a vial using a glass Pasteur pipette. Analysis was performed in the same manner as for the GSLs described in chapter 3. Internal standards were used to normalise the peaks and the ratio of this given where appropriate.

8.3.7 Analysis of Organic Acids by GC

Eleven acids, acetate, butyrate, formate, heptanoate, hexanoate, isobutyrate, isovalerate, lactate, propionate, succinate and valerate were analysed. The concentrations of organic acids were quantified using GC [1]. Samples were prepared as follows: Caecum digesta were homogenized in 0.01 M phosphate buffered saline with 2-ethylbutyric acid (5 mM) as an internal standard. The sample was then centrifuged at 3000 g for 5 min (4°C). The supernatant was acidified with concentrated hydrochloric acid and diethyl ether added, and then following vortexing, was centrifuged at 10,000 g for 5 min (4°C). The upper diethyl ether phase was collected and derivatised with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1 % tert-butyldimethylchlorosilane (MTBSTFA + TBDMSCI, 99:1; Sigma-Aldrich) by heating to 80 °C for 20 min. To allow complete derivatisation the samples were left for 48 h at room temperature before analysis. Standards containing 2-ethylbutyric acid (5 mM) as an internal standard were prepared alongside the samples. Analysis was performed on a Shimadzu gas chromatograph system (GC-17A, Kyoto, Japan) equipped with a flame ionization detector and fitted with a HP-1 column (10 m × 0.53 mm ID × 2.65 µm) (Agilent Technologies,

Santa Clara, CA, USA). The carrier gas was helium with a total flow rate of 37 mL/min and pressure of 7 kPa. The temperature program began at 70°C, increasing to 80°C at 10°C/min, with a final increase to 255°C at 20°C/min, holding for 5 min. The pressure program was set to 7 kPa, increasing to 15 kPa at 0.8 kPa /min, holding for 4 min. Injector and detector temperatures were set at 260°C. Samples were injected (1 µL) with a splitless injection. The instrument was controlled and chromatograms acquired using GCsolution software (Shimadzu). The acquired GC data was used to plot standard curves, providing a sample result of µmol SCFA/mL caecum or colon digesta.

8.3 Results: The metabolism of glucosinolates by caecal bacteria

8.3.1 Glucosinolate consumption (transformation)

GSLs added to the rat cecum *ex vivo*, were interconverted as well as transformed. Alkylsulfinyl GSLs glucoraphanin and glucoiberin were consumed almost entirely by the BSP fed group (B) and 93.3% consumed by caecal bacteria from the control diet group (A). The next most efficient consumer of GSLs was group C, which were given KF147 in their diets. The other five groups consumed around 60-70% of the GSLs but the reduced species increased or remained at the starting concentration suggesting that redox shifting from alkylsulfinyl to alkylthiol was occurring (Figure 8.2).

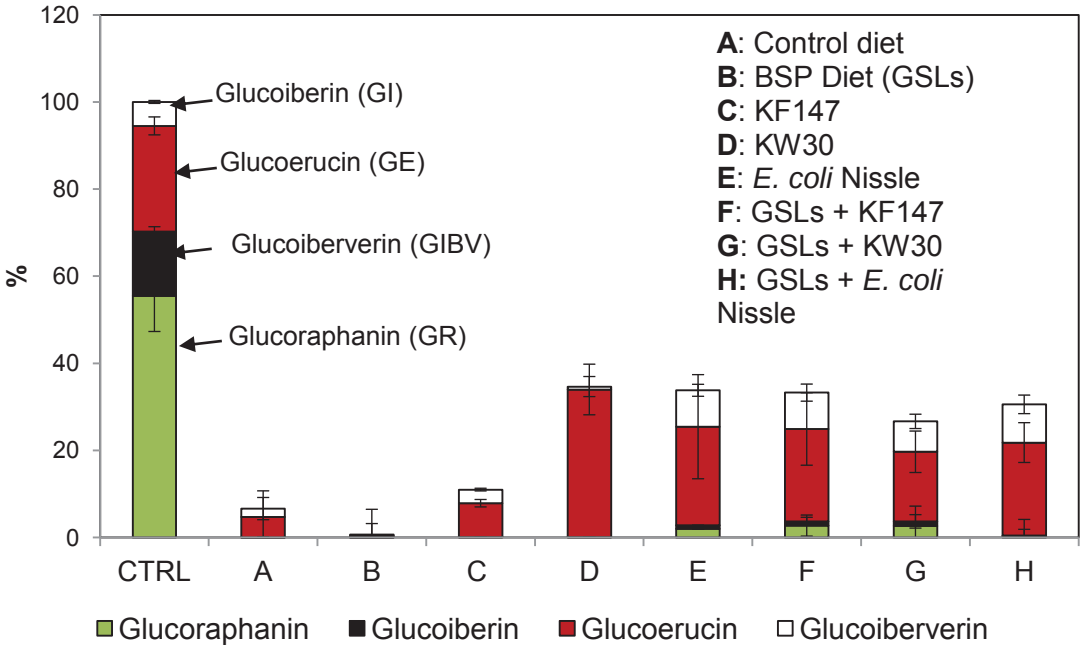


Figure 8.2. Caecal bacteria consumed almost all of the glucoraphanin (GR) and glucoiberin (GI). Glucoerucin (GE) and glucoiberverin (GIBV) was unchanged for E-H and increased for D. Endogenous microbiota (A and B) consumed the most glucosinolates, GSL group B consumed more than A perhaps due to a GSL adaptive response. (N=10) Error bars = SEM

8.3.8 Glucosinolate hydrolysis (metabolism)

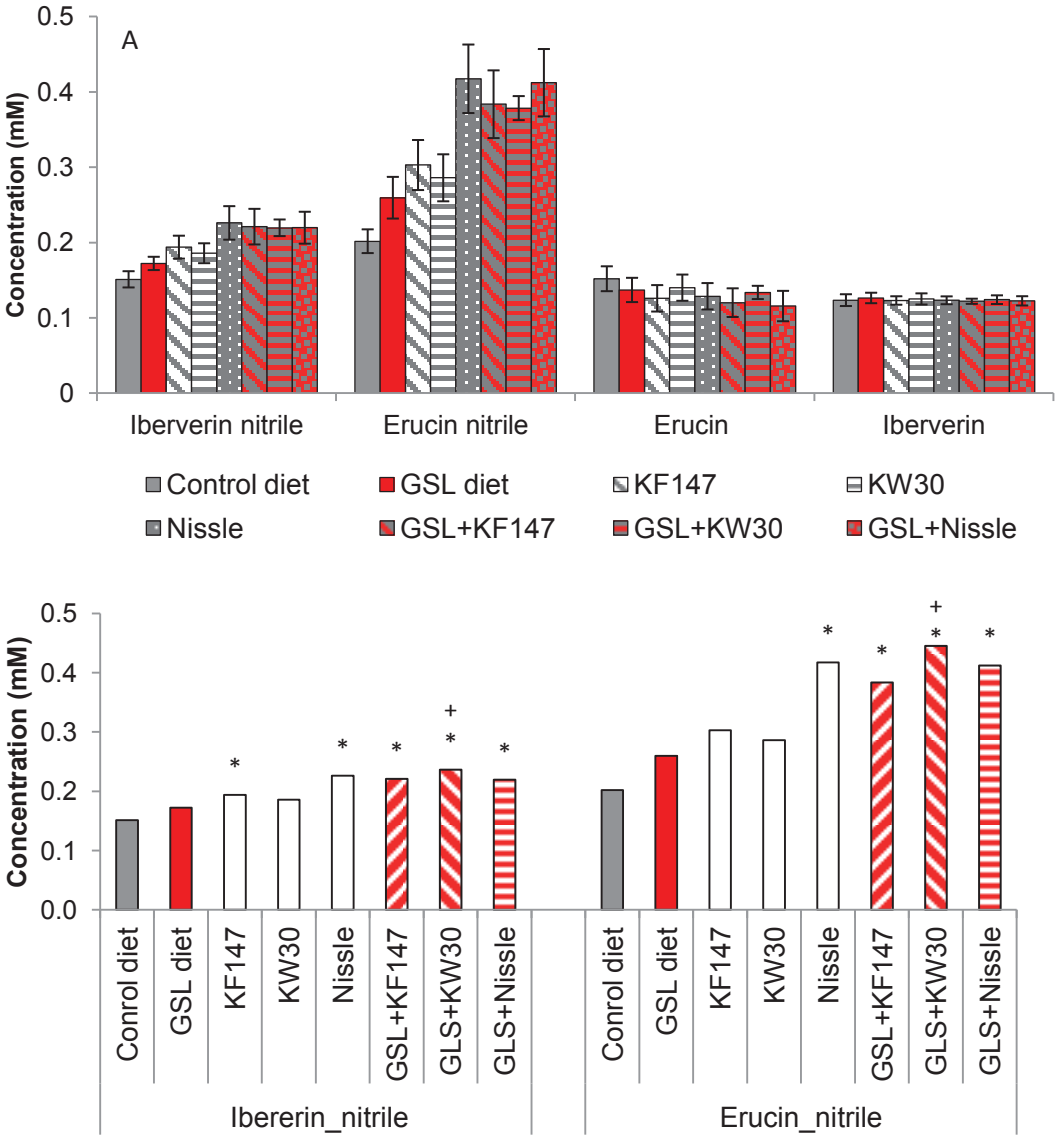
The products produced from the incubation of GSLs for 24 hours with caeca were iberverin nitrile, erucin nitrile, erucin and iberverin as shown in Figure 8.3, with groups pre-exposed to GSLs, coloured red. GSLs (0.3% (w/v)) incubated in buffer without caeca for the same time did not accumulate detectable degradation products.

The reduced species of isothiocyanate (ITC) and nitriles were the only products detected and these were iberverin nitrile, erucin nitrile, iberverin and erucin, (Figure 8.3). While the ITCs produced were in similar concentrations across groups, ANOVA (see Table 8.1) revealed differences in the nitrile concentrations between groups (iberverin nitrile $p<0.001$ and erucin nitrile $p=0.006$ respectively). As Figure 8.3 shows (except for *E. coli* Nissle which did not demonstrate a GSL-adaptive effect), groups that had previous exposure to GSLs (GSL-adapted), produced more iberverin nitrile and erucin nitrile than the GSL-naive groups. Groups that had previous exposure to beneficial bacteria also produced more iberverin nitrile and erucin nitrile than the control diet group.

Table 8.1. Analysis of Variance (ANOVA) - *p* value for groups

	Iberverin nitrile	Erucin nitrile	Erucin	Iberverin
Treatment Group	<.001	0.004	0.184	0.316

Significant (p<0-05) differences are highlighted



* Indicates higher than the control
\+ indicates higher than the corresponding treatment without glucosinolate

Figure 8.3. (A): Iberverin nitrile, erucin nitrile erucin and iberverin were the only products identified from GSL metabolism by all treatment groups. GSLs in buffer produced no degradation products after 24 h. (B): Iberverin nitrile and erucin nitrile with the significant differences between the eight treatment groups shown (N=10) Error bars = SEM

Because there were differences between groups suggesting that changes in bacterial metabolic activity were occurring or had occurred, the organic acid profiles were sought. Short chain fatty acids (SCFAs) are produced by gut bacteria during the fermentation of dietary fibre, and known to be important in maintaining colonic integrity and metabolism[2-4]. The SCFA profile from the colon or caecal contents of an individual, allows identification of the groups or classes of bacteria present as well as which of these are the most metabolically active. SCFA analysis enables identification of the bacteria present by the by-products that are generated (the three main ones are acetate, butyrate, and propionate) and gives an estimation of the metabolic rates. Epidemiological data suggests that there is a link between which SCFAs predominate (acetate or butyrate) and reduced cancer risk[5].

8.3.9 Organic acid profiles

The short chain fatty acids (SCFAs) were profiled and the results given in Table 8.2[*]. The least significant differences (l.s.d.) are also displayed.

Table 8.2. SCFA mean values shown in µmol SCFA/g digesta. In general, formate, lactate and succinate values were increased while isobutyrate, isovalerate and valerate values were reduced in the groups receiving GSLs and beneficial bacteria Grey shaded areas are low while red are high

Treatment Group	Acetate	Butyrate	Formate	Heptanoate	Hexanoate	Isobutyrate	Isovalerate	Lactate	Propionate	Succinate	Valerate
Control diet	130.26	52.89	0.08	0.00	0.18	7.83	11.25	1.62	67.20	1.06	7.53
GSLs	141.42	54.92	0.05	0.03	0.46	6.84	10.26	1.63	77.64	1.07	8.20
KF147	142.79	60.41	0.18	0.06	0.35	8.91	12.76	0.96	76.35	0.88	9.40
KW30	152.93	66.37	0.06	0.08	0.48	9.38	14.00	1.41	84.57	0.60	10.94
E. coli Nissle	160.40	67.64	0.41	0.00	0.05	4.92	7.16	10.33	92.13	5.64	4.42
GSL+KF147	148.46	74.61	0.49	0.00	0.09	3.45	5.37	25.06	73.40	2.59	6.18
GSL+KW30	146.93	56.52	1.24	0.00	0.13	3.03	4.49	29.92	69.49	9.55	2.99
GSL+*E. coli* Nissle	143.32	65.96	0.91	0.00	0.06	2.44	4.41	25.88	68.55	4.27	4.11
l.s.d.	112%	120%	405%	297%	262%	131%	128%	193%	115%	230%	129%

[*] Author acknowledges Halina Stoklosinksi (Plant & Food Research, Palmerston North) who processed GC samples then from the raw data generated, calculated the concentrations from standard curve data and provided the author with the results

8.3.10 Analysis of variance (ANOVA)

Statistical analyses were conducted using analysis of variance (ANOVA) and results were expressed as means of each group, each comprising ten biological replicate determinations ± standard deviation (SD). Genstat software (version 14.2) (VSN International Ltd, Hemel Hempstead, UK) was used for discriminant analyses and histograms[†].

The unit of analysis was the animal. Results are displayed as the mean of each group which compromised ten animals +/- standard deviation. Group means were compared using analysis of variance (ANOVA). Residuals were inspected to ensure distributional assumptions were met. For lactate, succinate and formate log-transforming stabilized the variance for ANOVA. Data for heptanoate are shown but were not analysed because of the large number of zeros.

Table 8.3. Analysis of Variance (ANOVA) - P value for treatment Group

	Acetate	Butyrate	Formate	Heptanoate	Hexanoate	Isobutyrate	Isovalerate	Lactate	Propionate	Succinate	Valerate
Treatment Group	0.048	0.003	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
Number of zeros	0	0	21	53	3	0	0	0	0	0	0

Significant (p<0-05) differences are highlighted

8.3.11 Discriminant Analysis

Discriminant analyses were performed on the data to see whether there were interactions between GSLs and the beneficial bacteria, whether an adaptive response to either was occurring and also whether the metabolic changes were reflected in the SCFA profiles of the groups (GSL-naïve versus GSL-adapted). It was hoped that just as the measurement of SCFAs can enable identification of the metabolic activity due to microbiota, changes in diet may also be reflected by changes of SCFA profiles from one fatty acid to another.

[†] Author acknowledges Duncan Hedderley (Plant & Food Research, Palmerston North) for assistance with statistical methods and interpretation of data

Discriminant analyses (DA) of the organic acid (SCFAs) and metabolite (nitriles and ITCs) data were applied separately and then combined to see if there were correlations between the two and the treatment groups[‡].

The SCFA data was log-transformed for the discriminant analyses. The organic acid discriminant dimension correlated most strongly with the first dimension.

Dimension 1 separated treatment Groups control diet (A), GSL diet (B), KF147 (C) and KW30 (D) from the rest *E. coli* Nissle (E), GSL + KF147 (F), GSL + KW30 (G) and GSL + *E. coli* Nissle (H). Groups A - D were higher in the acids valerate, hexanoate, isovalerate and isobutyrate, whereas Groups E - H were higher in the acids lactate, formate and succinate (Figure 8.4).

Dimension 2 separated treatment control diet (A) from KW30 (D). Group D was higher than group A in acids acetate, butyrate and propionate.

Dimension 3 separated *E. coli* Nissle (E) from Groups GSLs + KF147 (F), GSLs + KW30 (G) and GSLs + *E. coli* Nissle (H). Group E was higher in acetate and propionate, compared to groups F, G and H which were higher in the acids formate and lactate (Figure 8.5).

There was clear separation between the groups' organic acid profiles and all groups were significantly different from each other with acetate, p=0.048, butyrate, p=0.003 and for all other acids p<0.001 (see Table 8.1).

The discriminant analyses highlighted differences in the groups although they did cluster together. Dimension 1 separated groups A, B, C and D from E, F, G and H. Furthermore, for dimension 2 and 3, the control diet group (A) separated further from GSLs (B), KF147 (C), and KW30 (D), while *E. coli* Nissle (E) separated from the GSLs groups F, G and H.

Less apparent was separation between groups for the GSL hydrolysis products however, some trends were observed (Table 8.3). Nitriles iberverin nitrile and erucin nitrile differed significantly in all treatment groups (p<0.001 and p=0.004 respectively).

However, the discriminant analyses produced a clear separation of groups A, B, C and D from E, F, G and H and this was observed to a greater

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[‡] Author acknowledges the contribution of Halina Stoklosinski and Duncan Hedderley (Plant & Food Research) who provided discriminant analyses of the data

degree on the discriminant analysis that combined organic acid with GSL hydrolysis products data.

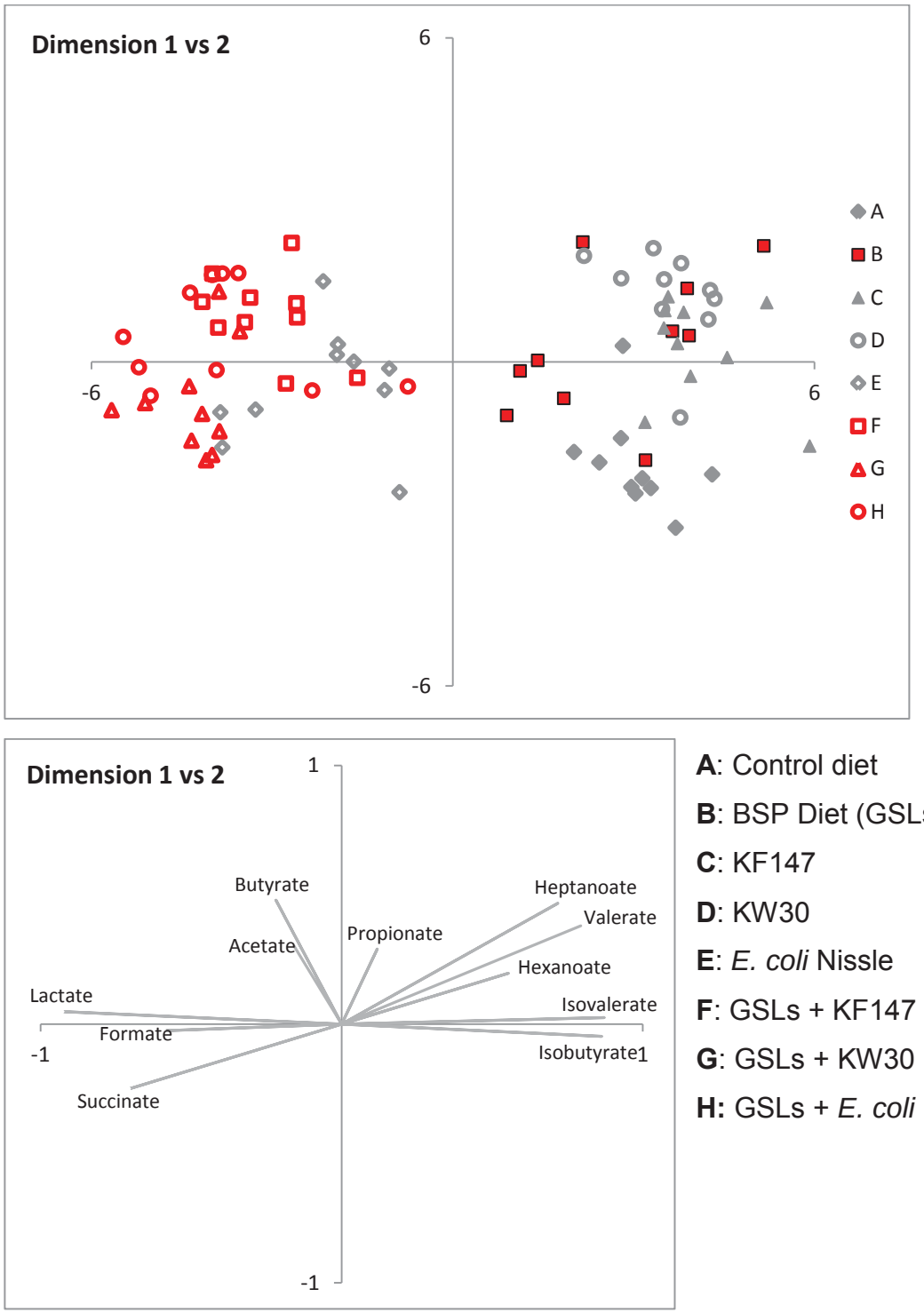
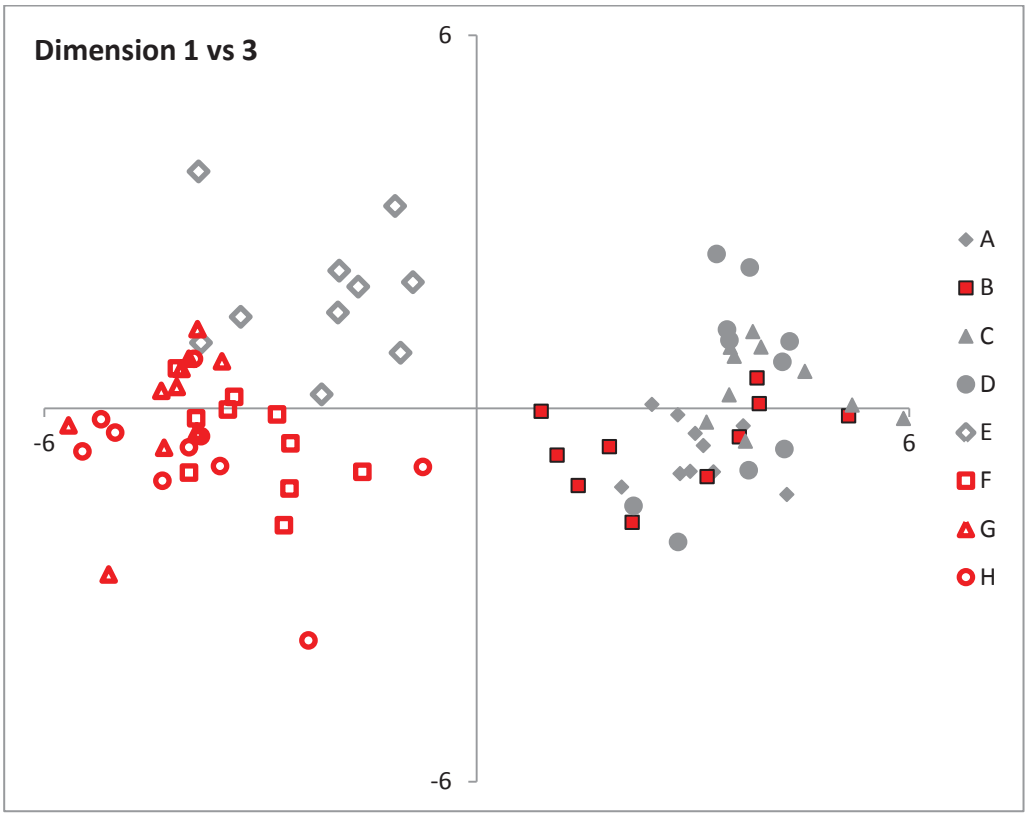
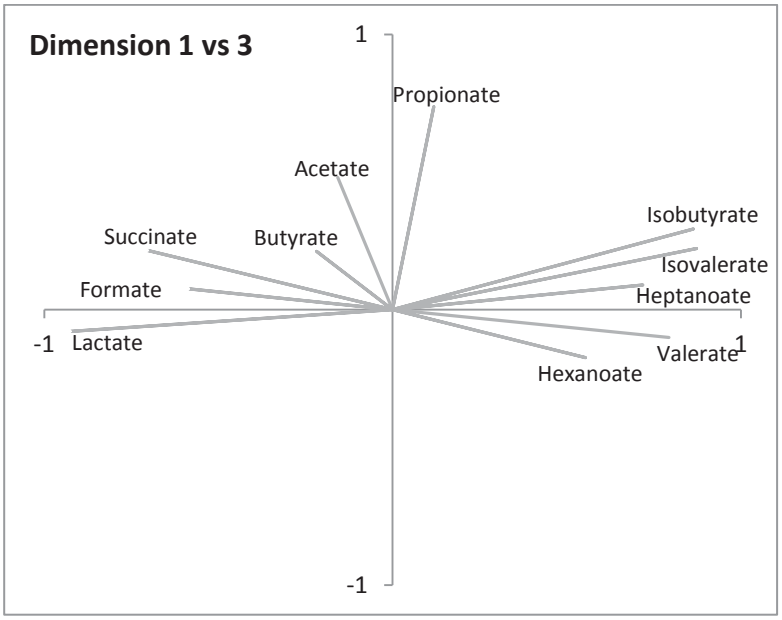


Figure 8.4. SCFAs discriminant analysis of dimension 1 and 2 separates groups A-D from groups E-F The top box shows the predefined treatment groups in an attempt to find the best low dimensional representation of the differences between these groups. The vector figure indicates the biggest single difference in the 1st dimension. Formate, lactate and succinate are high opposed to heptanoate, valerate, hexanoate, isovalerate and isobutyrate (N=10)





A: Control diet

B: BSP Diet (GSLs)

C: KF147

D: KW30

E: *E. coli* Nissle

F: GSLs + KF147

G: GSLs + KW30

H: GSLs + *E. coli* Nissle

Figure 8.5. Discriminant analysis of dimensions 1 versus 3 separates group E from groups F – H. Here the vector figure shows that the main drivers differentiating *E. coli E. coli* Nissle naive versus adapted were propionate and acetate (N=10)

8.3.12 Discriminant analysis of glucosinolate hydrolysis products

Like the organic acids, the metabolite discriminant dimension correlated most strongly with the first dimension. The metabolites DA had two significant dimensions:

Dimension 1 separated Treatment Groups Control diet (A), GSLs diet (B), KF147 (C) and KW30 (D) from *E. coli* Nissle (E), GSLs + KF147 (F), GSLs + KW30 (G) and GSLs + *E. coli* Nissle (H). Groups Groups Control diet (A), GSLs diet (B), KF147 (C) and KW30 (D) were higher in erucin and iberverin, whereas Groups *E. coli* Nissle (E), GSLs + KF147 (F), GSLs + KW30 (G) and GSLs + *E. coli* Nissle (H) were higher in erucin nitrile and iberverin nitrile.

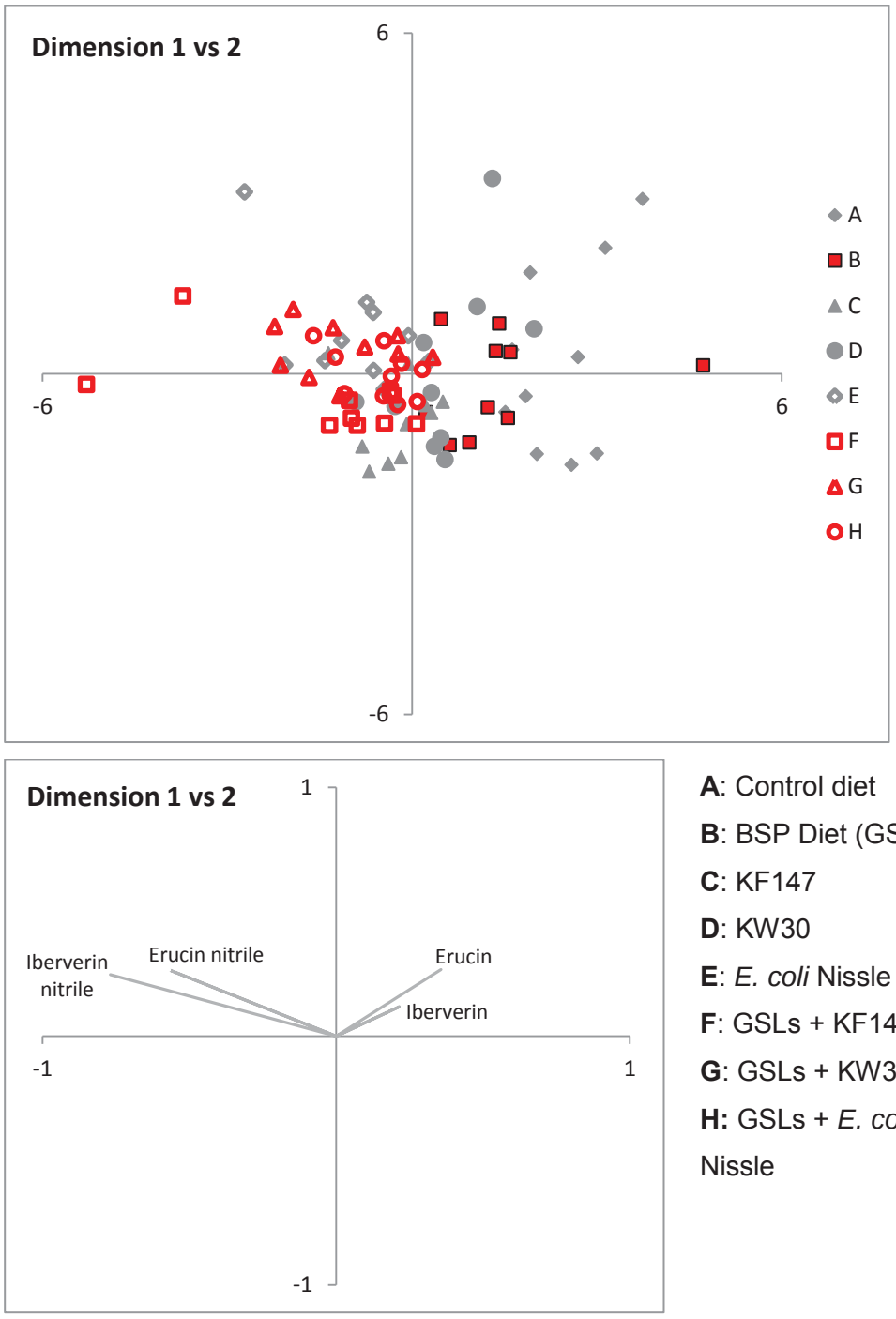


Figure 8.6. The correlations of the GSL hydrolysis products concentrations with two-dimensional discriminant analysis from 8 treatment groups. IT was not possible to find separation based on these measures (N=10)

8.3.13 Discriminant analysis combining organic acids with the glucosinolate hydrolysis products

The discriminant analysis with organic acids and metabolites combined had three significant dimensions (Figure 8.7 and Figure 8.8). While similar to the organic acid analysis, it further separated the groups Control diet (A), GSLs diet (B), KF147 (C) and KW30 (D) from Groups *E. coli* Nissle (E), GSLs + KF147 (F), GSLs + KW30 (G) and GSLs + *E. coli* Nissle (H).

Dimension 1 separated treatment groups Control diet (A), GSLs diet (B), KF147 (C) and KW30 (D) from groups *E. coli* Nissle (E), GSLs + KF147 (F), GSLs + KW30 (G) and GSLs + *E. coli* Nissle (H). Groups A - D were higher in acids isovalerate, isobutyrate, hexanoate and the metabolites iberverin and erucin. Groups E - H were higher in acids formate, succinate, lactate and nitrile metabolites erucin nitrile and iberverin nitrile.

Dimension 2 separated the control diet group (A) from KF147 (C) and KW30 (D), with C and D higher in propionate acetate and butyrate and also heptanoate, valerate, isovalerate and isobutyrate. Group A was lower in these acids but higher in erucin than Groups C and D

Dimension 3 separated *E. coli* Nissle (E) from GSLs + KF147 (F) and GSLs + *E. coli* Nissle (H). Group E was higher in acids acetate and succinate, formate, erucin nitrile and iberverin nitrile, while F and H were higher in acids butyrate and lactate.

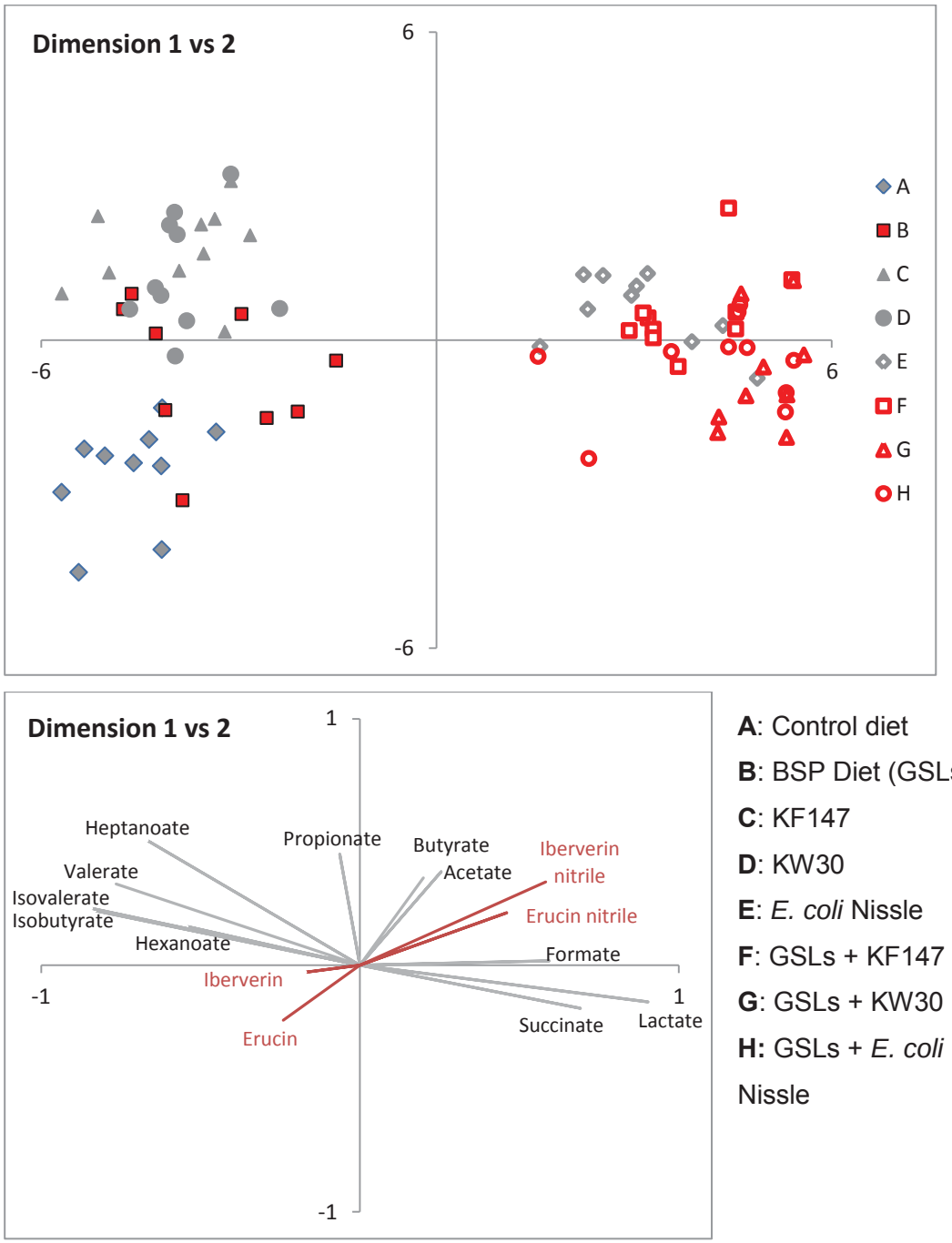


Figure 8.7. Combining both treatments of SCFAs and glucosinolates: Dimension 1 separated groups A – D from groups E – H while dimension 2 separated the group A from C and D. The nitriles show a trend of being greater for GSL adapted

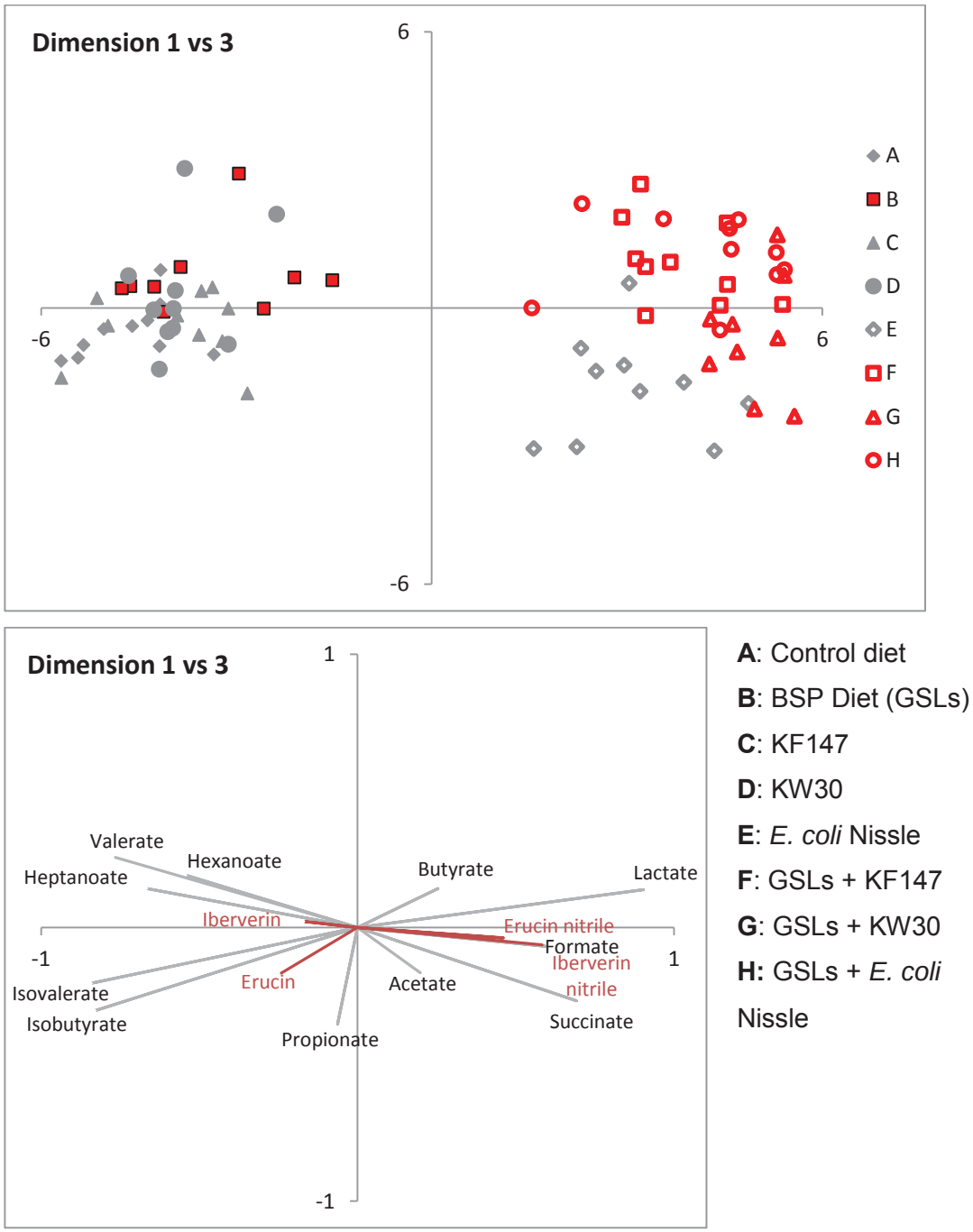


Figure 8.8. Discriminant analysis of dimensions 1 versus 3 separated group E (higher in acetate, succinate, formate, erucin nitrile and iberverin nitrile) from groups F and H which were higher in butyrate and lactate. Again there is a trend of nitriles being greater for GSL adapted but only a weak association with GSL naive and ITCs

8.4 Discussion

The passage of phytochemicals through the gut is important for health. Separating out the microbiota contribution from host metabolism is difficult *in vivo* as many metabolites are absorbed rapidly. This experiment aimed to separate the microbiota from the host metabolism of GSLs because while the GSLs and hydrolysis products were the same between *in vitro* and *in vivo*, it was apparent that the metabolites found in urine and blood were proportionally in different states of redox than expected based on the results obtained in chapter 5. These results showed that the microbiota produced the same products as the *in vitro* experiments with KF147, KW30, *E. coli* Nissle and *E. cloacae* in chapter 5 and do not oxidise the hydrolysis products. This the first time it has been shown that the microbiota of rats reduce alkylsulfinyl GSLs to thiols and supports the hypothesis of bacterial reduction followed by host oxidation of the GSL hydrolysis products These results also support the *in vitro* findings that confirmed for the first time that Enterobacteriaceae reduce alkylsulfinyl GSLs (chapters 5 and 6).

The implications for this are that consumption of glucoraphanin does not mean sulforaphane is going to be produced at equivalent concentrations when the host microbiota are the first point of contact. It suggests that erucin and erucin nitrile are going to be the major products and also that subsequent oxidation by the host could transform these to sulforaphane and sulforaphane nitrile. Where and when this happens and how long the products circulate will determine the host phase II response.

It offers some explanation for the observed differences in bioactivity studies to date since most of these studies used cultured cells.

It explains why several *in vivo* studies that have measured the GSLs going in and what comes out via urine and blood have found this phenomenon, the “glucoerucin effect”. Where a mixture of GSLs has been used from broccoli, it is not so obvious but when pure glucoraphanin has been introduced to microbiota, the glucoerucin effect stands out[6].

pH may be a factor in nitrile formation but microbiota most certainly cause the glucoerucin effect while host oxidation reverses its hydrolysis

products erucin and erucin nitrile back to sulforaphane and sulforaphane nitrile, or oxidising again irreversibly to erysolin species.

8.4.1 There was an adaptive response to glucosinolates

Consumption of GSLs was greatest for the groups which had not been given beneficial bacteria but this did not translate into more hydrolysis products which were greatest in the groups that had previously been exposed to GSLs (Figure 8.2). *E. coli* Nissle was the exception showing no GSL-adaptive effect. *In vitro, E. coli* Nissle behaved similarly (chapter 5). The disappearance of alkylsulfinyl GSLs to a greater degree than alkylthiol GSLs also supports those results and also in chapter 6 when seleno-GSLs were used *in vitro*.

The only GSL hydrolysis products detected were the reduced species, iberverin nitrile, erucin nitrile, iberverin and erucin (Figure 8.3) supporting previous results from *in vitro* experiments in chapter 4 and chapter 6.

From these results it is apparent endogenous host microbiotas consume more GSLs than host microbiotas that have been 'manipulated' or perturbed by feeding with beneficial bacteria but this does not produce the equivalent hydrolysis products (Figure 8.2 and Figure 8.3).

Also it was confirmed that while Figure 8.2 and Figure 8.3 show there was no differences between GSL consumption or metabolism for GSL-naïve or adapted *E. coli* Nissle, discriminant analysis separated *E. coli* Nissle's GSL-naive group (E) from its GSL-adapted group (H) (see Figure 8.8).

8.4.2 Short Chain Fatty Acids and bacterial fermentation

Short chain fatty acids (SCFAs) are produced in the colon by bacteria during their fermentation of carbohydrates. SCFAs have a physiological effect and are important anions in the colonic lumen serving as nutrients for the mucosa cells, stimulating mucosal proliferation and blood flow. Three main SCFAs. butyrate, acetate and propionate are often compared as biomarkers of health. Butyric acid is a major carbon source for colonocytes and butyrate has been shown to inhibit colon cancer cells in culture[7] and more recently has been implicated in blood pressure regulation[8].

SCFA production decreases the luminal pH, which in turn may stimulate mineral absorption and reduce secondary bile acid formation in the colon.

Dietary changes have an impact on the diversity and density of microbiota and the proportions of the major three, acetate, propionate and butyrate are altered by diet[9]. It is more recently however that studies (human and mice) have begun to reveal the effects that a gut microbiota has on host metabolism in improving the energy yield from food and also through modulating dietary or the host-derived compounds that alter host metabolic pathways[10]. Shown recently was that butyrate, propionate, and acetate all protected against diet-induced obesity and insulin resistance while butyrate and propionate, but not acetate, induced gut hormones and reduced food intake[11].

The SCFA analyses separated the groups into two clusters. The endogenous microbiota groups GSL-adapted and GSL-naive clustered together, the lactic acid and endogenous microbiota groups clustered together and the GSL+beneficial bacteria and *E. coli* Nissle clustered together in their SCFA profiles. From the table of SCFA values (Table 8.2), the differences were marked for some metabolites such as the branched fatty acids isobutyrate and isovalerate. These are synthesised from branched chain amino acids rather than carbohydrate degradation and may be an indicator of the bacteria present[12] or reflecting changes in metabolism and population shifts within the microbiota. For example, lactate the 3 carbon intermediary of carbohydrate metabolism is produced from the reduction of pyruvate which under physiological conditions exists in its conjugate base form lactate. Lactate utilising bacteria are able to use lactate and acetate producing from these, butyrate via the butyryl CoA : acetate CoA transferase route[13].

The groups fed lactic acid bacteria that were GSL-adapted had lactate concentrations of twenty-five times those of the GSL naive groups (with or without beneficial bacteria) (Table 8.2). This on its own is not unusual as lactic acid bacteria produce lactic acid which could account for lactate. *E. coli* Nissle is also a member of *E. coli* and *E. coli* produce lactate[14]. However one publication called the Townsend Letter, a journal subtitled “Practical Use of Probiotics and Prebiotics[15]” states “Non-lactic-acid producing probiotics include a specific healthful strain of Escherichia coli called *E. coli* Nissle 1917” but offers no citation to support this statement. Results from this study do not necessarily prove otherwise but they do show that *E. coli* Nissle is associated with increased lactate.

What was unexpected about the lactate concentrations was that it was not related to the presence of lactic acid bacteria because it was only raised for the GSL-adapted lactic acid bacteria groups. This suggests that GSL-adaptation may have resulted in either the inhibition of lactate utilising bacteria or it may have promoted growth of or the metabolic activities of lactate producing bacteria. *E. coli* Nissle it should be noted, had relatively high lactate concentrations without GSLs at six-fold concentration to the other non-GSL diet groups but the lactate concentration for GSL-adapted was still double that of *E. coli* Nissle GSL-naive.

This serves again to demonstrate how complex the interactions are. While beneficial bacteria did appear to change the metabolic activity and the GSL degradative ability of caecal microbiota, the non-manipulated endogenous group performed well, consuming and transforming GSLs as did all the groups. Hydrolysis product erucin nitrile concentrations ranged from 0.2 – 0.4 mM; endogenous microflora managing the lower value while GSL-adapted *E. coli* Nissle, the highest, yet for ITCs, all were within range of one another (Figure 8.3). While the differences may have been statistically significant, it is doubtful whether they were large enough to be truly significant. This study also demonstrates the value that *in vitro*, *in vivo* and *ex vivo* experiments have in separating complex systems and underlines the importance of this approach in research relating to biotransformation of phytochemicals.

Furthermore these results suggest that bacteria tolerate and reduce to thiol the alkylsulfinyl GSLss and their hydrolysis products whereas the host tolerates either form of GSL but oxidises alkylthiol hydrolysis products to alkylsulfinyl products. The reason for the host response is most likely related to hydrophobicity and clearance whereas the reason for bacterial reduction is most likely because alkylsulfinyl species are targeted by bacterial sulfoxide reductases which are detoxification mechanisms in bacteria that deal with oxidative stress. They apparently recognise the sulfoxide group and don't differentiate between GSLs and the ITCs and nitriles.

How all this bacterial interference combined with host intervention during the metabolism of GSLs relates to health may be simpler than it seems. If the products are all bioactive but we are only seeing part of the story or the end of the line products, we might think that in the absence of plant active myrosinase,

the products produced were ‘inconsequential’, poor phase II inducers or or only weakly effective in promoting health benefits.

Indeed, the activation of broccoli by the addition of myrosinase prior to consuming can produce ITCs in millimolar proportions, sulforaphane a major component if glucoraphanin was the major GSL.

But if the body’s microbiota reduces the sulforaphane to a tolerated erucin, how will this affect the bioactivity? If the body then shifts erucin back to sulforaphane is the effect going to be at the tissue level for example, the gut or will it be occurring in the liver and be circulating in the blood?

If nitriles are formed through microbiotal metabolism of GSLs, is there a concentration that assures bioactivity and is sulforaphane nitrile really not as effective as sulforaphane if it’s at twenty times the concentration, remains stable, circulates longer and becomes even more concentrated over time in the bladder?

This is a point worth considering because bladder cancers are known to be one of the cancers that are reduced by dietary GSLs which presumes that the bioactive components are exposed to the bladder probably in concentrated form.

8.5 Summary

This experiment was designed to study the products of caecal metabolism of GSLs and whether GSL-adaptation occurs. Results indicate that the fate of the alkylsulfinyl GSLs by the host microbiota is consumption followed by transformation into reduced species, erucin and iberverin. Caeca from groups fed KW30 or KF147 displayed adaptive responses to GSLs but *E. coli* Nissle did not. Also, further evidence of GSL-adaptation was found as prior exposure to dietary GSLs primed the host microbiota making them more efficient at GSL hydrolysis but they were not necessarily better at GSL consumption. However, consumption is not the same as production of hydrolysis products. Those that consumed the most without the corresponding transformation to GSL hydrolysis products probably degraded the hydrolysis products to other compounds that could not be identified.

The beneficial bacteria that were fed to the groups described in chapter 7 caused an increase in the QR activity from bladder tissue. The caecal

microbiota of these groups also had different SCFA profiles from the groups with endogenous caecal microbiota. Similarly, the groups that consumed beneficial bacteria produced more GSL hydrolysis products than those that did not consume beneficial bacteria with their diet. This may have translated into a health benefit in that an increase in activity for the phase II enzyme QR for all groups that received beneficial bacteria as well as those that received GSLs represents a host response that confers increased protection for the bladder against toxins.

Metabolically, the microbiota from the beneficial bacteria fed groups had different SCFA profiles from endogenous, indicating population shifts and metabolic shifts and all (KF147, KW30 and *E. coli* Nissle) elevated QR in the bladder even without the addition of GSLs. The groups that had consumed beneficial bacteria in combination with GSLs had a microbiota adapted to GSLs and although the combination of GSLs and beneficial bacteria did not translate into additive phase II induction, there appeared to be synergy and neither interfered with one another's phase II induction potential. As GSL metabolisers, the adaptive groups outperformed the GSL-naive but both performed well, with the control diet group containing the endogenous microbiota, producing the lowest concentrations of erucin nitrile at 0.2 mM, while the best performers were the GSL-adapted groups KF147 + GSLs, KW30 + GSLs, *E. coli* Nissle + GSLs and *E. coli* Nissle (no added GSLs) which all produced ~ 0.4 mM of erucin nitrile (Figure 8.3).

8.6 References

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NINE

9.0 Putting it all together

The first aim of this thesis was to identify efficient glucosinolate (GSL) transformers from beneficial bacteria

Although previous studies are published on lactobacilli and bifidobacteria, to this author's knowledge nothing is published regarding the efficiency of GSL transformation by *E. coli* Nissle.

Nor has a study of the combination of beneficial bacteria with GSLs spanning *in vitro, in vivo* and *ex vivo* been published.

While there were several bacteria that could have formed the basis for the bacterial work in this thesis, based on the preliminary experiments in chapter 4, the beneficial bacteria that were identified and selected were *Lactococcus lactis* KF147, *Lactobacillus plantarum* KW30 and *Escherichia coli* Nissle.

9.1 Beneficial bacteria adapt to glucosinolates

It was apparent after both *in vitro and ex vivo* experiments, all showed a GSL adaptive response. KW30 was a more efficient GSL transformer after pre-exposure *in vitro* while *ex-vivo*, the metabolic activity of KF147, *E. coli* Nissle and the microbiota was also affected by pre-exposure to GSLs suggesting adaptation to GSLs. This was evident from the short chain fatty acid (SCFA) profiles obtained from GC analysis in chapter 8. While Nissle did not show an increase in GSL transformation efficiency after adaptation, its metabolic SCFA profile was altered as reflected in the discriminant analyses.

9.1.1 How and why do these bacteria metabolise glucosinolates?

The next question this thesis aimed to answer was how these bacteria metabolise GSLs. KW30 and KF147 were selected from a number of candidate lactic acid bacteria after identification of putative glycoside hydrolase genes found within an operon which also contained a number of carbohydrate phosphotransferase system (PTS) genes. Because of the glycoside sugar attachment, it was hypothesised that a sugar transport system for active transport of GSLs into cells may be involved but, of the transporter systems

tested, none were involved. GSLs were not a carbon source either because in sugar-free minimal media with GSLs (0.3% (w/v)), bacterial growth stalled. When MRS media was used, the lactic acid bacteria consumed the same amount of GSLs whether the MRS media had sugar added or not. In contrast, the known myrosinase-producing bacterium *Enterobacter cloacae* cultivated in RCM media without added sugar, consumed less glucoraphanin and glucoiberin than when any of the sugars tested was added. It did not produce more glucoerucin or glucoiberverin however, so this reduction reaction apparently occurs whether GSLs are metabolised or not.

It is possible that GSLs do not require active transport into cells but may simply passively diffuse through. Inside the cells they become reduced but are still a GSL, soluble and able to diffuse out again. Why the lactic acid bacteria do not reduce the remaining GSLs could not be solely due to pH because when we incubated the GSLs in media with a pH of less than 3 without bacteria (acid hydrolysis), we got all the expected products, both oxidised and reduced that matched the species of GSLs. The bacteria also most likely transported the reduced hydrolysis products out of the cell again because the nitriles and ITCs were detected in the culture medium. How this is achieved is still not known but their chemistry (hydrophilic and lipophilic) suggests that they are would not diffuse out in a passive manner.

This raises the question of why these bacteria (the Enterobacteriaceae) reduce GSLs or why lactic acid bacteria do not.

Enterobacteriaceae are facultative anaerobes and anaerobic environments are reducing environments. Enterobacteriaceae possess methionine sulfoxide reductase A, (MsrA) EC 1.8.4.11 (ExPASy) and their involvement in oxidative stress responses has previously been reported[1] and is known to be present in all aerobic organisms[2]. Although genomic data for Nissle is not available in the public domain, the structure and function of the *E. coli* peptide methionine reductase is known[3], and *E. cloacae* ATCC13047 also carries a gene encoding the MsrA enzyme (NCBI ref: YP_003611138.1). Although KF147 and KW30 did not carry out this reduction reaction, sequence data identified an MsrA enzyme for *Lb. plantarum* ATCC14917 (NCBI ref: ZP_07078054.1), *Lb. plantarum* JDM1 (NCBI ref: YP_003063253.1) and an MsrB-like enzyme for KF147 (NCBI ref: YP_003352658.1). Also, the change in

GSL redox was observed only for the alkylsulfinyl GSLs glucoraphanin, glucoiberin and their nitriles.

Although sulfoxidation can be reversible, no evidence of this was seen and the amounts of the reduced products always increased over and above the oxidized species.

The evidence while not conclusive, suggests that the Enterobacteriaceae have the ability to reduce the alkylsulfinyl GSLs (and their hydrolysis products) because of their methionine sulfoxide reductase enzymes and they do so to detoxify perhaps because the sulfinyl group resembles methionine sulfoxide which has the potential to cause oxidative damage.

9.1.2 Consumption versus metabolism: not the same thing

At the beginning of this project words like degradation, metabolism and transformation were used to describe how GSLs were processed by bacteria. It became apparent that what was being described, observed and commented on was actually consumption and metabolism of GSLs. It is important to separate the two because consumption does not translate to equal products of metabolism. For example, KF147, KW30, Nissle and *E. cloacae* consumed similar amounts of GSLs but Nissle and *E. cloacae* metabolised theirs into identifiable products while only around 10% of metabolites were found for KF147 and KW30 from the results of the experiments described in chapter 5.

It was hypothesised that lactic acid bacteria further degrade the hydrolysis products into as yet unknown compounds. One possibility is that KF147 and KW30 produce nitrilases which break down nitriles to carboxylic acid and ammonia. Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase nitrilases from the Uniprot database have been identified in *Lb. reuteri, Lb. fermentum* and *Lb. buchneri* and although BLAST (NCBI) did not give specific hits for the bacteria in this thesis, they may exist and the target nitrilase should be one with a preference for aliphatic nitriles.

The missing hydrolysis products remain unidentified. Time course sampling at four-hour intervals failed to detect any. If bacterial nitrilases catalysed the removal of a nitrile group leaving behind carboxylic acid and ammonia this would account for the missing nitriles in the lactic acid bacteria spent media. It may be simpler to look for nitrilases than looking for degradation

products too small to be distinguished from background products as this indirect approach can be measured. For example, if nitrilases are present and their activity increases in the presence of GSLs we might be able to link the disappearance of nitriles with their activity.

9.1.3 Selenoglucosinolates

GSLs (including seleno-GSLs) from high selenium broccoli were found at 18% of the concentration extracted from the same cultivar without selenium fertilisation. This confirms that selenium fertilisation reduces the total GSL content significantly. Selenoglucoiberverin and selenoglucoerucin represented 0.3% and 0.8% of the GSLs assayed and it was not possible to be certain that they were consumed due to the high standard deviation values. It appeared however that seleno-GSLs were still in the media at the end of 24 hours while much of the other alkylsulfinyl GSLs were consumed and no selenium-GSL hydrolysis products were detected. Repeating the experiment with more replicates would not have helped tighten the error bars which were large. Also, seleno-GSLs are inseparable from sulfur GSLs using current anion-exchange methods because they have the same charge or affinity for columns so even concentrating 100-fold; there would still be a ratio of 99:1 of GSLs to seleno-GSLs. Bacteria can only process so much in 24 hours and there is no reason to expect that they would select more seleno-GSLs if they were available in preference to sulfur GSLs.

This highlights the consequences of trying to increase the bioactivity by manipulating the plant's nutrients. For whatever reason, selenium supplementation appears to impact on the plant's GSL biosynthesis pathways resulting in a substantial reduction (over 80%) of the desired products. It remains to be seen whether the increased bioactivity is worth the reduction in quantity.

9.2 Do the bacterial products of glucosinolate metabolism confer health benefits?

The final aim of this thesis was how hydrolysis products of GSL metabolism confer health benefits. A review of GSLs in health and disease was published recently[4] which leaves one in no doubt that the potential for benefits are there for GSLs and isothiocyanates. Allowing that bacteria are efficient GSL

metabolisers, do the bacterial products of GSL metabolism confer health benefits? Epidemiological studies point towards a relationship between the consumption of dietary GSLs and a reduction in certain types of cancer. This was known before sulforaphane became famous. Sulforaphane has been found to be produced from hydrolysis of GSLs *in vivo* but the experimental data from this thesis indicate that nitriles are the only products detected in quantity; are nitriles passing on the health benefits that would be usually attributed to sulforaphane?

Table A1 (Appendix A) summarises the known bioactivities of the 4-carbon alkyl ITCs and nitriles, sulforaphane, sulforaphene, erucin, erysolin, sulforaphane nitrile and erucin nitrile. As expected, ITCs induce glutathione S transferase (GST) almost certainly entirely due to the conjugation with glutathione (GSH). However erucin nitrile has also been shown to induce GST so GST conjugation cannot be the only way to induce gene expression of GSTs. Sulforaphane nitrile does not demonstrate induction of GST so perhaps it is erucin nitrile's thiol group in combination with the long alkane chain that adds hydrophobicity which in turn induces GSTs.

Table A2 (Appendix A) shows the comparative bioactivities of some other ITCs and nitriles, the aromatic and some with hydroxyl groups. All except cheirolin have GST inducing capacity and cheirolin is the only sulfone suggesting that the hydrophobicity, the oxidation state and any other reactive groups are involved.

When cell cycle arrest potency is considered, sulforaphene and erucin are only half as potent as sulforaphane. Sulforaphene is sulforaphane with an alkene group between the sulfinyl and the ITC end while erucin is the thiol ITC. Why these two should have similar profiles is unknown. Apoptosis via induction of the cysteine-aspartic proteases (caspases) shows sulforaphane and erysolin are effective while erucin is not. Perhaps sulfinyl and sulfonyl are more potent in apoptosis-inducing potential than thiol.

To enable cross comparisons sulforaphane was used as the reference point. Doing this, it is apparent that some ITCs or nitriles that share some identical features, either the same side chain, or the same reactive groups and oxidation state, have different degrees of bioactivity from one another.

A case in point is erysolin which is sulforaphane in its highest, stable and irreversible oxidation state, the sulfone. While erysolin has been shown to be more potent than sulforaphane in murine hepatoma cells[5], the reason for this may be due as much to the cells used as the compound itself. Erysolin has three potentially active groups, the isothiocyanate active site, the alkane chain length for hydrophobicity which must be somewhat mitigated by the highly polar sulfone.

What is apparent from these tables is that the differences in bioactivity cannot be predicted solely by the presence of an ITC group or alkyl chain length or even the oxidation state.

Also these results need to be taken in context. The data summarised in Tables A1 and A2 (Appendix A) represent studies that relied on results obtained from cell culture and as bacteria and host modify the oxidation state *in vivo*, ultimately what we see may not be the molecule that was the most bioactive.

We still do not have the answers about how nitriles might induce host responses that confer health benefits.

9.2.1 *In vitro* does not always reflect life

While it is accepted that *in vitro* needs to be taken in context because often results cannot be applied to life, it is not always possible to use real-life models. For the bioactivity studies results are different between *in vitro* and *in vivo*. One animal trial was published to this effect where nitrile crambene was found to be potent *in vivo* yet only weakly phase II-inducing in cell culture[6].

In another animal feeding trial it was found that cheirolin, identical to erysolin but one carbon shorter in the side chain, showed no phase II induction potency when measured in gut tissue and bladder yet AITC, sulforaphane, erucin, iberin and iberverin did[7].

So the degree of bioactivity appears to be a complex combination of the oxidation state, presence of an isothiocyanate group, the chain length and composition of the side chain and hydrophobicity. Also, depending on the hydrophobicity profile, the products may circulate in blood serum or be taken up into the membranes of cells that line the gut.

Again it needs to be stressed that *in vitro* and *ex vivo* experiments have their place and may be the only way to look at a biological system by its separate components but the results do not necessarily relate to the living host.

The past has also taught us that while animal trials are also necessary if we are to avoid experimenting on humans, animals are not always the best model for human physiological systems because even though we may share similarities at the cellular level, we may be too dissimilar to apply the results to our physiology.

One example is the epithelial E-cadherin protein involved in the control of many functions including cell to cell adhesion, signalling and apoptosis. The pathogen *Listeria monocytogenes* produces a virulence protein called internalin A (InlA) which has a leucine-rich domain which binds specifically to E-cadherin exploiting the nature of E-cadherin to be able to interact with several different signalling molecules[8].

When InlA binds with E-cadherin, it results in signalling to the cell to initiate 'ruffling' which precedes phagocytosis[8]. Once engulfed by the phagosome, *L. monocytogenes* is able to escape and invades the cell, coming out only to invade the next and the next which causes the disease listeriosis.

Mouse E-cadherin on the other hand, while having the same function, differs from human by a single amino acid substitution of glutamic acid for proline at residue 16. This one substitution prevents InlA from invading mouse epithelia which means that mice could not be models for human listeriosis[8,9].

It is interesting that E-cadherin is also a tumour suppressor gene[10] controlling apoptosis and its mutation or loss of function is often implicated in cancers including colorectal[11]. It would be interesting to see whether GSL hydrolysis products can interact with E-cadherin and if so what the implications for this might be.

Bridging *in vitro* and *in vivo*, there are gut models under continual development and being improved on to more closely model a human intestine[12]. From glassware to a human 'gut on a chip' the physiological conditions of an intestine are being modelled to enable study of a human digestive system without actually being one[13].

These examples all illustrate that to study a biological system sometimes a combination approach is the only approach and even then, something that works in one system may be completely inadequate for another.

9.2.2 Selenium glucosinolates

If the oxidation state of ITCs and nitriles is an important bioactivity determinant, why are selenoisothiocyanates which are not oxidised, more potent than their sulfur analogues? Either another mechanism is responsible for bioactivity, or selenoisothiocyanates are not managed by the body in the same way as their sulfur analogues. The bacteria in the experiments from chapter 6 consumed hardly any seleno-GSLs and the seleno-GSLs were only a small percentage of the total GSLs present to begin with. Based on these findings it is hard to see how seleno-GSLs in selenium fertilised broccoli could have been responsible for increases in phase II enzymes in the previous studies described in this chapter.

In contrast, seleno-aminoacids such as selenocysteine and selenomethionine, or even inorganic selenate also become incorporated into the broccoli tissue and these could be entirely responsible for the observed phase II effects. Based on this it is concluded that another mechanism, the selenium itself, is responsible for bioactivity and the seleno-GSLs or selenoisothiocyanates make only a minor contribution.

9.2.3 Are nitriles able to confer protective effects similar to ITCs *in vivo*?

There are several possibilities, one being that nitriles are not bioactive and it falls to the ITCs such as sulforaphane which is produced during digestion of GSLs. Sulforaphane becomes rapidly absorbed due to its conjugation with GSH and goes through to excretion inducing phase II enzymes all the way.

A further possibility is that nitriles are not bioactive but during the process of metabolism, bacterial reductions of alkylsulfinyls to alkylthiols are countered by a host response which is to oxidise these back again after absorption.

Bacteria may be producing erucin from hydrolysis of glucoerucin, which they had generated through the reduction of glucoraphanin. This erucin is oxidised to sulforaphane subsequently conjugated with GSH, goes out the excretion route while inducing phase II enzymes all the way.

Another possibility is that nitriles are exerting cytoprotective effects but not through the mechanisms ITCs follow. In support of this premise, the results of the quinone reductase (QR) assays showed that in the bladder there was an increase of QR for GSL diet groups.

9.2.4 Beneficial bacteria raised quinone reductase alone

The group receiving GSLs alone had elevated QR activity for the bladder compared to the control diet group. The surprise finding was that KF147, KW30 and Nissle raised QR activity with or without GSLs. This can be explained by previous studies which have shown that some SCFAs, butyrate for example, has been shown to inhibit colon cancer cells in culture[14] and more recently has been implicated in blood pressure regulation[15]. It is apparent that SCFAs have the potential to modulate the host phase II enzymes along with other metabolic pathways and in this instance, beneficial bacteria added to the GSL diet caused an increase not only in the production of some SCFAs, but a reduction in others and a GSL-adaptive response to GSLs. All of these results indicate that GSL-adapted and GSL-naive beneficial bacteria raised QR activity in the bladder, the place where metabolites concentrate. The SCFA metabolites, butyrate for example were not markedly dissimilar between groups and while isobutyrate and isovalerate were changed, it was towards less not more and this was seen for GSL-naive Nissle and GSL-adapted Nissle, GSL-adapted KF147 and GSL-adapted KW30. While the differences between groups was significant, the only SCFA that was significantly lower in the control diet group was acetate so is acetate responsible for the elevated QR?

Not according to one study. In this, sodium butyrate increased phase II enzyme activities in rat small intestine epithelial cells in a dose-dependent manner;, while the other short-chain fatty acids did not[16]. This group found that sodium butyrate enhanced the activities of phase 2 enzymes through Nrf2 dependent mechanisms with a concomitant decrease in the mRNA and protein levels of p53. In a different study, rat distal colon cells that were treated with acetate and butyrate were able to reduce the induction of DNA damage by up to 500 μM H_2O_2, whereas isobutyrate and propionate did not[17].

9.2.5 Glucosinolate-adapted microbiota were metabolically different

An unexpected finding was the changes in the profiles of SCFAs for the GSL-adapted groups indicating metabolic shifts in either bacterial populations or in metabolic capabilities. One of these was a marked decrease of the branched chain fatty acids isovalerate and isobutyrate. These branched chain fatty acids are a by-product of the metabolism of proteins (as a carbon source) by bacteria such as Clostridia which are members of the Firmicutes class.

Another was lactate the concentration of which was significantly higher in Nissle and GSL-adapted groups. If we consider these results as a whole, it clearly shows bacterial GSL adaptation. Whether this resulted in changes to the bacterial ratios or whether the bacteria were being more or less metabolically active, cannot be determined without further work to identify to genus level which bacteria were present, their numbers and their metabolic contribution. 'Next Generation Sequencing' would enable identification of these groups.

Something that is known from a past study is that a human trial showed that the composition of the bacterial community from the gut was altered by consumption of the cruciferous vegetables and the bacterial community response to cruciferous vegetables was individual-specific[18]. Because of this the authors speculated that the differences may affect the gut metabolism of bioactives and from this, the host exposure. The results from this study certainly demonstrate bacterial differences and show that the bioactives, in this case ITCs and nitriles are affected.

9.2.6 The host contribution

Carbohydrates are a major energy source for human and microbial cells but humans do not produce enzymes that can degrade complex polysaccharides (this includes GSLs) so must rely on the host microbiota to do this.

Non-digestible carbohydrates such as cellulose or inulin reach the host colon intact where the microbiota ferment them for their own energy needs and in doing so, release SCFAs as their end or by-product. It is the SCFAs that exert biological effects as not only are they an energy source for host enterocytes, the epithelial cells that line the gut, but SCFAs have also been

shown to elevate phase II enzymes, to modulate blood pressure, inflammation and promote wound healing.

The composition of the microbiota and how much is eaten will determine the SCFAs that are produced. This has a direct effect on health even health issues such as obesity when more carbohydrate fermentation results in more absorption by enterocytes with the host balancing the interactions with microbiota and nutrients[19].

Metabolism in the host is therefore not an isolated event but involves a complex system that is continuously responding to a dynamic environment requiring a rapid response to changes and redox flux. Just as the SCFAs, the by-products of bacterial metabolism change according to the metabolic activity of the microbiota, so does the host respond to the availability of these SCFAs which are used by enterocytes, the host epithelial cells that absorb and use the available short chain fatty acids for energy.

This is where GSLs and SCFAs paths cross. The reduced species of GSLs dominated over oxidised indicating that only the bacteria reduced the GSLs. Without a host (*ex* vivo) the microbiota went one step further and reduced the alkylsulfinyl GSL hydrolysis products (either sulforaphane nitrile or iberin nitrile) into predominantly their reduced species erucin nitrile and iberverin nitrile.

In contrast, the host was modifying by oxidation the hydrolysis products.

The bacterial reduction of the GSLs and their products may have been due to a requirement for an electron sink by the microbiota rather than to reduce reactive oxygen species (ROS) whereas the oxidation of the hydrolysis products by the host could be related to hormesis where a biphasic response occurs when the host response is to facilitate elimination based on the degree of 'perceived' toxicity[20]. Although not toxic, nitriles tend to be hydrophobic and lipophilic, so perhaps they are taken up in cell lipid bi-layers rather than circulating in blood serum but we don't know for certain what nitriles from GSLs are doing because no one has ever studied them. This is probably because the ITCs such as sulforaphane, allyl ITC, benzyl ITC and phenyl ethyl ITC perform so well as bioactives whereas nitriles are stable so appear inactive. *In vitro* experiments support this premise where nitriles have had to be in concentrations 100-fold of their corresponding ITCs to elicit the same effects

and it could be argued that anything in that concentration would have an effect. The only studies published on nitriles seem to be on the metabolism of the toxic ones; hydrogen cyanide, acetonitrile and butyronitrile (or butanenitrile) for example where the pathway to elimination causes severe GSH depletion followed by death[21]. GSL derived nitriles do not appear to be toxic in that manner but are they taken up in tissue where accumulation generates a host response of oxidation to increase solubility, followed by elimination? The focus on sulforaphane while the nitriles go unstudied is understandable given that discovery of such a potent bioactive in food can suggest that it is the only one. But if sulforaphane is only produced under certain optimal conditions while erucin nitrile is really the default product of glucoraphanin hydrolysis in the gut, the question begs asking; is sulforaphane as biologically relevant as erucin nitrile? Epidemiological studies show only an association with the consumption of crucifers and reduced risk of cancer. Research has revealed that products produced from brassica GSLs have potent bioactivity and now it remains to establish whether the epidemiological association is because of these products.

9.3 Summary

From the results of this thesis the path taken by alkyl-GSLs, glucoraphanin and glucoiberin leading to excretion is proposed as follows: After ingestion, some GSLs are simply expelled again in the urine unmodified. Alkylsulfinyl GSLs may be taken up by bacteria from the microbiota in the caecum or colon and be reduced via redox reduction to the alkylthiol GSL. In the reducing environment of the gut, this is to be expected just as lactate may be produced from reduction of pyruvate through the redox activities of bacteria.

Next the alkyl GSLs, are absorbed through the caecal or colon walls where they are processed through the liver without modification and go straight to the bladder. If alkyl-GSLs are hydrolysed due to bacterial metabolism, they may become ITCs but more likely nitriles and either way, this changes their properties considerably, making them hydrophobic.

Both ITCs and nitriles require further oxidation to increase solubility for elimination. Further oxidation of sulfinyl ITCs or sulfinyl nitriles to the highly polar sulfone erysolin or erysolin nitrile is irreversible. This accumulates in the urine and was detected in both urine and blood (Figures 7.7 – 7.8).The reduced

thiol GSLs did not undergo host-oxidation like the hydrolysis products did because GSLs are highly soluble already, this property being conferred by the glycoside and sulfate groups. Analysis of the urine confirmed that the predominant product was glucoerucin rather than glucoraphanin (Figure 7.7).

Evidence to support this is that the major GSLS were sulfinyl; they became thiol, and then were transformed into thiol ITCs or nitriles. It was expected that the ITCs and nitriles found in urine and blood would be the same, thiol and for the *ex vivo* caecal incubation, they were (Figure 8.3). However, after uptake and after transit through the liver, the major products were sulfinyl again; sulforaphane nitrile and erysolin nitrile followed by iberin nitrile and erucin nitrile (Figure 7-8).

The most likely reason for this change back again relates to modification of the molecule to increase its solubility and decrease its lipophilicity, both results relating to elimination from the host. Further evidence to support this was the appearance of the sulfone, erysolin nitrile which can only be explained by further oxidation of either the sulfinyl or thiol part of the R group in sulforaphane, sulforaphane nitrile, erucin and erucin nitrile. Based on this premise, it might be possible for iberin, iberin nitrile, iberverin and iberverin nitrile to be irreversibly oxidized into sulfone cheirolin nitrile. While cheirolin nitrile could not be found, it is accepted that if it was present, the concentrations would have been down at the limits of detection allowing that sulforaphane was found at these concentrations. However, at one carbon shorter in the alkyl chain, iberin, iberin nitrile, iberverin and iberverin nitrile were already more soluble than the sulforaphane, erucin based molecules and were probably excreted without requiring further solubilisation.

While it may not reflect life, there were obvious benefits to *in vitro* studies. Experimenting with the beneficial bacteria in this way enabled study of the bacteria in isolation and in a controlled environment where products could be measured quantitatively. This was how it was discovered for the first time that the Enterobacteriaceae reduced GSLs and their hydrolysis products. These results were supported by the *ex vivo* study where even when a host microbiota was involved, still the erucin effect was apparent. The *ex vivo* studies were then able to validate that the host and the microbiota were both contributing to the products generated from the biotransformation of GSLs.

By studying this relationship *in vivo*, for the first time, a relationship between beneficial bacteria and GSLs could be seen. Not only did the dietary GSLs increase the QR activity, the beneficial bacteria did also and they did so when GSLs were added in combination as well. It could be seen that GSL consumption was not an accurate indicator of GSL hydrolysis products. The presence of hydrolysis products were a measure of bioactivity as measured by QR but the QR activity did not increase proportionally more when beneficial bacteria were added with the GSLs suggesting tolerance of one another but not synergy.

9.4 Future directions

There are always further experiments that could be done. Labelled GSLs would enable accurate monitoring of their fate. Also, searching for nitrilase activity to discover the missing products from the lactic acid bacterial metabolism of GSLs would confirm that they were degraded further and identifying the transport mechanism (if it exists) for bacterial GSL uptake and excretion could be another experiment for another day.

The SCFAs produced by the caeca *ex vivo* may provide clues as to why the beneficial bacteria raised QR and it may be related to the flux of butyrate, propionate and acetate. 'Next-Generation sequencing' would be a good way to find out more about the composition of the microbiota.

9.4.1 Human trials

The health benefits were the reason for this study and to that end, the experiment worth doing is the one that involves humans; a human feeding trial.

Given time, this is the experiment that should be done. The same product, the broccoli powder, could be given in combination with beneficial bacteria. While QR could not be measured as it is an invasive measure of cells, it is possible to collect urine and blood which could be assessed for the products of GSL metabolism. Faecal samples could be analysed for the bacterial products of metabolism (SCFAs) and if we were to make the assumption that the bacteria present in the faecal material include a representation of the host microbiota, this material could be incubated with GSLs much like the *ex vivo* work from chapter 8. Maintaining 100% anaerobic conditions would not be possible but it may not be critical for assessing the

beneficial bacterial contributions as they are either facultative anaerobes or oxygen tolerant.

9.4.2 Methane mitigation

There may be other implications that consuming beneficial bacteria with dietary GSLs brings and it involves the New Zealand policy response to climate change; the New Zealand Emissions Trading Scheme (ETS).

The following information (italicised) was taken directly from the Ministry for the Environment's website http://www.mfe.govt.nz:

> *"In 1990, New Zealand's total greenhouse gas emissions were 59,797.2 Gg carbon dioxide equivalent (CO_2-e). In 2010, total greenhouse gas emissions had increased by 11,860.0 Gg CO_2-e (19.8 per cent) to 71,657.2 Gg CO_2-e. The four emission sources that contributed the most to this increase in total emissions were road transport, dairy enteric fermentation, agricultural soils, and public electricity and heat production."*

With New Zealand's reliance on agriculture, mitigation of methane emissions due to dairy enteric fermentation has been a target for AgResearch scientists for over a decade now. There is no denying that the cause of the methane emissions from ruminants is due to the metabolic activity of the microbiota residing in these animals' rumens and strategies have been developed to reduce this source of methane. Rumen microbes include methane producing microbes from the archaea domain of life. Known as methanogens, they utilise the byproducts from other microbes releasing methane as their by-product of anaerobic respiration.

There have been a number of strategies proposed to reduce methane and some of them specifically target methanogens such as vaccination, feed supplements that reduce the number of methanogens, and animal selection[22].

Feed supplements have included brassica, rapeseed and rapeseed oil which resulted in a reduction of methane production by both sheep and cattle[23,24]. Rapeseed oil supplements show promise as there is now metatranscriptomic evidence that lactating dairy cows given rapeseed oil supplements resulted in the reduction of a novel group of methylotrophic methanogens known as Thermoplasmata which incidentally coincided with methane mitigation[25].

These results while not definitive, hint that the GSLs might be an electron sink after all and if given as a feed supplement, may be helping to reduce methane emissions. While this is an oversimplification, if in an anaerobic environment, carbon dioxide, acetic acid and hydrogen are the only possible electron acceptors left, the transfer of electrons would result in methane carbon dioxide and water as shown below:

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O$$

Or $$CH_3COOH \rightarrow CH_4 + CO_2$$

GSLs or their hydrolysis products may provide an alternative electron sink and in combination with beneficial bacteria, provide several benefits. First, in the production of SCFAs that help modulate metabolic pathways, secondly, GSL-derived enhancement of phase II enzymes and thirdly, because GSLs modify the microbiota, this in turn enhancing some metabolic activities and or prevalence of beneficial bacteria over others. If GSLs and their hydrolysis products are providing an alternative electron sink, this could also reduce the requirement for carbon dioxide as an electron acceptor, resulting in the reduction of methane production. While mutton that spends its final days foraging on kale does result in distinctively tainted meat (personal experience), this could be overcome by a washing out period whereby the animals are taken off brassica supplementation for a period of time prior to slaughter.

9.4.3 Watercress development

The next thought for the future involves watercress. While not discussed in this thesis, watercress is another member of the brassica family and it contains gluconasturtiin which produces the ITC phenylethylisothiocyanate (PEITC). Not only that but watercress also contains longer chained alkylsulfinyl GSLs such as methylsulfinylheptyl GSL and 8-methylsulfinyloctyl GSL which hydrolyse to methylsulfinylheptyl-ITC and 8-methylsulfinyloctyl-ITC, both even more potent phase II inducers than sulforaphane[26] . There is an opportunity to develop and market within New Zealand an industry for watercress products as a healthy food supplement for consumption much as broccoli sprouts have been marketed for their glucoraphanin content. This has never been developed beyond some initial work some years back within the then Crop and Food Crown Research Institute (CRI) where at the time, contamination issues hindered further development[*].

I hope that the results from this thesis stimulates opportunities for future development in bioactives, methane mitigation and new markets or industry for fresh New Zealand products because while this is the end of one story, it shows us only a fraction of the story about how intrinsically human health, diet and microbiota entwine.

9.4.4 Bladder cancer

Prior to this project I studied the bioengineering of nano-beads by using microbes as cell factories to produce tailor-made, immobilised, functional proteins for bio separation. The main reason I left this field to pursue a PhD in food and nutrition was because of a personal desire to find effective tools against bladder cancer.

This project has been about health outcomes not disease models and it is rightly so. However I cannot end this thesis without reference to how dietary GSLs and their hydrolysis products might improve the health outcome for people with bladder cancer. If given the opportunity, I would like to know whether the GSLs and or their hydrolysis products can substitute for the apoptotic function of E-cadherin as cancers are often associated with the loss of function of this molecule[27,28]. If the results support the premise that GSL nitriles

[*] Personal communication

are capable of activating apoptosis, this will provide more science to back up the health claims for consumers of broccoli. It would also be great opportunity to cross compare GSLs (broccoli glucoraphanin - glucoerucin) against watercress (PEITC or other) in a gut model or otherwise.

9.4.5 Final words

My final offering is Figure 9.1 which summarises the hydrolysis of GSLs with host modified products in bold and bacterial-modified products in bold italics. For simplification purposes, only glucoraphanin is shown which is only fair because glucoraphanin is the GSL everyone wants to have since it is the GSL precursor of sulforaphane. Perhaps however, glucoraphanin is sulforaphane only in myrosinase worlds. It could be argued now that in the bacterial-influenced world everyone should have glucoerucin. That's the 'erucin effect'.

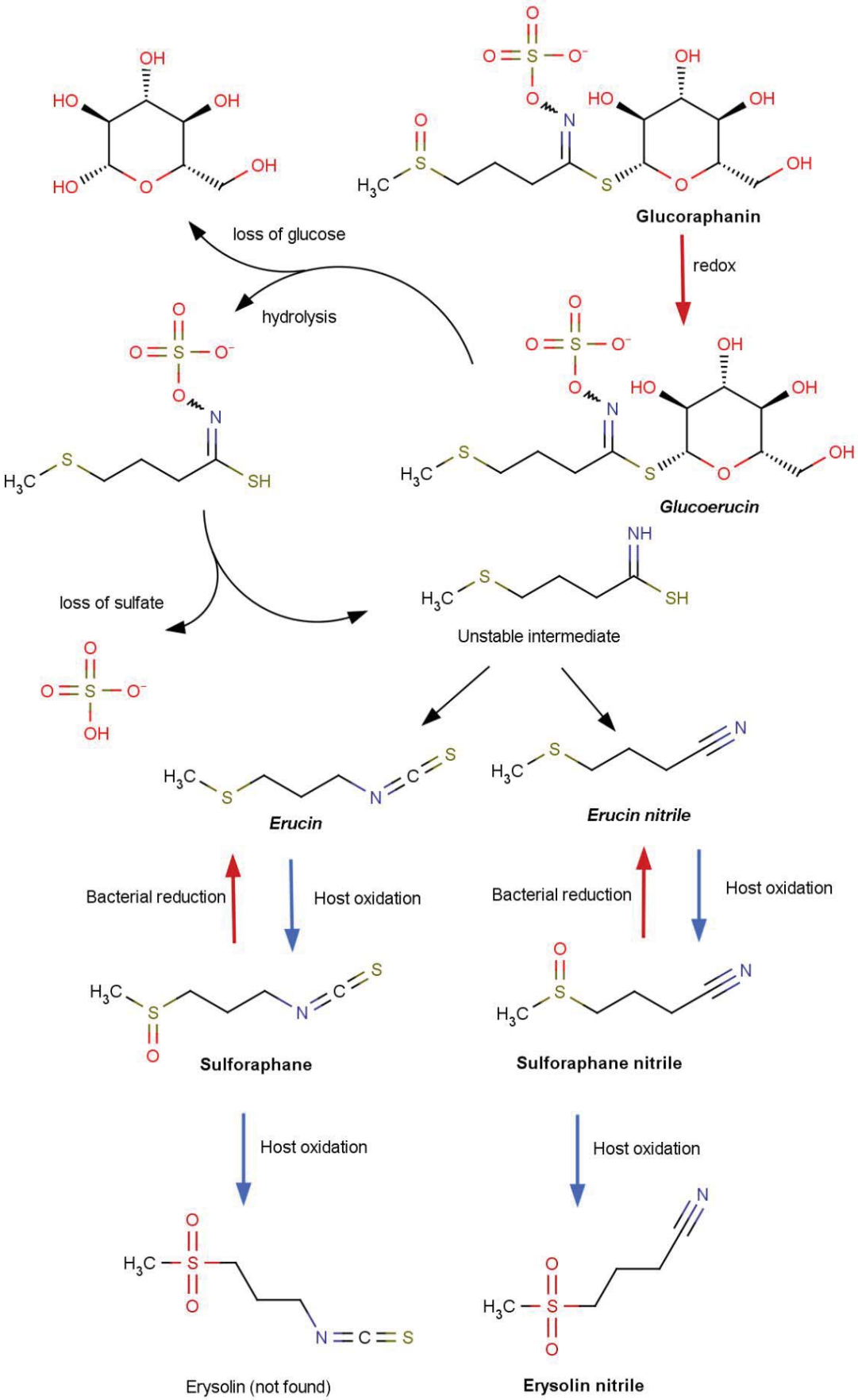

Figure 9.1 The erucin effect

9.5 References

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APPENDIX A

List of Figures and Tables

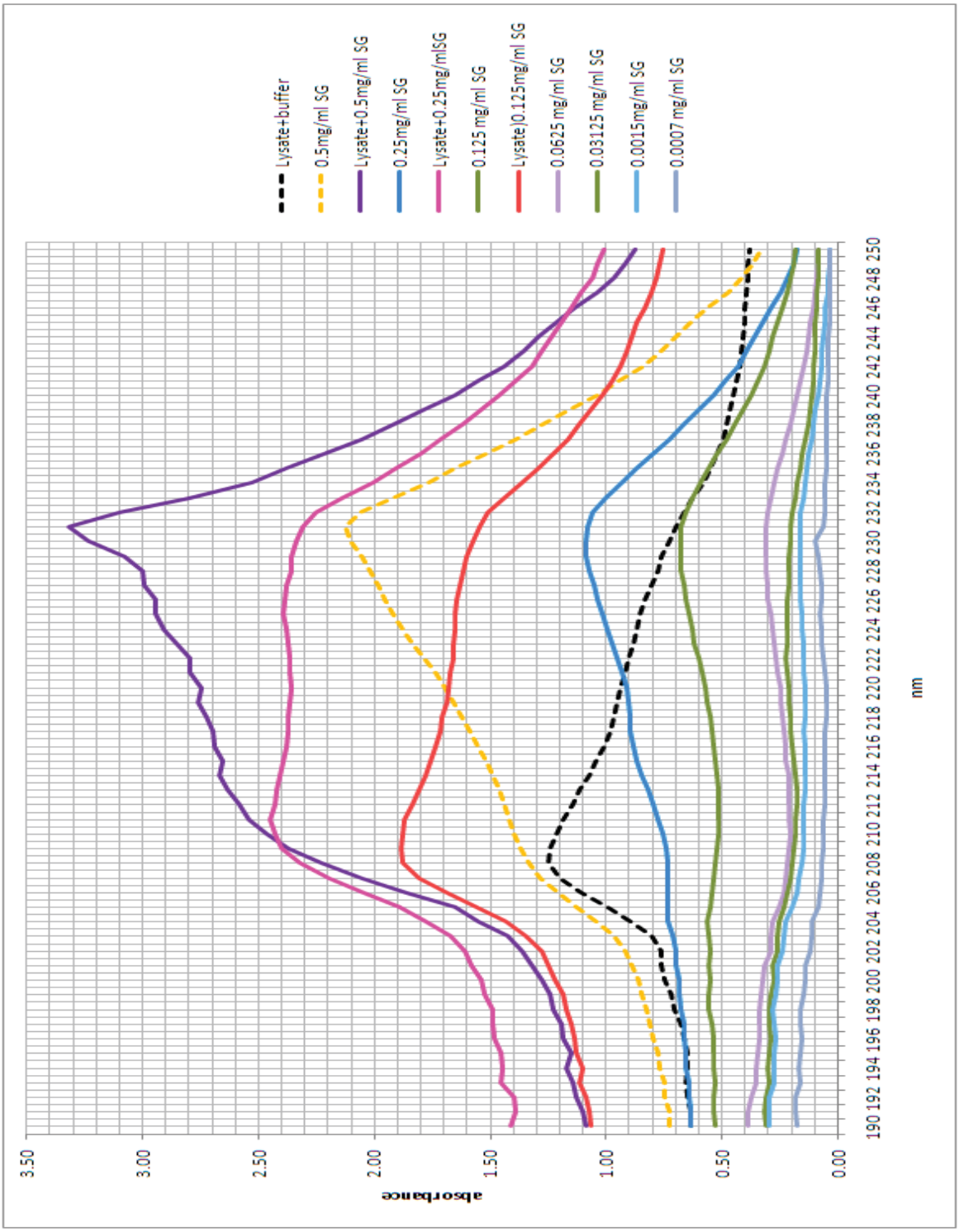


Figure A1. Scanning the absorbance from 190 – 250 nm showed that proteins (bacterial cell lysate) could interfere with the glucosinolate measurement, particularly at low concentration. SG=sinigrin

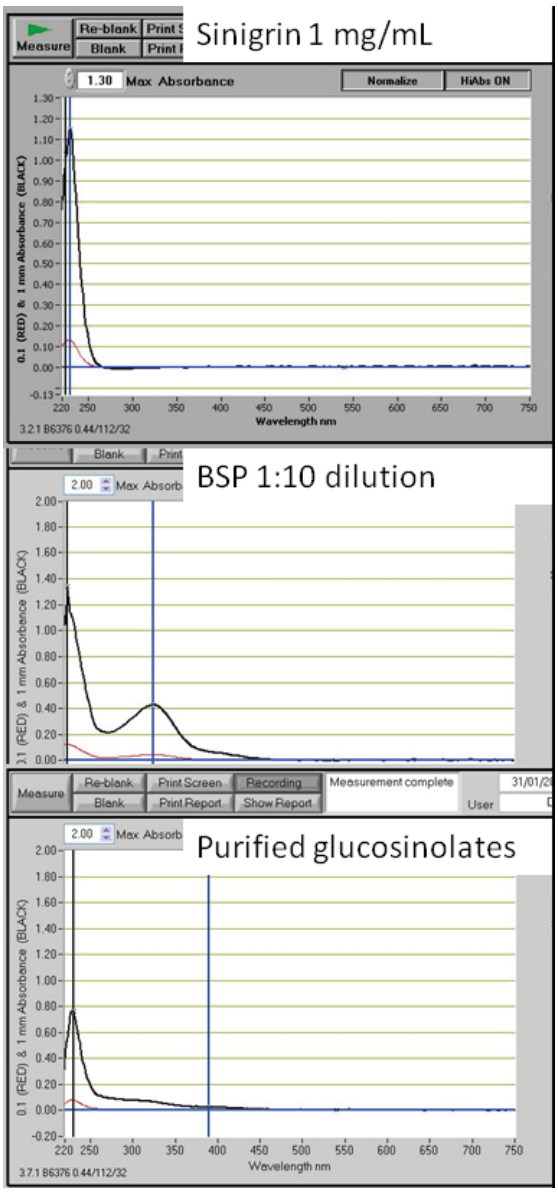


Figure A2. Nanodrop quantification (A) Allyl glucosinolate (sinigrin) 1 mg/mL, (B) BSP extract (crude) 1:10, (C) BSP extract after purification with SPE columns shows a single peak

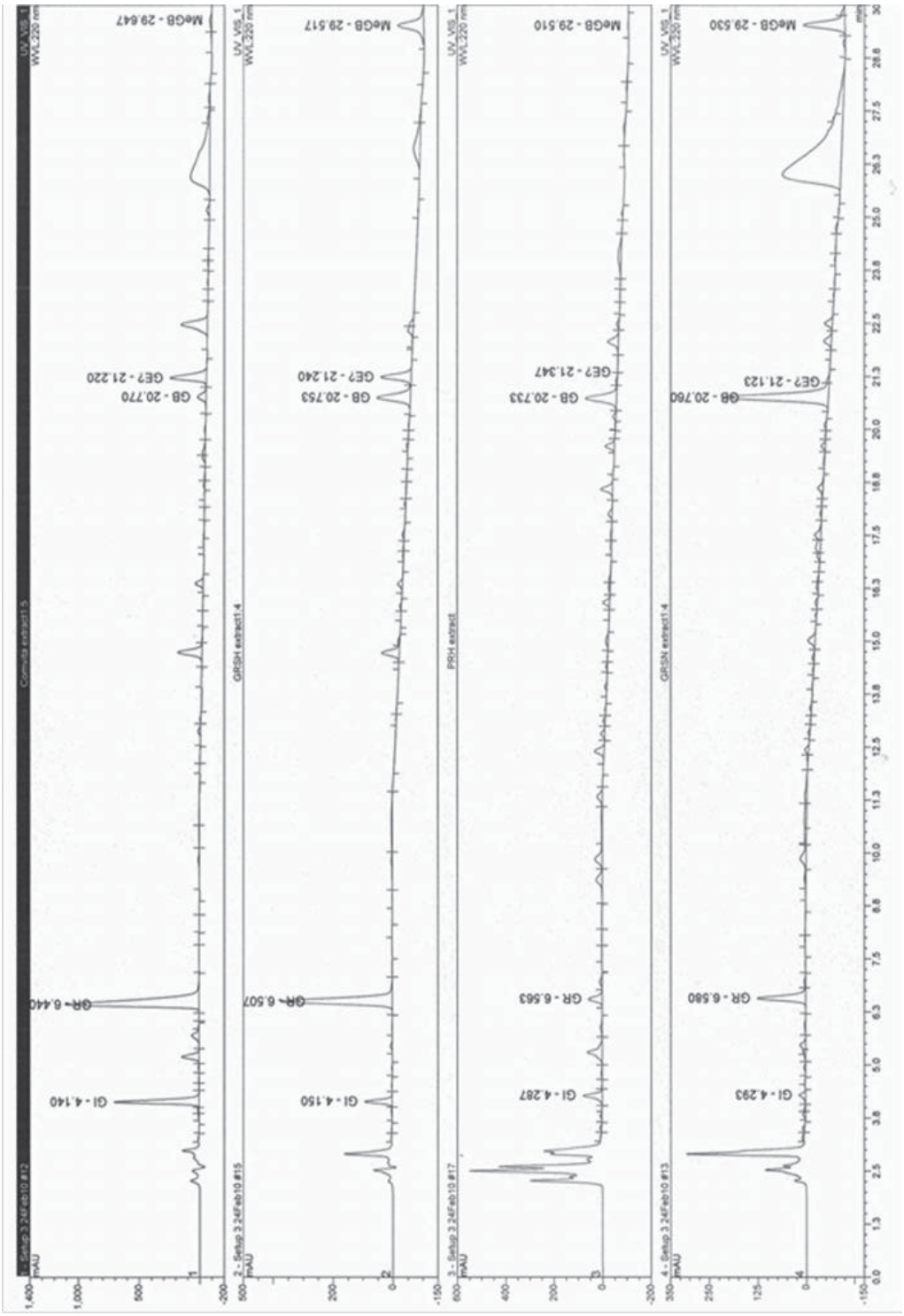


Figure A3. The comparison of known GSLs with the quality control (QC) standard (PRH extract), Comvita (from seed), GRSH (freeze dried blanched broccoli sprouts) and GRSN (freeze dried non blanched broccoli sprouts). Key: GI, glucoiberin, GR, glucoraphanin, GE, glucoerucin, GB, glucobrassican and MeGB, methylglucobrassican

Southern Cross UNIVERSITY

Centre for Phytochemistry & Pharmacology

CERTIFICATE OF ANALYSIS

SAMPLE	Broccoli
CUSTOMER	Comvita
CERTIFICATION DATE	10 August 2010
CUSTOMER LOT BATCH No.	Batch # TB 107534
LABORATORY REF. No.	CPA101455
JOB No.	A10548

TEST	RESULTS	TEST METHOD
Assay – Glucoraphanin (% w/w)*	12.95	CPP-TM165

*** Assay by HPLC (DAD detection @ 225nm)**

.....

MR. ASHLEY DOWELL
MANAGER-ANALYTICAL DIVISION

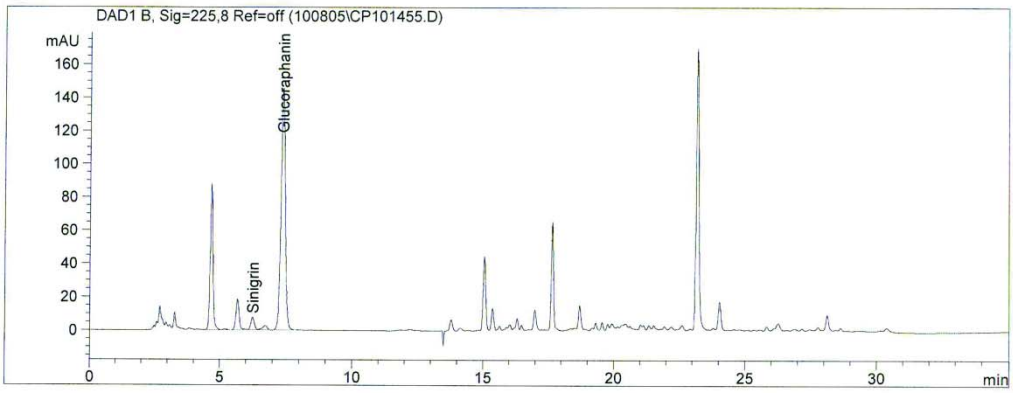
T (61 2) 6622 3211 F (61 2) 6622 3459 Military Road Lismore NSW 2480 PO Box 157 Lismore NSW 2480 Australia
www.scu.edu.au/research/cp ABN 41995 651 524 TGA Licence No. 181048

Figure A4. Validation of the glucoraphanin content of the broccoli seed powder

Injection Date : 8/5/2010 9:25:24 PM
Sample Name : brassica
Acq. Operator : PM

Seq. Line : 8
Location : Vial 17
Inj : 1
Inj Volume : 10 µl

Acq. Method : E:\DATA\METHODS\QAGLUCPH.M
Last changed : 8/5/2010 8:46:07 PM by PM
Analysis Method : E:\DATA\METHODS\QAGLUCPH.M
Last changed : 8/9/2010 4:40:54 PM by GAD
(modified after loading)



ESTD Percent Report

Sorted By : Signal
Calib. Data Modified : 8/9/2010 4:40:56 PM
Multiplier : 1.0000
Dilution : 10.0000
Sample Amount : 21.08000 [mg]
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 B, Sig=225,8 Ref=off
Uncalibrated Peaks : using compound Sinigrin

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
4.369	VV	1.15821	1.07977e-4	5.93263e-3		?
4.674	VV	653.84210	1.07977e-4	3.349142		?
5.203	VP	7.06258	1.07977e-4	3.61763e-2		?
5.679	BB	159.26543	1.07977e-4	0.815797		?
6.253	BV	72.36760	1.07977e-4	0.370685		Sinigrin
6.735	VP	33.85599	1.07977e-4	0.173419		?
7.375	VB	1644.12329	1.07977e-4	8.421585		Glucoraphanin
Totals :				13.172737		

Results obtained with enhanced integrator!

*** End of Report ***

CHROMO-1 8/9/2010 4:41:11 PM GAD

Page 1 of 1

Figure A5. Broccoli seed powder analysis of glucosinolates by Southern Cross University

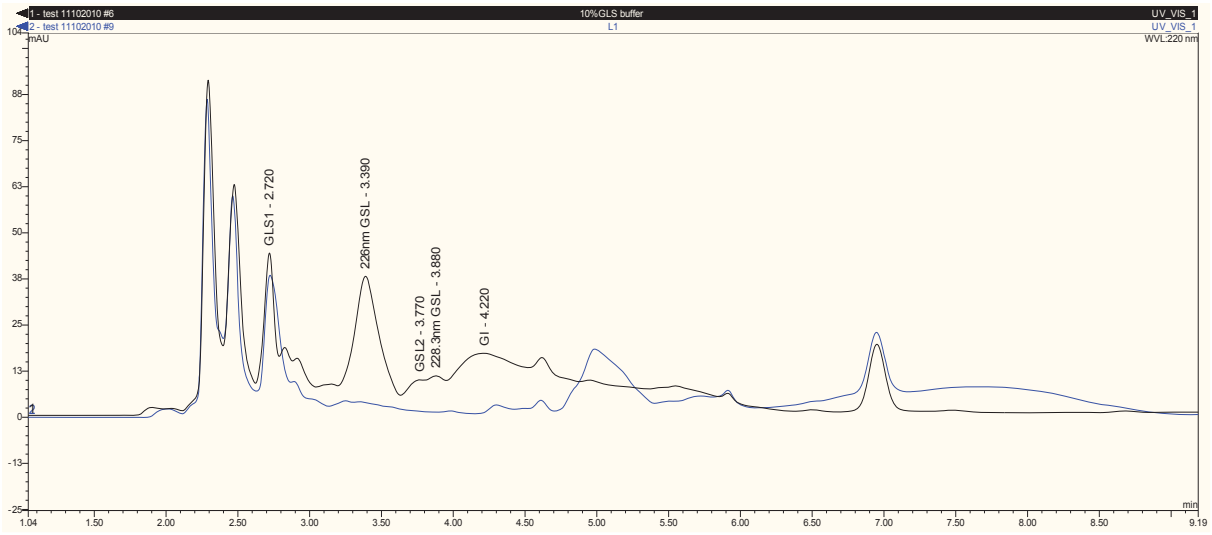


Figure A6. *B. animalis* subsp. *lactis* incubated with GSLs had transformed the GSL that eluted at 3.9 minutes but other peaks had increased and it was not possible to identify them. Key: Black lines are the starting material at time = 0 min, the blue lines the test sample after 24 h.

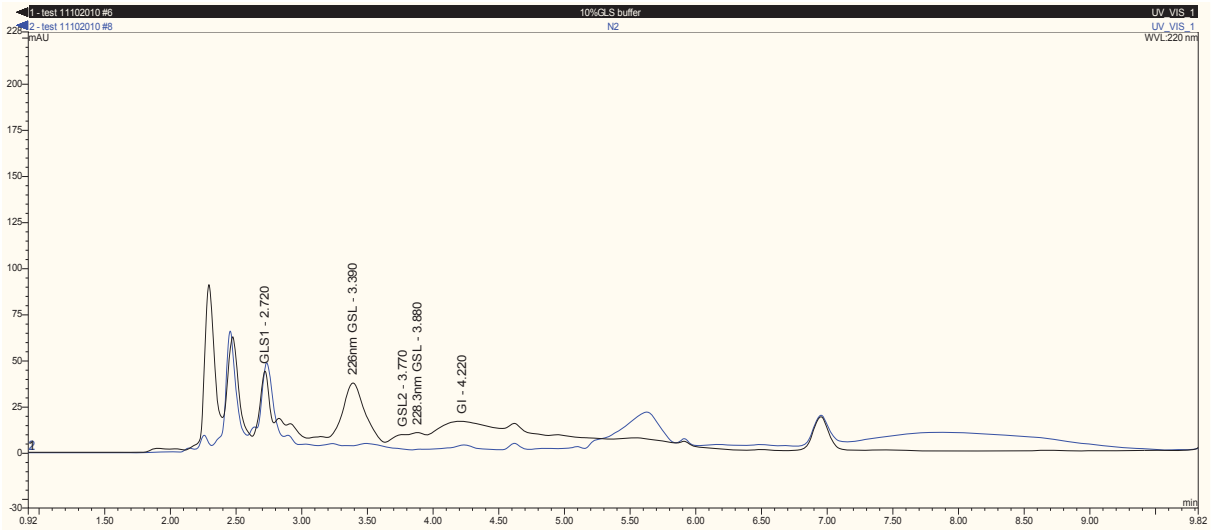


Figure A7. *E. coli* Nissle supernatant after 24 h incubation with 10% GSLs in buffered media from 0 min - 9.5 min shows a similar pattern

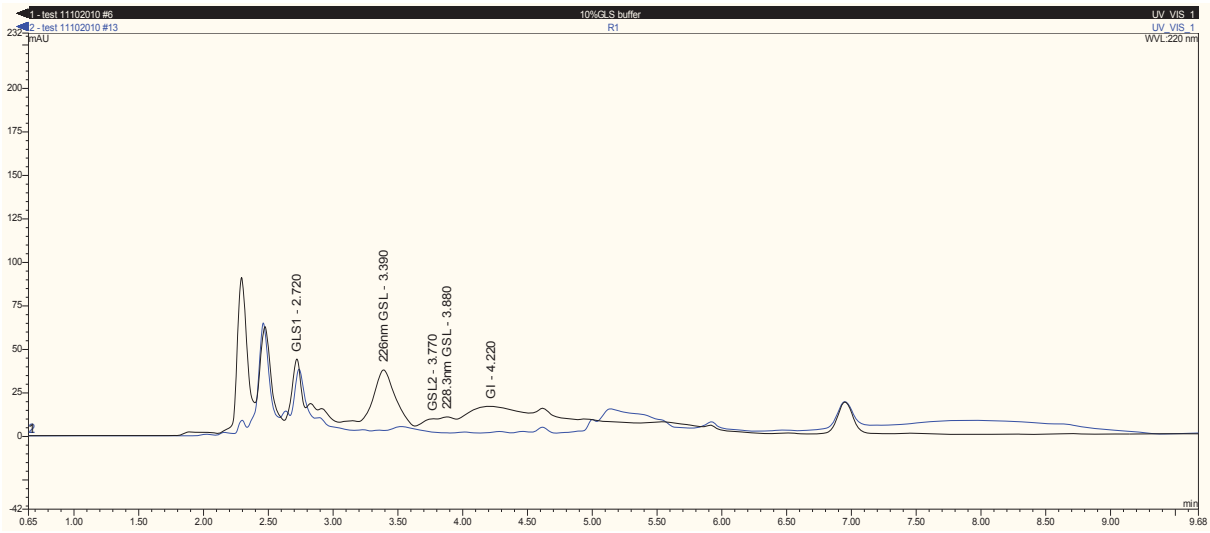


Figure A8. *Lb. rhamnosus* appeared to be transforming an unknown GSL at 3.39 min. Peaks that were not annotated were not of the correct UV value (GLSs characteristic UV spectra is ~230 nm) so are not thought to be GSLs

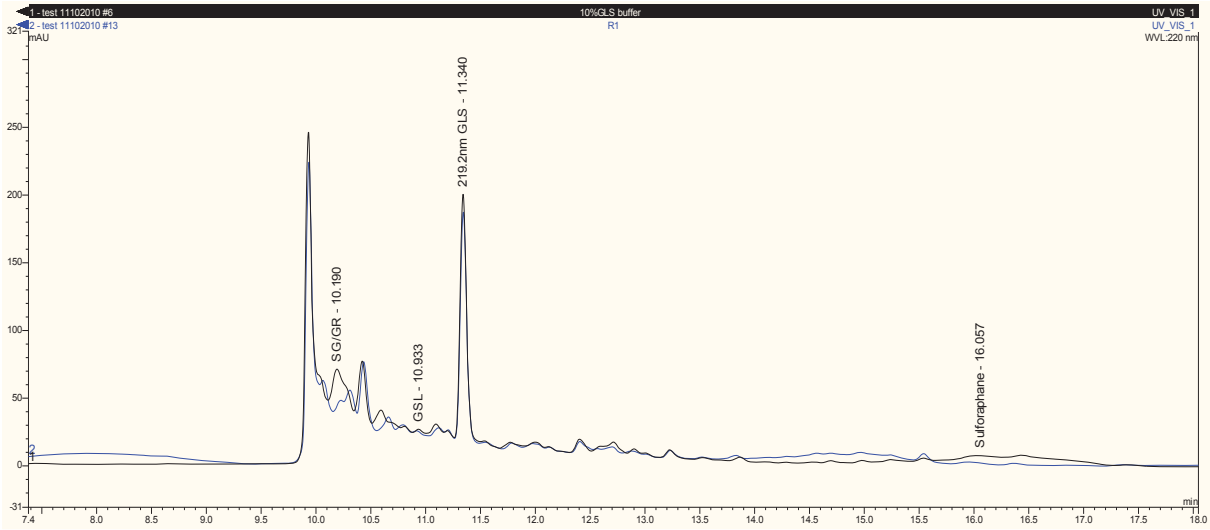


Figure A9. But *Lb. rhamnosus* made no changes to the later glucosinolate peaks like glucoraphanin

RT, min	m/z, $[M-H]^-$	$ms^2(ms^3(ms^4))$, PQD	glucosinolate
6.36	422	358, 259, 97	**glucoiberin (3-methylsulphinylpropyl)**
8.18	436	372, 259, 97	**glucoraphanin (4-methylsuphinylbutyl)**
9.22	388	97, 259, 275	epiprogoitrin ((S)-2-hydroxy-3-butenyl)
11.12	358	97, 259, 275, 162	Sinigrin (2-propenyl)
18.91	372	97, 259, 275, 292, 195	3-butenyl or 2-methyl-2propenyl glucosinolate
21.45	406	97, 275, 259	Glucoiberverin (3-methylthiopropyl)
24.32	420	97, 259, 275, 340	**Glucoerucin (4-methylthiobutyl)**
25.65	388	97, 259, 275, 210	n-pentyl

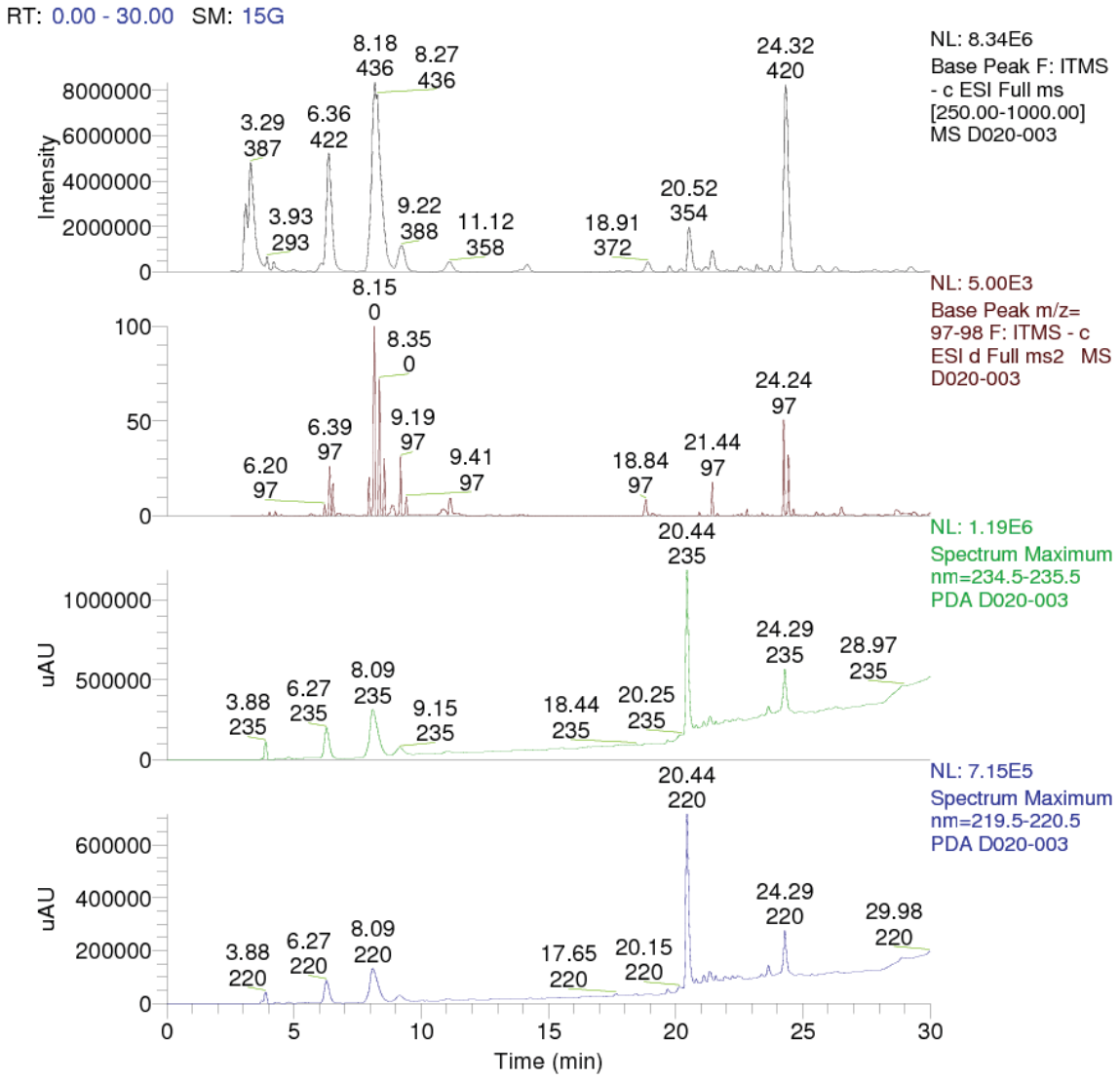


Figure 1 Base peak (parent mass) chromatogram (top), m/z 97 mass filter indicating glucosinolate (second), UV 235nm (third) and 220nm (bottom).

Figure A10. LC-MS/MS analsyis of BSP extract

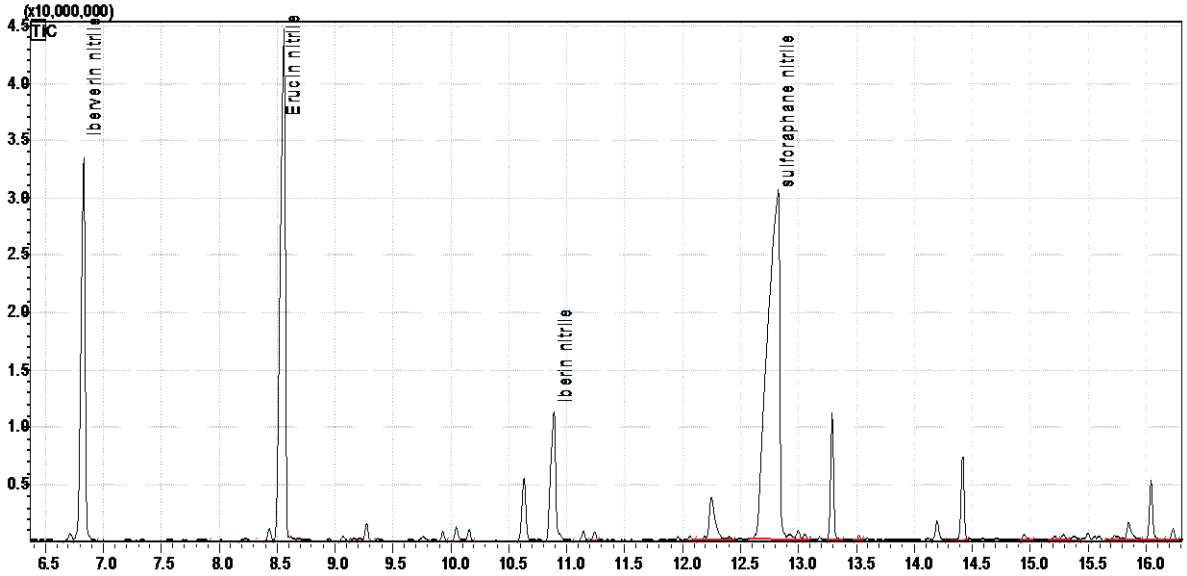


Figure A11. GC-MS chromatogram of individual nitriles after hydrolysis of BSP with myrosinase at pH<3

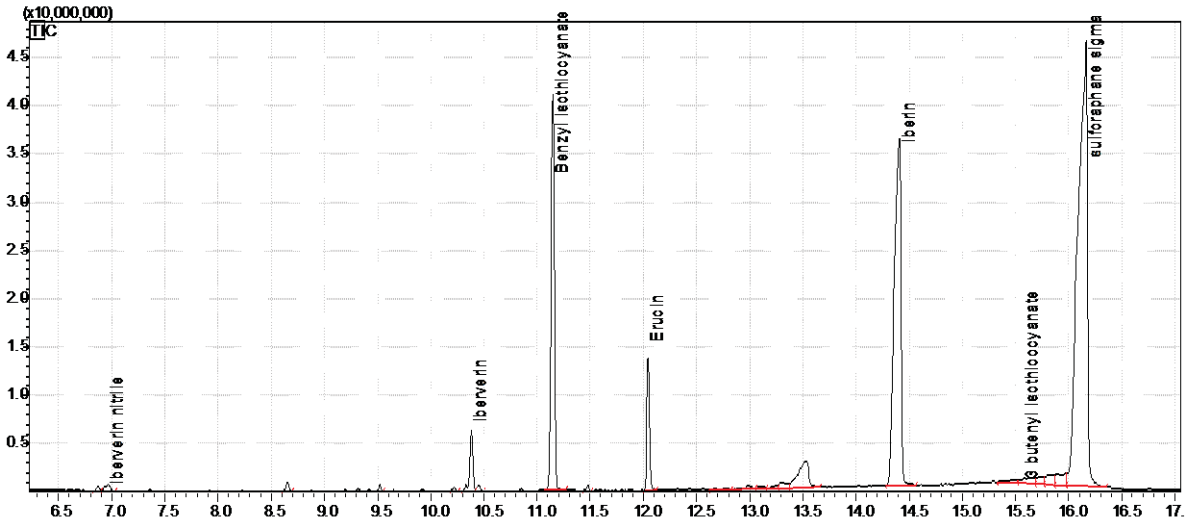


Figure A12. Myrosinase hydrolysis at pH9 after hydrolysis with myrosinase at pH 9. Benzyl isothiocyanates was added as an internal standard

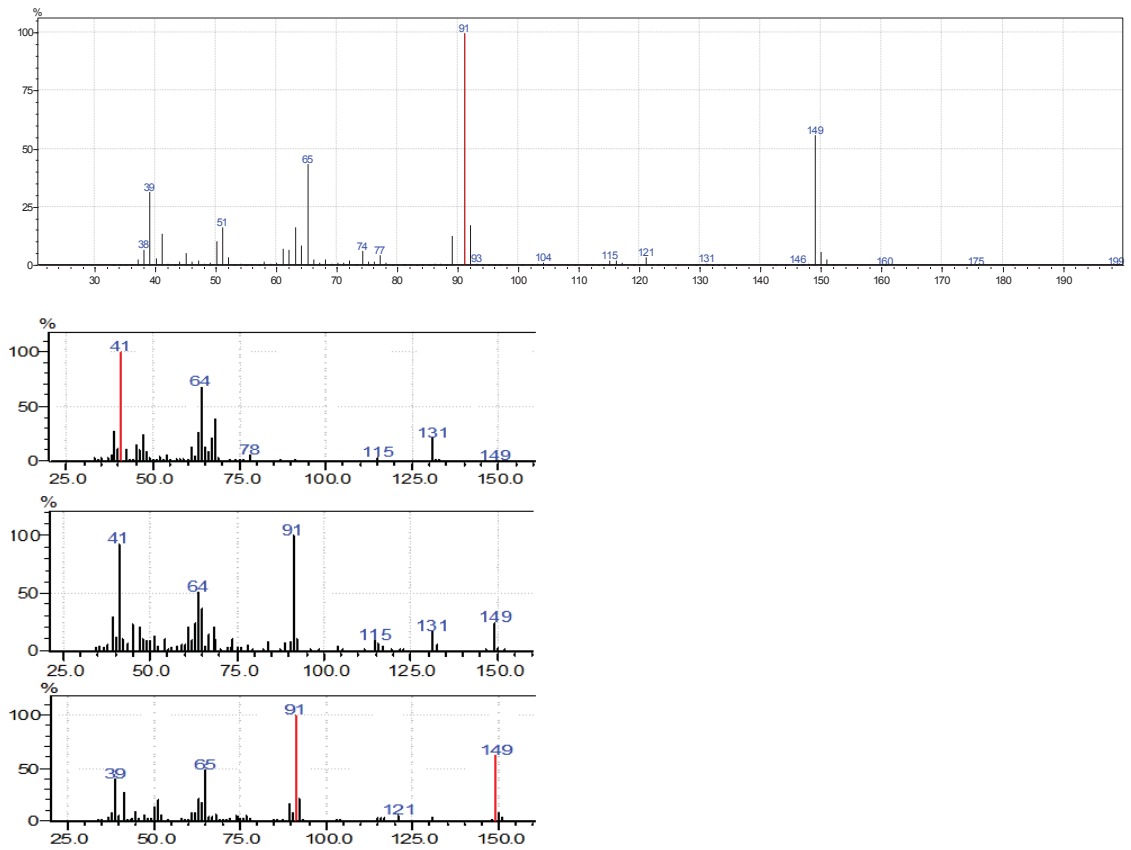
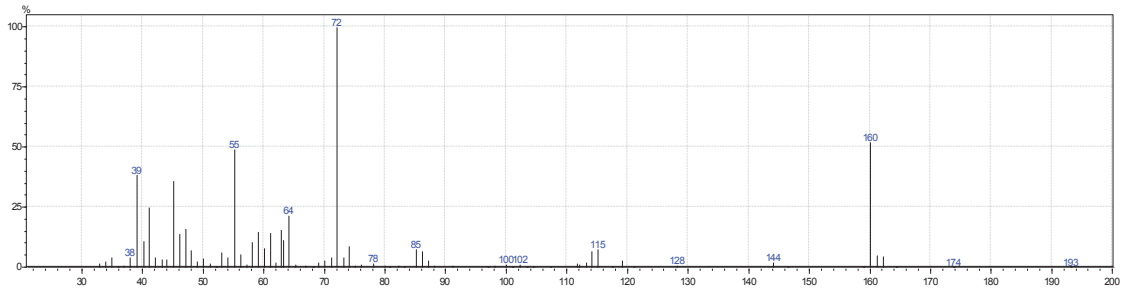
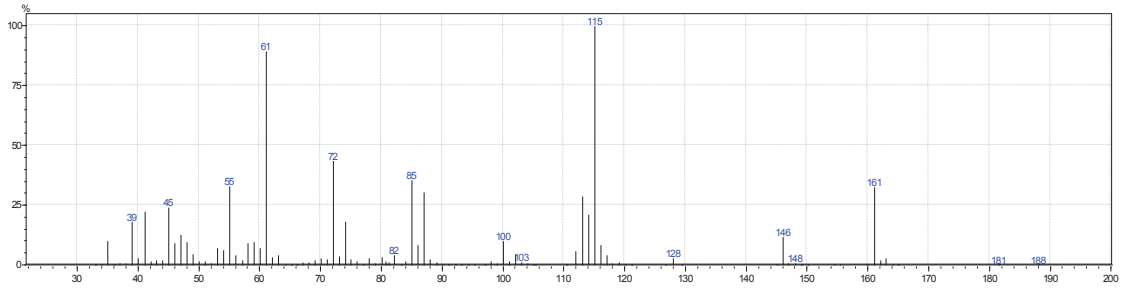


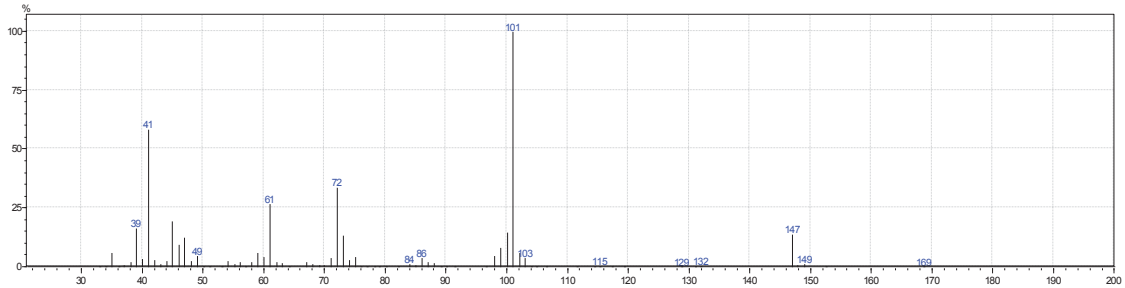
Figure A13. .Top Ion spectra for benzyl isothiocyanate (internal standard) Bottom. Iberin nitrile co-eluted with benzyl isothiocyanates. The benzyl isothiocyanate internal standard and iberin nitrile peaks were partly overlapping one another as can be seen from scanning these spectra. Red lines are saturated ions



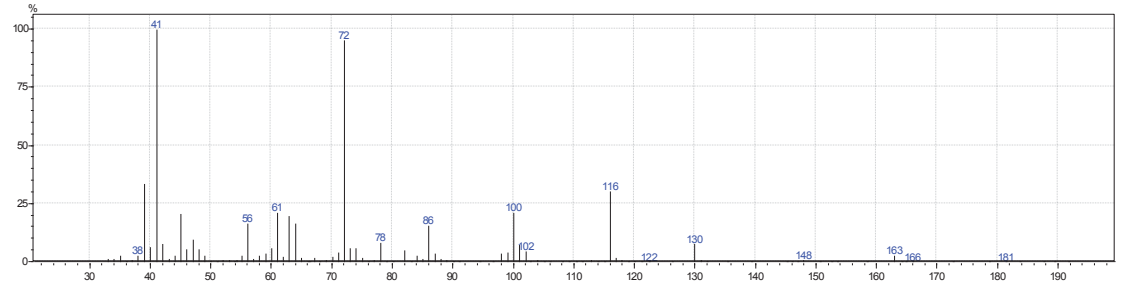
Sulforaphane (m/z 177.28)



Erucin (m/z 161.28)

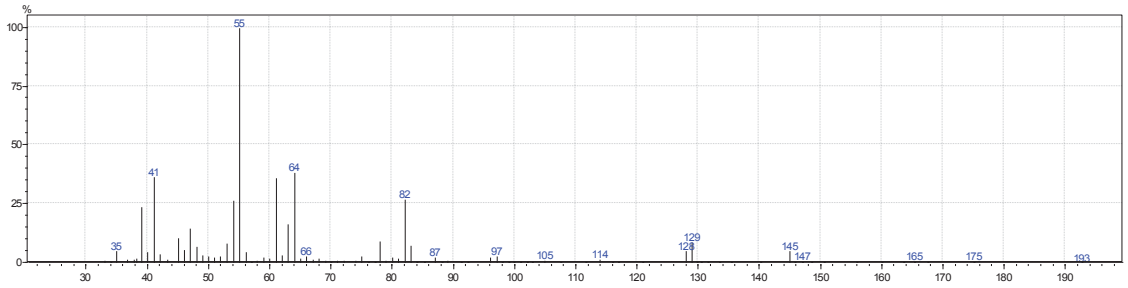


Iberverin (m/z 147.26)

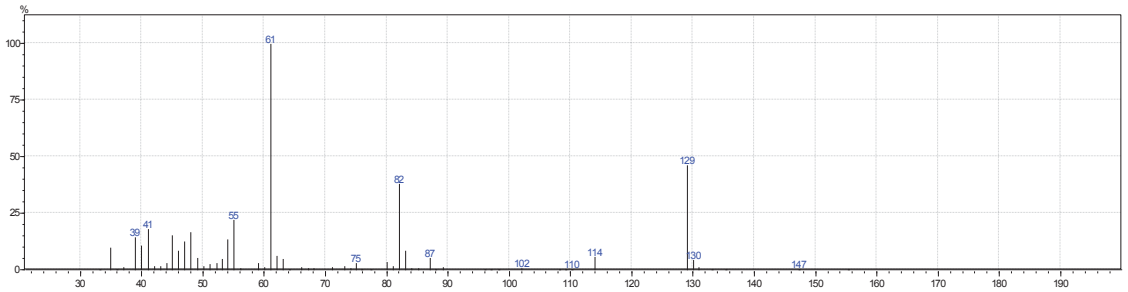


Iberin (m/z 163.26)

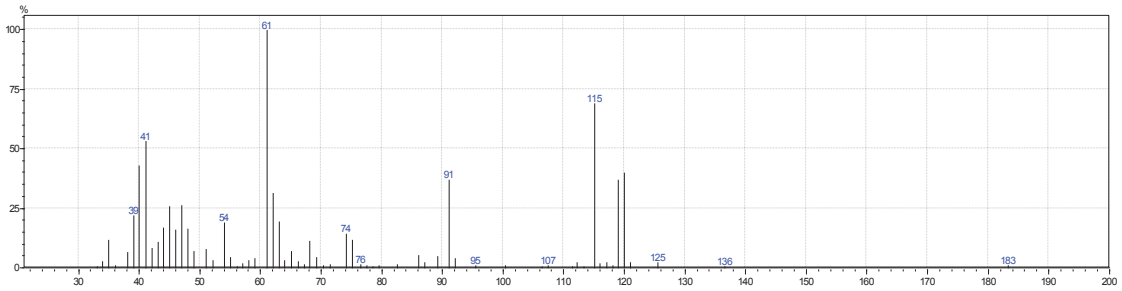
Figure A14. Ion spectra for the isothiocyanates found in the broccoli material used in this thesis



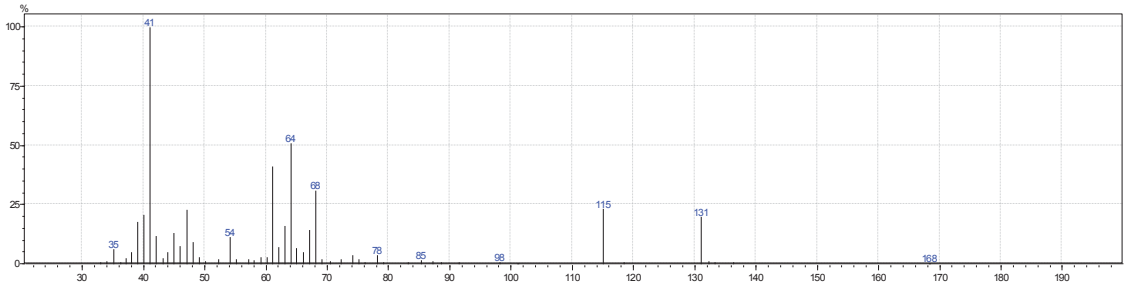
Sulforaphane nitrile (m/z 145.22)



Erucin nitrile (m/z 129.22)



Iberverin nitrile (m/z 115.20)



Iberin nitrile (m/z 131.19)

Figure A15. Ion spectra of the nitriles found in the broccoli material used in this thesis

Residue	Colour	Property
AVFPMILW	RED	Small (small+ hydrophobic (incl.aromatic -Y)
DE	BLUE	Acidic
RK	MAGENTA	Basic - H
STYHCNGQ	GREEN	Hydroxyl + sulfhydryl + amine + G
GR	glucose recognition site	
Zn	zinc binding site for dimerisation (myrosinase)	
X	Catalytic nucleophile site	
@	General Acid/base site in related O-glycosidases	

Figure A16. Alignment of *B. animalis* subsp. *lactis* beta glucosidase with myrosinase from *Brassica oleracae*

Figure A17. Alignment using ClustalW2 of myrosinase from 1CCGB P26205-1CBG, *Brassica oleracea* ABS30827.1, *Enterobacter* YP_001174777.1, *Lactobacillus* YP_003064398.1 and ZP07078860.1

Red = rare Arg codons **AGG, AGA, CGA**
Green = rare Leu codon **CTA**
Blue = rare Ile codon **ATA**
Orange = rare Pro codon **CCC**

for the following input sequence:

atg acg atg acg ttc ccg aag ggc ttc cag ttc ggc acc gcg act gcc gcc tac cag atc gaa ggc
gcg gtg gac gaa gac ggc cgc acg ccg tcg atc tgg gat gtg ttc tcg cac gcc ccg ggc cgc gtg
ctg aat ggc gac acc gga gac aag gcc gac gat ttc tac cac cgc tgg cag gac gat ctc aag ctc
gtg cgc gat ctc ggc gtg aac gca tac cgg ttc tcg att ggc gtg ccg cgc gtc atc CCC acc ccg
gac ggc aag ccg aac gag aag ggc ctc gat ttc tac gag cgc att gtc gac cag ctg ctc gaa tac
ggc atc gac ccg att gtg acg ctc tac cat tgg gat ctg ccg cag tat ctg aac gaa gat ccg tac
cgg gat ggc tgg ctg aac cgt gag acc gcg ttc cgc atg gcg gag tat gcc ggc att gtg gcc aag
cgc ctc ggc gac cgt gtg cac acc tac acc acg ctc aac gaa ccg tgg tgc tcg gcg cac ctg agc
tac ggc ggc acc gag cat gcc CCC ggc ctg ggc gcc ggc ccg ctc gcg ttc cgc gcc gcc cat cac
ctg aat ctg gca cat ggt ctg atg tgc gag gca gtg cgt gcc gag gcc gga gcg aag ccg gat ctc
tcg gtg acg ctg aat ctg cag gtg aac cgc ggt gat gcg gat gcc gtg cac cgc gtg gat ctc att
gcc aac cgc gtg ttc ctc gat ccg atg ctg cgc ggc tac tac ccg gac gag ctg ttc gca atc acc
aag gga atc tgc gat tgg gac ttc gtg cat gac ggc gat ctc aag ctc atc aac cag ccg att gac
gtc ctg ggg ctt aat tat tac tcg acg aat ctg ctc gcc atg agc gac cgc ccg cag ttc ccg cag
agc acc gag gcc tcc acc gcg ccg ggc gcc agc gac atc gac tgg ctg cct acc gac ggc ccg cac
acg cag atg ggg tgg aac atc gac ccg gat gcg ctt tat aac acg ctg gtt cgc ctg aac gac gac
tac gac cac att ccg ctc gtc gtc act gaa aac ggc atg gcg tgc CCC gac gag gtg gaa gtc ggc
ccg gat ggt gtg aag atg gtg cac gac gac gac cgc atc gac tac ctg cgt cgc cat ctc gag gcc
gtc cac cgc gcg atc gag gag ggg gcg aat gtc atc gga tac ttc gtg tgg tcg ctg atg gat aat
ttc gag tgg gcg ttc ggc tac gac cgc cgc ttc ggc ctg acc tac gtg gac tac gac acc gag gag
cgc ATA cgg aag gac agc tac aac tgg tac cgt aac ttc atc gcc gag cac tcc gcc aag tag

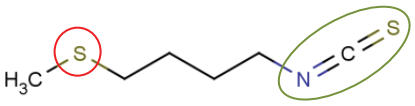
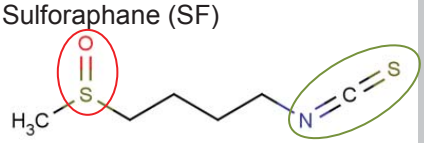
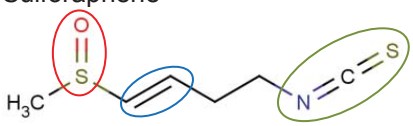
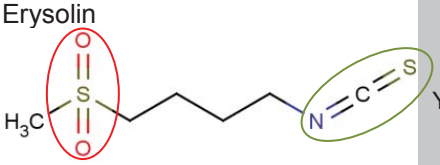
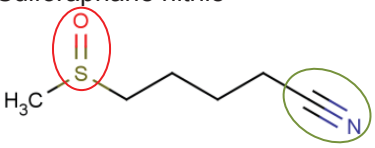
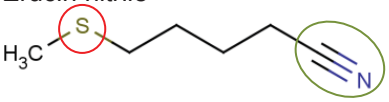
The length is: 1383 nucleotides

Number of total single rare Arg codons: 0
Number of tandem rare Arg codon double repeats: 0
Number of tandem rare Arg codon triple repeats: 0

Figure A.18. The web based software RaCC (rare codon calculator) was used to check for codon bias for the lactobacilli genes encoding putative glycoside hydrolases. Here is an example of one of these genes showing three rare proline codons (orange) and one rare isoleucine (blue). Website: http://nihserver.mbi.ucla.edu/RACC/ . Last accessed February 2010

Table A1. Comparison of the bioactivity of 6-carbon ITCs that vary in oxidation state and side chain structure.

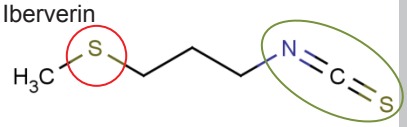
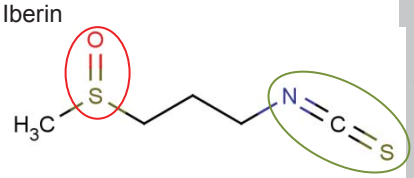
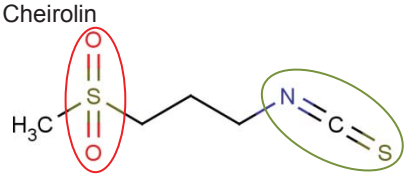
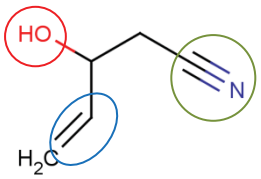
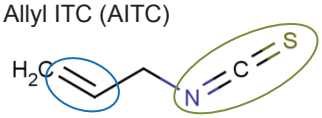
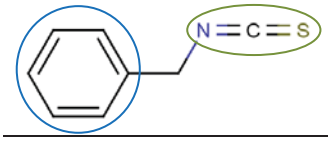
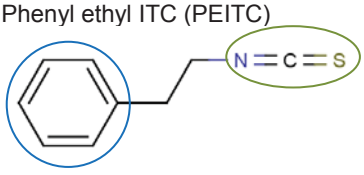
Oxidation state
Reactive element
ITC or nitrile

	Phase II Induction potential			Apoptosis	Anti-proliferative
	Quinone reductase (QR) [1-3]	Glutathione-S-transferase (GST) [2,4-7]	Thioredoxin reductase	Inducing via (caspase 8 or 9)	Cell cycle arrest (IC_{50})* µM
Erucin 	Yes [1-3]	Yes similar to SF [2,4-7]	1 mM	Poor (2%)	15 [8]
Sulforaphane (SF) 	Yes [1-3]	Yes [2,4-7]	1 mM	Good (10%)	6.6 [9]
Sulforaphene 	Yes [6]	Yes [6]			15 [10]
Erysolin 	Yes	Yes		Yes	
Sulforaphane nitrile 	Poor inducer [11,12]	Poor inducer [11,12]			
Erucin nitrile 	ND	Yes			8

*IC_{50} defined as inhibitory to 50% *in vitro* K562 cells [10] Blank: No Data available

Table A2. Comparison of the bioactivity of 5-carbon ITCs, aromatic ITCs and 3-Hydroxy-4-pentenenitrile (crambene).

Oxidation state
Side chain
Active site

	Phase II Induction potential			Apoptosis	Antiproliferative
	Quinone reductase [1-3]	Glutathione-S-transferase [2,4-7]	Thioredoxin reductase [13]	Inducing via (caspase 8 or 9) [8,14-18]	Cell cycle arrest (IC_{50})* µM [8,10]
Iberverin 	Yes [2]	Yes [2]			
Iberin 	Yes [2]	Yes [2]	Yes [13]		
Cheirolin 	No [2]	No [2]		No [2]	Yes [10]
Crambene 	Yes [2]	Yes [2]		Yes [14]	Yes 164
Allyl ITC (AITC) 	Yes [2,19]	Yes [2,19]		Yes [18]	
Benzyl ITC (BITC) 	Yes 1 mM	Yes 5 µM [3]		Yes [17]	Yes 1.5
Phenyl ethyl ITC (PEITC) 		Yes [3]			

*IC_{50} defined as inhibitory to 50% *in vitro* K562 cells [10]

Blank: No Data available

References

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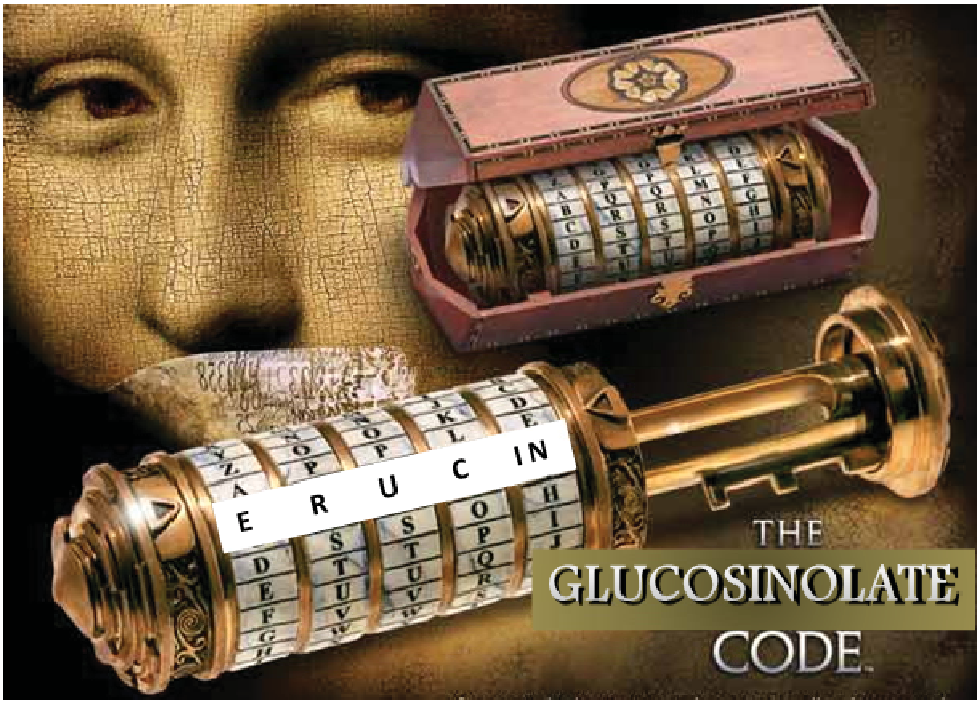

Figure (4X). Suggested solution to the problem

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Jane Adair Mullaney

Name/Title of Principal Supervisor: Julian Heyes

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Experimental design, carried out experiments, data analysis
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Lactic Acid Bacteria Convert Glucosinolates to Nitriles Efficiently Yet Differently from Enterobacteriaceae

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ABSTRACT: Glucosinolates from the genus *Brassica* can be converted into bioactive compounds known to induce phase II enzymes, which may decrease the risk of cancers. Conversion via hydrolysis is usually by the brassica enzyme myrosinase, which can be inactivated by cooking or storage. We examined the potential of three beneficial bacteria, *Lactobacillus plantarum* KW30, *Lactococcus lactis* subsp. *lactis* KF147, and *Escherichia coli* Nissle 1917, and known myrosinase-producer *Enterobacter cloacae* to catalyze the conversion of glucosinolates in broccoli extract. Enterobacteriaceae consumed on average 65% glucoiberin and 78% glucoraphanin, transforming them into glucoiberverin and glucoerucin, respectively, and small amounts of iberverin nitrile and erucin nitrile. The lactic acid bacteria did not accumulate reduced glucosinolates, consuming all at 30−33% and transforming these into iberverin nitrile, erucin nitrile, sulforaphane nitrile, and further unidentified metabolites. Adding beneficial bacteria to a glucosinolate-rich diet may increase glucosinolate transformation, thereby increasing host exposure to bioactives.

KEYWORDS: *Bacterial myrosinase, glucosinolates, broccoli, isothiocyanates, gut bacteria*

INTRODUCTION

Epidemiological studies have shown that people who consume *Brassica* vegetables have a reduced risk of certain types of cancer, in particular pancreatic, bladder, colorectal, and prostate cancers.[1] This is thought to be due to cruciferous plant *β*-thioglucoside-*N*-hydroxysulfates or glucosinolates (GSLs), which, when consumed, can be converted into bioactive compounds capable of inducing a phase II response from the host. It is this induction of phase II antioxidant enzymes that helps to provide protection from carcinogens and oxidative stress.[2]

Making GSLs bioactive requires removal of the sulfur-linked glucose. Enzymatic removal of the glucose is performed by the thioglucosidase enzyme myrosinase (EC 3.2.1.147). If endogenous plant myrosinase is inactivated by cooking or long-term storage,[3−5] hydrolysis of the GSLs in the gut relies on activity by either the host enzymes or the resident microflora.

The isothiocyanates (ITCs), which are one of the possible products of GSL transformation, have been investigated extensively for their role in cancer chemoprevention.[6,7] Their mode of action is thought to be due to either a chemopreventative (delaying or reversing damage) or a therapeutic effect by promoting cancer cell death (apoptosis), or both. Broccoli contains several GSLs,[7] and the one at highest concentration, glucoraphanin, can be converted to the ITC sulforaphane, a known potent inducer of phase II enzymes.

A number of microorganisms are capable of metabolizing GSLs, including *Escherichia coli*,[8] *Enterobacter cloacae*,[9] *Bacillus cereus*,[10] *Lactobacillus agilis*,[11] *Lb. gasseri*, *Lb. acidophilus*, *Lb. casei*, *Lb. plantarum*,[12] *Bifidobacterium pseudocatenulatum*, *B. adolescentis*, *B. longum*,[13] and *Bacteroides thetaiotaomicron*.[14] In the absence of active plant myrosinase, intestinal microbiota with myrosinase activity have the potential to transform dietary GSLs into bioactive compounds that in turn increase the beneficial chemopreventative or therapeutic effects.

The aim of this study was to identify beneficial lactic acid bacteria that were efficient at GSL metabolism. We screened several lactic acid bacteria[15−18] (Table 1) for myrosinase activity, and when we found that all had similar activity (data not shown), we selected *Lactobacillus plantarum* KW30 (KW30) and *Lactococcus lactis* subsp. *lactis* KF147 (KF147) to continue with. We then compared the *in vitro* transformational efficiency of these against two members of Enterobacteriaceae, *Escherichia coli* Nissle 1917 (Nissle), which is also considered beneficial, and *Enterobacter cloacae* ATCC13047 (*E. cloacae*), a known myrosinase-producing organism, by culturing each in a GSL extract derived from broccoli seed.

MATERIALS AND METHODS

Glucosinolate Purification. Broccoli extract was prepared using freeze-dried broccoli seeds (kindly provided by Comvita NZ Ltd.,

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Table 1. List of Bacterial Strains in This Study[a]

	strain	YP_003064398.1	ZP_07078860.1	ref
Lactic Acid Bacteria				
Lactobacillus plantarum	KW30	×	×	15
Lactobacillus plantarum	WCFS1	×	×	BAA-793
Lactobacillus plantarum	ATCC8014		×	ATCC8014
Lactobacillus plantarum	ATCC14917	×	×	ATCC14917
Lactobacillus plantarum	NC8	×	×	16
Lactobacillus brevis	LMG11437		×	ATCC367
Lactobacillus sakei	LB790	×	×	17
Lactococcus lactis subsp. *lactis*	KF147		×	18
Lactococcus lactis subsp. *lactis*	KF152		×	18
Lactococcus lactis subsp. *lactis*	KF282		×	18
Enterobacteriaceae				
Escherichia coli	1917 Nissle			DSM6601
Enterobacter cloacae	ATCC13047			ATCC 13047

[a]GH1 family genes YP_003064398.1 and ZP_07078860.1 from lactic acid bacteria were used to help select which bacteria were screened for glucosinolate metabolism.

Paengaroa, NZ). Powdered seed was chosen over broccoli sprouts or mature broccoli because it provided a quality source of GSLs having been fully characterized for purity and glucoraphanin content (~12% w/v). The powdered seed was defatted using a method previously described[19] but adapted as follows: To 40 g of powder was added 300 mL of *n*-hexane (Mallinckrodt), and the mixture stirred for 30 min. The liquid was decanted and replaced with another 300 mL of *n*-hexane and stirred for a further 30 min, after which the liquid was removed and the residue evaporated to dryness. The dry residue was then dissolved in 100 mL of 50 mM potassium phosphate buffer (pH 7.2) preheated to 80 °C, and the solution incubated at this temperature for 45 min to inactivate any endogenous myrosinase. Zinc/barium acetate (1:1) in a final concentration of 20 mM was added to the cooled extract, and this was left to precipitate for 30 min at 4 °C. The extract was then centrifuged at 4000*g* for 30 min at 4 °C, and the supernatant collected. Further purification was performed using a method described by Rochfort et al.[20] The extracted solution was evaporated under vacuum at 40 °C until a yellow, clear residue remained, and this residue was dissolved in Milli-Q purified water. Quantification of the GSL concentration was obtained by reading the absorbance at 229 nm using sinigrin (Sigma S1647) as a reference standard. All reagents were purchased from Thermo Fisher Scientific New Zealand Ltd. except for the zinc acetate, which was purchased from Merck Ltd., New Zealand. Due to pure standards not being available for all of the GSLs, we used the GSL sinigrin as a standard to normalize the LC-QTOF-HRMS data. The composition of our GSL extract is shown in Table 2.

Bacterial Growth. The bacterial strains used are shown in Table 1. Strains were cultured at 28 °C for *Lc. lactis*, 30 °C for *E. cloacae*, and 37 °C for *E. coli* and the *Lactobacillus* species. For the GSL assays, bacteria were grown at 37 °C to simulate body temperature and started from a 1% inoculum into the appropriate medium: either 'de Man, Rogosa and Sharpe' or reinforced clostridia medium (both from Oxoid, Auckland, NZ). The chosen medium was used with or without additional broccoli GSL extract. A minimal medium was also used for testing sugar preference for GSL degradation. This medium was adapted[21] and contained trypticase peptone 2%, sodium acetate 0.1%, ascorbate 0.05%, $MgCl_2$ 0.2%, $MnCl_2$ 0.02%, and cysteine 0.5% in 50 mM potassium phosphate buffer pH 7.2.

Bacterial Selection. Plant myrosinase (thioglucosidase) is a member of glycoside hydrolase family 1 (GH1) (www.cazy.org), but while GH1 family enzymes with diverse substrate specificities are commonly found in bacteria, none have been functionally characterized as having thioglucosidase activity. *Lb. plantarum* KW30 has recently been shown to produce a glycopeptide bacteriocin in which sugars are S-linked to a cysteine,[22] and because of this, we hypothesized that KW30 would be a candidate for a potential thioglucosidase producer. Analysis of the draft genome sequence of *Lb. plantarum* KW30 highlighted a region encoding GH1 family enzymes, together with beta-glucoside-specific PTS system transporters. The genes for two GH1 family enzymes with best Blast matches to 6-phospho-beta-glucosidase (YP_003064398.1) and beta-glucosidase (ZP_07078860.1) were selected as likely candidates. As listed in Table 1, several lactobacilli and lactococci that had one or both of these genes were screened and found to be similar at GSL metabolism (data not shown), and so we selected *Lb. plantarum* KW30 and *Lc. lactis* KF147 as representative species for this study. Previous work by Tani and co-workers[9,23] identified *Enterobacter cloacae* #506 as a myrosinase-producing organism, and the genome sequence for *E. cloacae* ATCC 13047 contains genes for GH1 family enzymes (GenBank accession numbers CP001918, CP001919, and CP001920).[24] We therefore included this strain as a putative myrosinase-positive organism to compare with the lactic acid bacteria. We also included *E. coli* Nissle because it is considered to be beneficial to the host,[25] to see whether it also had GSL-degradation capability.

Table 2. HPLC Analysis of Glucosinolates in Broccoli Seed Extract

chemical name and common name (italics)	MW	relative abundance (%)	retention time (min)
3-methylsulfinylpropyl glucosinolate (*glucoiberin*)	422.025	17.2	1.2
2-hydroxy-3-butenyl glucosinolate (*progoitrin*)	388.042	4.8	1.5
2-propenyl glucosinolate (*sinigrin*)	358.026	2.0	1.7
4-methylsulfinylbutyl glucosinolate (*glucoraphanin*)	436.041	48.0	1.8
5-methylsulfinylpentyl glucosinolate (*glucoalyssin*)	450.056	0.4	2.9
3-butenylglucosinolate (*gluconapin*)	372.044	1.3	3.0
3-methylthiopropylglucosinolate (*glucoiberverin*)	406.032	1.7	3.7
4-methylthiobutyl DS glucosinolate(*desulfoglucoerucin*)	341.092	1.4	4.3
4-methylthiobutyl glucosinolate (*glucoerucin*)	420.046	15.0	5.0
3-indolylmethyl glucosinolate (*glucobrassicin*)	447.053	0.4	5.1
3-butenyl DS glucosinolate (*desulfogluconapin*)	294.513	4.9	6.9
n-hexyl glucosinolate	402.089	0.6	8.3
1-methoxy-3-indolylmethyl glucosinolate (*neoglucobrassicin*)	477.063	0.2	8.6

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Table 3. MS Spectral Signatures of Identified End Products of Glucosinolate Metabolism after Incubation of Broccoli Seed Extract with Bacterial Cultures or in Acid Medium for 24 h

name	retention time (min)	mass	MS spectral data m/z (% relative abundance)
iberverin nitrile, *4-(methylthio)butanenitrile*	6.7	115.20	115 (40), 88 (0.1), 74 (5.3), 68 (12), 62, (12), 61, (100), 48(17), 45 (48), 44 (10), 41 (36)
erucin nitrile, *5-(methylthio)pentanenitrile*	8.4	129.22	129 (72), 114 (11), 82 (68), 61 (100)
iberverin, *3-(methylthio)propyl isothiocyanate*	10.4	147.26	147(11), 101(100), 72(38), 61(43), 45(26), 41(38)
erucin, *4-(methylthio)butyl isothiocyanate*	11.5	161.28	161 (18), 115 (66), 85 (20), 72 (57), 61 (100)
iberin nitrile, *4-(methylsulfinyl)butanenitrile*	11.3	131.19	131(21), 115(3), 87(2), 68(39), 64(68), 41(100)
sulforaphane nitrile, *5-(methylsulfinyl) pentanenitrile*	12.8	145.22	145 (19), 129 (9), 82 (42), 64 (54), 55(100)
iberin, *3-(methylsulfinyl)propyl isothiocyanate*	13.7	163.26	130 (5), 116 (20), 100 (15), 86 (9), 72 (100), 63 (34), 61 (18), 56 (13), 41 (98), 39 (56)
sulforaphane, *4-(methylsulfinyl)butyl isothiocyanate*	15.3	177.28	160 (47), 114 (6), 72 (100), 64 (16), 55 (45)

For all GSL degradation experiments, 10 mL of an overnight culture was pelleted, washed twice by resuspending the pellet in sterile 50 mM potassium phosphate buffer, and then centrifuged at 10000*g* for 10 min, and the cells were resuspended in 10 mL of sterile potassium phosphate buffer (pH 7.2). Two percent inocula were used for cultivation in the minimal medium supplemented with 0.3% (w/v) sugar, 0.3% (w/v) GSLs, or both. Growth was determined by measuring optical density at 600 nm (OD_{600}) and by the most probable number method.[26] For GSL metabolism experiments, all bacteria were incubated in the appropriate growth medium supplemented with GSL extract (0.3 (w/v)) for 24 h, and then the spent medium was clarified by centrifugation at 10000*g* for 10 min. The supernatant was removed and filtered, and the products of GSL metabolism were extracted and assessed by GC-MS and LC-QTOF-HRMS.

LC-QTOF-HRMS. LC-MS grade acetonitrile was from Fischer Scientific, methanol (ChromAR) was from Mallinckrodt Chemicals, and ethanol (95%) was from LabServ. The LC-MS system was composed of a Dionex Ultimate 3000 Rapid Separation LC system and a microTOF QII mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an electrospray source operating in positive mode. The LC system contained an SRD-3400 solvent rack/degasser, an HPR-3400RS binary pump, a WPS-3000RS thermostated autosampler, and a TCC-3000RS thermostated column compartment. The analytical column was a Zorbax SB-C18 2.1 × 100 mm, 1.8 μm (Agilent, Melbourne, Australia) maintained at 50 °C and operated in gradient mode. Solvents were A = 0.5% formic acid and B = methanol/water (90:10) at a flow of 400 μL/min. The gradient was 99% A, 1% B, 0−0.5 min; linear gradient to 70% A, 30% B, 0.5−8 min; linear gradient to 25% A, 75% B, 8−13 min; linear gradient to 100% B, 13−15 min; composition held at 100% B, 15−17 min; linear gradient to 99% A, 1% B, 17−17.2 min; to return to the initial conditions before another sample injection at 21 min. The injection volume for samples and standards was 1 μL. The microTOF QII source parameters were as follows: temperature 200 °C; drying N_2 flow 8 L/min; nebulizer N_2 1.5 bar; end plate offset −500 V; capillary voltage −3500 V; mass range 100−1500 Da; acquired at 2 scans/s. Postacquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis. GSL components were quantified using QuantAnalysis (Bruker Daltonics) by extracting ion chromatograms with mass windows of 10 mDa. Samples were prepared for analysis by adding 100 μL of supernatant to a vial containing 900 μL of 5 μg/mL epicatechin in water.

GC-MS. GC-MS analyses employed a Shimadzu 17A GC coupled to a Shimadzu 5050A quadrupole mass detector spectrometer with a source temperature of 240 °C. One microliter injections were made into the injection port at 220 °C. Standard separations of compounds used a 30 m × 0.25 mm i.d. × 0.18 μm film thickness Rxi-5 ms (Restek) capillary column with a helium flow of 1 mL min^{-1}. The oven temperature program was 60 °C for 1 min, 10 °C min^{-1} to 300 °C, and held for 1 min. Samples were prepared for analysis as follows. One milliliter of aqueous extract was added to 2 mL of dichloromethane (Sigma-Aldrich 650463) containing either internal standard BITC (Aldrich 252492) or tetradecane (Aldrich 17246) at a concentration of 1:2000; the tube was tightly capped, shaken, and mixed by inversion for 15 min. The samples were centrifuged for 5 min at 2000*g* to separate the phases, and the organic phase (bottom layer) was carefully transferred to a vial using a glass Pasteur pipet. To generate the individual compounds, we hydrolyzed our broccoli extract with and without myrosinase, at pH 9 and 4. For myrosinase hydrolysis, the 50 mM potassium phosphate buffer was adjusted to either pH 4 or 9 and contained 0.5 mM ascorbic acid. To improve GSL conversion to nitriles, 10% of a 100 mM ferric chloride (Sigma F2877) solution was included in the pH 4 myrosinase reaction. For acid hydrolysis the broccoli extract was added to pure water and the pH adjusted to 4 with HCl. Compounds were identified by matching peaks to our pure standards (sulforaphane (Sigma S6317), BITC (Sigma W510548), AITC (Sigma 377430)) and from our library constructed from published data for individual compounds. We also checked retention times against the previously published data, where the only differences were in the number of carbons.[27−29] The identified compounds, their spectral data, and retention times are shown in Table 2. The areas under the peaks identified were normalized to the internal standard, and the partitioning efficiency was validated empirically using triple solvent extraction. On the basis of the efficiency and where possible, sulforaphane was used to generate a standard curve for estimating nitrile concentration.

RESULTS AND DISCUSSION

Analysis of Broccoli and Composition. Analysis of the broccoli extract confirmed that the major GSLs present were glucoraphanin, glucoiberin, and glucoerucin with minor amounts of glucoiberverin, gluconapin, desulfogluconapin, progoitrin, desulfoglucoerucin, sinigrin, glucobrassicin, *n*-hexylglucosinolate, and neoglucobrassicin, as shown in Table 2. The GSLs that we chose to study in detail were glucoraphanin, glucoiberin, glucoerucin, and glucoiberverin.

Bacterial Growth Was Not Inhibited by the Presence of GSLs. Media supplemented with pure GSL extract (0.3% (w/v)) and incubated for 4 days with bacteria did not inhibit bacterial growth, as determined by taking optical density measurements at 600 nm and also by using the "most probable number" (MPN) method [25] (data not shown). Bacteria that were cultured in identical medium without GSLs increased in optical density similarly to those grown with GSLs, remained viable using MPN, but did not accumulate any GSL degradation products. When bacteria were cultivated in minimal medium without any added sugar, KW30 and KF147 grew poorly (as assessed by optical density at 600 nm);

however, all groups were still viable after 5 days when enumerated by MPN (data not shown).

Incubation of Lactic Acid Bacteria with Glucosinolates. Both representative lactic acid bacteria produced similar results. The concentration of the individual GSLs in the medium as determined by LCQTOF-HRMS decreased by up to 46% in the presence of either over 24 h (Figure 1).

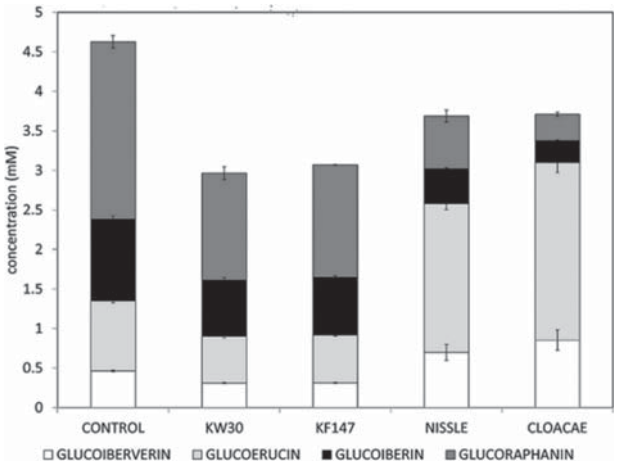

Figure 1. Glucosinolate profile of culture medium as determined by LC-QTOF-HRMS after 24 h anaerobic incubation of named bacteria with broccoli seed powder extract (BSP) at 37 °C. Control samples had broccoli seed extract added to uninoculated culture medium. The Enterobacteriaceae interconversion of GSLs can be seen as glucoraphanin and glucoiberin decrease, while their reduced forms glucoerucin and glucoiberverin increase. Error bars = standard deviation (n = 3).

Conversion from GSLs to nitriles was observed, and GC-MS analysis identified iberverin nitrile, erucin nitrile, and sulforaphane nitrile in the supernatant (Figure 2). Not all the

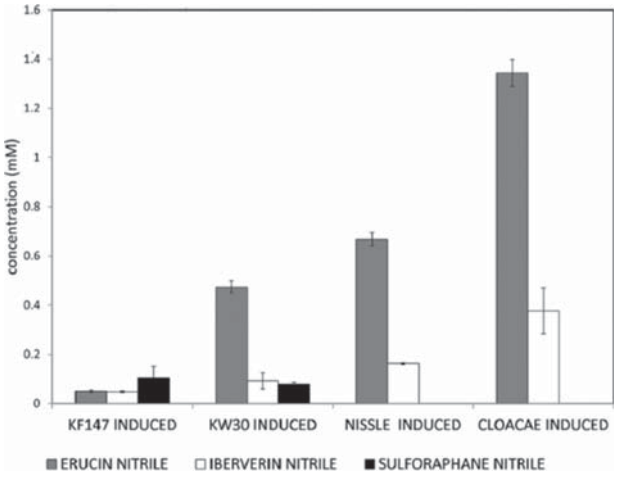

Figure 2. Concentration (mM) of individual nitriles in culture medium after 24 h incubation with named bacteria that had been induced by prior anaerobic culture with broccoli seed extract for 24 h. Erucin nitrile was the predominant product followed by iberverin nitrile, with *E. cloacae* producing the most. No sulforaphane nitrile was detected for Nissle or *E. cloacae*, whereas for lactic acid bacteria KW30 and KF147 we detected sulforaphane nitrile as well as erucin nitrile and iberverin nitrile. Error bars = standard deviation (n = 3).

GSLs were accounted for, and our results were similar to others, suggesting that further conversion had occurred.[12] Nitrilases ubiquitous among the plant kingdom are known to be produced by GSL-producing plants which, during catabolism of their GSLs, are able to recycle nitrogen as ammonia, and we hypothesize that some of the metabolites yielded from the consumption of GSLs were further metabolized by bacterial nitrilases.[30] Because previous literature had shown that sinigrin-degrading ability could be induced by culturing the organism in the presence of sinigrin,[11] we also precultured in the pure GSL extract and found that only KW30 transformed more GSLs into nitriles when precultured in media containing GSLs, increasing the nitrile production from 130 to 565 μM (Figure 3).

Incubation of Enterobacteriaceae with GSLs Resulted in Interconversion. Nissle and *E. cloacae* removed almost all of the glucoraphanin and glucoiberin from their media; however, glucoiberverin and glucoerucin increased concomitantly (Figure 1). The total nitriles detected in the supernatants of the Enterobacteriaceae were greater than for the lactic acid bacteria (Figure 2). As glucoiberverin and glucoiberin are the same molecules but in a different state of redox (as are glucoerucin and glucoraphanin), we concluded that the Enterobacteriaceae had not metabolized but had instead converted these GSLs by a reduction reaction, as interconversion between GSLs that are redox pairs has previously been observed.[31−33] To determine whether these bacteria were changing the sulfinyl GSLs glucoraphanin and glucoiberin to their reduced forms because of the anaerobic conditions, we cultivated Nissle and *E. cloacae* in media containing both GSLs and nitriles either anaerobically or aerobically by shaking for 24 h. Under both conditions, Nissle and *E. cloacae* grew similarly, as assessed by optical density (600 nm), and both interconverted glucoiberin and glucoraphanin to their reduced forms glucoiberverin and glucoerucin (Figure 4, anaerobic conditions). When we assessed the composition of nitriles formed by transformation of the GSLs, we found for Nissle that both aerobic and anaerobic conditions favored the production of iberverin nitrile and erucin nitrile, but for *E. cloacae*, aerobic conditions produced only trace amounts of iberverin nitrile and erucin nitrile (data not shown). We concluded that the presence of oxygen did not influence either the interconversion of sulfinyl GSLs or the end product favored, but for *E. cloacae*, aerobic conditions did appear to prevent the transformation of GSLs to nitriles.

Metabolism of the other GSLs, including progoitrin and sinigrin, was similar between the Enterobacteriaceae and the lactic acid bacteria (data not shown). Nissle produced indole with and without GSLs (as expected),[34] which was not due to transformation of any GSLs. Although low pH is known to promote the conversion of GSLs to nitriles rather than ITCs, it does not appear to be a determinant in formation of reduced over the oxidized species, as when we incubated the GSLs at pH < 3, the products were the expected ratios of oxidized and reduced (data not shown). We noticed that a previous study found also that controlling for pH throughout still resulted in erucin nitrile being the only product produced from glucoraphanin by microbiota.[35] As the GSLs did not degrade spontaneously when incubated at 37 °C and as bacteria did not accumulate any GSL degradation products unless they were cultivated in medium containing GSLs, the presence of these degradation products can be ascribed only to bacterial metabolism.

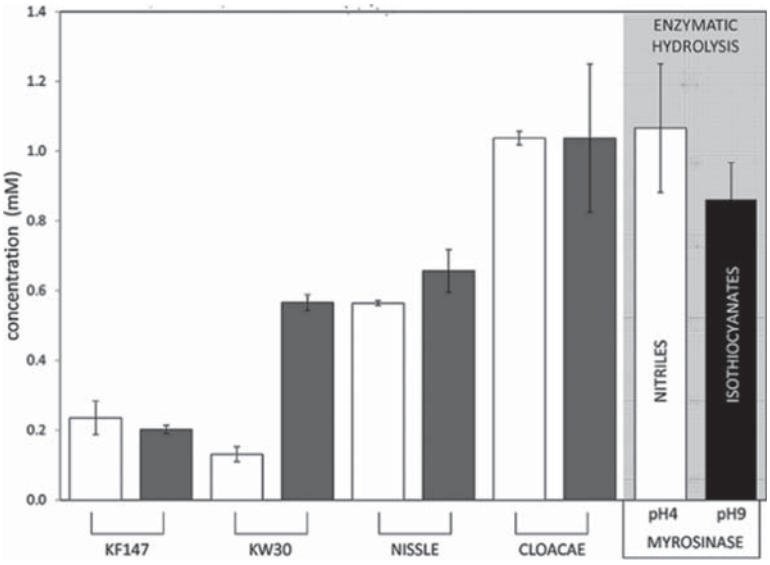

Figure 3. Total nitrile concentration in culture medium after anaerobic incubation of BSP with named bacteria after 24 h prior to incubation with or without BSP as assessed by GC-MS. An adaptive response was found only for KW30. Dark gray bars represent those precultured with GSLs (adapted); white bars without (GSL-naïve). The shaded box at the right contains the results from the enzymatic hydrolysis of BSP at pH 4 (nitriles) and pH 9 (isothiocyanates) after 4 h. Error bars = standard deviation (n = 3).

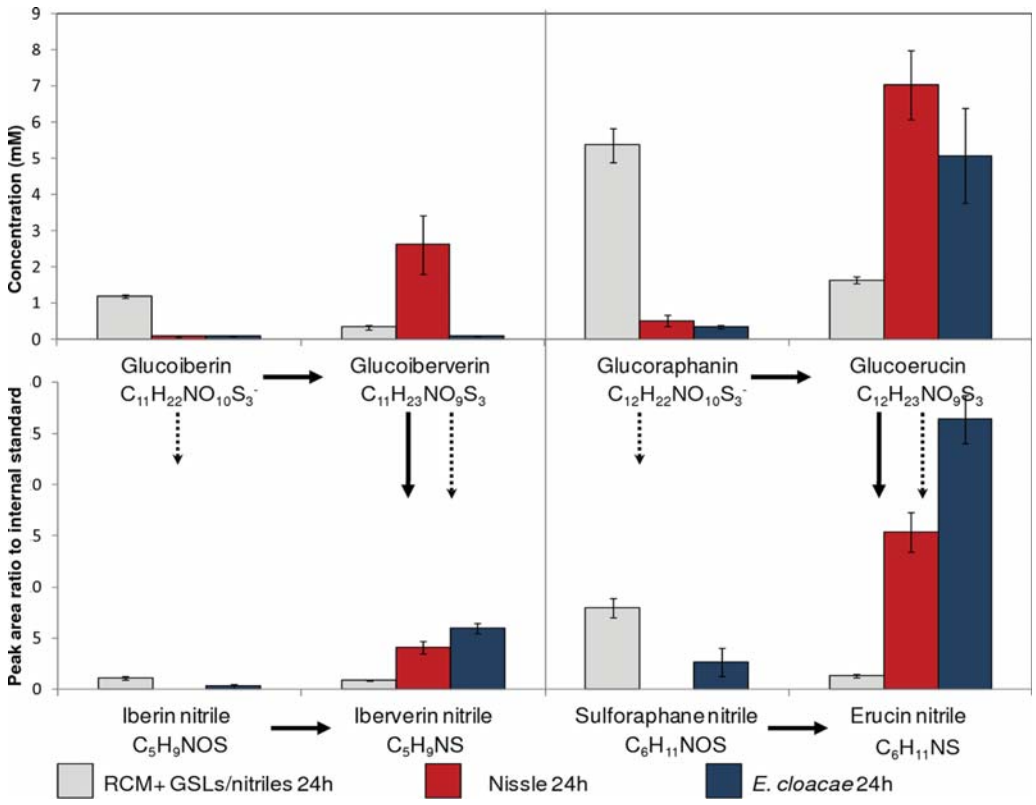

Figure 4. Comparison of the GSLs and nitriles shows that interconversion of GSLs occurred only for Nissle and *E. cloacae*. There were more of the reduced species iberverin nitrile and erucin nitrile than could be accounted for by hydrolysis of glucoerucin and glucoiberverin. This is because sulforaphane nitrile and iberin nitrile were also interconverted to erucin nitrile and iberverin nitrile. The dotted arrows indicate the conversions of glucosinolate to the corresponding nitrile when hydrolyzed under the appropriate acidic conditions. The emphasized solid arrows indicate the direction that interconversion of sulfinyl GSLs and nitriles actually took when bacteria were involved, which is only toward the thiol or reduced redox state. Nissle are in red, *E. cloacae* in blue, glucosinolates at the top, and nitriles beneath. All chemical structures were drawn using MarvinSketch (ChemAxon). Error bars = standard deviation (n = 5).

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Enterobacteriaceae and Oxidative Stress Response. The enzyme methionine sulfoxide reductase A (MsrA) EC 1.8.4.11 (ExPASy) involved in oxidative stress responses has previously been reported[36] and is known to be present in all aerobic organisms.[37] Although genomic data for Nissle are not available in the public domain, the structure and function of the *E. coli* peptide methionine reductase are known,[38] and *E. cloacae* ATCC13047 also carries a gene encoding the MsrA enzyme (NCBI ref: YP_003611138.1). Although KF147 and KW30 did not carry out this reduction reaction, we found sequence data for an MsrA enzyme for *Lb. plantarum* ATCC14917 (NCBI ref: ZP_07078054.1), *Lb. plantarum* JDM1 (NCBI ref: YP_003063253.1), and a MsrB-like enzyme for KF147 (NCBI ref: YP_003352658.1). The change in GSL redox was observed only for the sulfinylalkyl GSLs glucoraphanin, glucoiberin, and their nitriles. Although sulfoxidation can be reversible, we saw no evidence of it, and the amounts of the reduced products always increased over and above the oxidized species (Figure 4).

Bacterial Myrosinase and Beta-Glucoside-Specific PTS System Transporters. The results from the lactic acid bacterium KW30 (Figures 2 and 3) indicated that an increased GSL conversion through pre-exposure to GSLs had occurred (induction effect). The possibility that these bacteria recognized, bound, or catabolized the sugar moiety of the GSLs through an alternative sugar catabolism or uptake pathway was investigated. As our choice of selecting KW30 was based on the genes encoding GH1 family enzymes and these were found together with beta-glucoside-specific PTS system transporters, we looked further to see whether a particular sugar transport system was involved in this induction effect. Minimal media were supplemented with glucose, galactose, arabinose, sucrose, lactose, or trehalose at 0.3%, and while growth was similar between groups (as verified by MPN and OD_{600}), no differences in GSL transformation were observed (data not shown). We concluded that the bacteria could not use the glucose linked to the GSL directly as a carbon source and that the carbohydrate transporter mechanism used for these sugars is not related to the mechanism used by the bacteria to take up GSLs.

GSLs Were Transformed into Nitriles. It is not nitriles but the ITCs that have been shown to elicit a chemoprotective and therapeutic effect by the induction of regulatory mechanisms including apoptosis pathways and the induction of phase II enzymes quinone reductase and glutathione S-transferase.[39] For example, sulforaphane is known to activate the transcriptional antioxidant and antinflammatory response element nuclear response factor (Nrf2) through interaction with inhibitor Keap1 and its associated ubiquitin ligase Cullen 3 (Cul3).[40]

GSLs are transformed into ITCs or nitriles by myrosinase, and previous studies have shown that in the absence of active plant myrosinase, bacteria are capable of this conversion of GSL to ITC.[14,35] Conversely, other studies have found that in the absence of plant myrosinase, very little conversion of GSLs occurs, and what does transform, does so into nitriles. This is thought to be due to the low pH conditions.[13] Sulforaphane nitrile is considered to have only poor phase II induction potential,[41,42] with only the nitrile crambene produced from progoitrin and nitrile 4-hydroxybenzyl cyanide from sinalbin shown to have phase II induction properties similar to ITCs *in vitro*.[43] We detected nitriles in our study not ITCs, and nitriles have been shown to have less phase II induction potential than ITCs. However, the hydrolysis products of glucosinolates, whether ITCs or nitriles, retain their R group, and this R group also has an impact on bioactivity.[44,45] Like erucin nitrile, iberin nitrile and iberverin nitrile share common features with sulforaphane nitrile and vary only by the number of carbons in their alkane chain and their oxidation state (see Figure 4). It may be assumed that they too will have poor phase II induction potential, but this may not be the same thing as having no bioactivity. *In vivo*, a host response independent of any phase II induction may still be occurring depending on the species of nitrile produced and the oxidation state of the sulfur in the R group.[31,46] GSLs, in particular glucoerucin, have been previously shown to have antioxidant properties,[47] and GSLs do pass through the body intact,[48] so during the digestion process the host may still gain increased antioxidant benefits from the GSLs if host gut bacteria interconvert GSLs to their reduced species and then transform these further to nitriles. The lack of ITCs in our products of biotransformation shows that the process is more complex than anticipated. However, this study supports the hypothesis that bacterial biotransformation of GSLs is performed by both lactic acid bacteria and Enterobacteriaceae. This shows for the first time that the GSL degradation capability of KW30 can be induced by preculturing in GSL-rich media and also that the interconversion of GSLs glucoraphanin to glucoerucin, glucoiberin to glucoiberverin, nitriles sulforaphane nitrile into erucin nitrile, and iberin nitrile into iberverin nitrile was due to the presence of facultative anaerobes Nissle and *E. cloacae*. The enzyme MsrA produced by Nissle and *E. cloacae* is the most likely explanation for these interconversions, as these reductases are known to reduce sulfoxides, which in turn enable the organism to inactivate reactive oxygen species (ROS). We propose that as all facultative anaerobic gut bacteria carry these genes, not only will the proportion and metabolic rates of facultative anaerobes in the gut determine the outcome of the metabolism of GSLs, but only GSLs, ITCs, or nitriles that contain a methyl sulfoxide at their terminal R group will be targets for bacterial sulfoxide reductases. Also, to gain the most benefit from the consumption of GSLs in the diet, the GSLs in our food should be those that can tolerate modification by our gut flora without losing significant bioactivity.

By combining extra beneficial bacteria with a GSL-rich diet, even in the absence of active plant myrosinase, it may be possible to increase the amount of GSL transformation and, in doing so, increase the protective effect the GSLs and their metabolites may confer against cancers. The reduced nitriles erucin nitrile and iberverin nitrile may have more or less effect on bioactivity in the host compared with sulforaphane nitrile and iberin nitrile, but as previous work has indicated that sulforaphane nitrile is less potent than sulforaphane[41] and erucin less potent than sulforaphane,[49] future work is planned to test these bacteria in an animal model to determine whether the consumption of these bacteria in combination with GSLs can increase the production of metabolites and determine what effect they may have on phase II enzymes in the host.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AITC, allyl isothiocyanate; BITC, benzyl isothiocyanate; BSP, broccoli seed powder extract; GC-MS, gas chromatography−mass spectrometry; GSL, glucosinolate; ITC, isothiocyanate; KF147, *Lactococcus lactis* subsp. *lactis* KF147; KW30, *Lactobacillus plantarum* KW30; LC-QTOF HRMS, liquid-chromatography quadrupole time of flight−high-resolution mass spectrometry

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Review

AQ1 # Biotransformation of glucosinolates from a bacterial perspective

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Abstract

Epidemiological studies have shown an association between the consumption of cruciferous vegetables and a reduced risk of certain types of cancers, in particular, pancreatic, bladder and colorectal. This is thought to be the result of the conversion of glucosinolates (GSLs) present in the vegetables into bioactive isothiocyanates (ITCs) that in turn stimulate a host response involving detoxification pathways. Conversion of GSLs is catalysed by the enzyme myrosinase, co-produced by the plant but stored in separate tissue compartments and brought together when the tissue is damaged. Myrosinase activity can be lost during storage of vegetables and is often inactivated by cooking. In the absence of active plant myrosinase the host's gut bacteria are capable of carrying out a myrosinase-like activity on GSLs in the lower gut. Several micro-organisms are known to be capable of metabolizing GSLs leading to the production of ITCs and nitriles, and this review examines the bacterial biotransformation of GSLs and a role for the microbiota in their bio-transformation.

Keywords: Bacterial myrosinase, Myrosinase, Sulphoraphane, Glucoraphanin, Gut bacteria, Human health, Cancer prevention.

Review Methodology: We searched databases within PubMed, Google Scholar, Elsevier, the Cambridge Journals, ACS publications, Wiley online library, ISI 'Web of Knowledge', 'Ovid' and Science Direct using keywords: glucosinolate; sulphoraphane, bacterial myrosinase and bacteria + myrosinase.

Eating Cruciferous Vegetables Reduces your Cancer Risk

Epidemiological studies have shown an association between the consumption of cruciferous vegetables and a reduced risk of certain types of cancers, in particular, pancreatic, bladder and colorectal [1–12]. A reason given for this is that these vegetables contain the bioactive compounds known as glucosinolates (GSLs). GSLs are secondary plant metabolites that have a biological effect on plants, fungi, insects and bacteria [13]. Originally named mustard oil glycosides, they occur in dicotyledons and in almost all species of the families Cruciferae, Resedaceae, Moringaceae and Capparidaceae. They have also been identified in species of Euphorbiaceae, Phytolaccaceae, Tropaeolaceae, Caricaceae and Rubiaceae [14, 15]. Some of the edible vegetables from the Cruciferae include cabbage, broccoli, kale, Brussels sprouts, mustard, radish and canola [4, 16].

The Structure of GSLs

GSLs are thought to provide plants with resistance to non-adapted pathogen and insect pests, and structural variations between GSLs are known to affect the plant's fitness in response to attack by pathogens or animal predators [17–19]. Genes that determine the variation

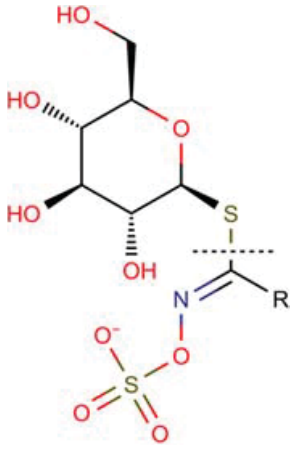

Figure 1 General structure for all GSLs, the dashed line showing the site where the glucose molecule is cleaved during hydrolysis.

have been identified, are known to be conserved across most Brassicaceae and seem to vary depending on the type of insect pest [20]. It has been suggested that the evolutionary changes are the result of reactions to the fluctuating insect populations and the diversity of GSLs reflect their differing biological actions. Another suggested possible function for GSLs has been as a nutrient storage system for sulphur and nitrogen [13, 18, 19].

Classification of GSLs

The number of reported GSLs described in the literature is reaching 200 [21] and they are classified according to their structural features which include alkyl, aromatic, benzoate, indole, multiple glycosylated and sulphur-containing side chains (for a comprehensive description, see reviews [4, 21]. There are also small groups of benzyl GSLs containing another glycosidic-linked sugar such as rhamnose or arabinose to the aromatic ring. While the significance of this is not clear, it is interesting that they are present in plants exploited for pharmacological properties [4].

Although there are other non-Brassicaceae including some Chinese herbal plants which also contain GSLs [15], interest has been focused on the Brassicaceae family, which contains around 350 genera and 3000 species because every member of this family tested to date has been found to contain GSLs [4].

Hydrolysis of GSLs Yields Nitriles, Thiocyanates and Isothiocyanates (ITCs)

Hydrolysis of GSLs occurs by enzymatic attack on the thioglucoside bond releasing D-glucose, a sulphate ion and an unstable aglycone that undergoes spontaneous rearrangement to form any one of several products as shown in Figure 1 [22–24]. The estimated activation energy for thermal degradation of allyl GSL (sinigrin) is 22.6 kcal/mole so under natural environment conditions spontaneous hydrolysis is unlikely to occur. GSLs will form nitriles, thiocyanates or ITCs if the sulphur-linked glucose molecule is removed either enzymatically or through acid hydrolysis or by non-enzymatic thermal degradation [24].

As Figure 2 shows, the end product is determined by several factors. Generally, hydrolysis at neutral pH leads to the formation of ITCs, whereas acid hydrolysis in the presence of ferrous ions and nitrile specifier proteins drives the reaction in favour of the formation of nitriles [18, 24, 25]. Other associated proteins referred to as 'epithiospecifier' proteins or 'epithiomodifier' proteins can direct the formation of epithionitriles [18, 26–29] while oxazolidine thiones (goitrin for example) are formed from cyclized hydrolysis products from GSLs such as progoitrin (2-hydroxy-3-butenyl glucosinolate).

Myrosinase

Enzymatic removal of the glucose is performed by myrosinase, an *S*-glucosidase enzyme [24]. Myrosinase demonstrates broad substrate specificity, and unlike *O*-glycosidases which are very common in nature, myrosinase is the only known *S*-glycosidase [16]. Glycosidases (or glycoside hydrolases) are classified according to whether they either retain or invert the end product and myrosinase is known to be a retaining enzyme consistent with its sequence similarity with family 1, the *O*-glycosidases [16]. Myrosinase is stored in plant tissue but compartmentalized away from GSLs, which prevents it from hydrolysing GSLs until the plant tissue becomes damaged [30, 31]. Damage by crushing or chewing breaks down the compartments and allows GSLs and myrosinase to come together.

GSLs to ITCs: ITCs and Toxicity

GSL-containing oilseed crops for many years have been produced as a supplementary feed for livestock (and more recently as fuel) [32, 33]. Cabbage feeding was first blamed for goitre in 1928 [34] and goitrin identified as the cause [35], while myrosinase had earlier been identified as 'myrosin, a thioglycosidase that hydrolyses mustard oil' [36].

A solution for preventing goitre was to reduce the toxicity of GSL hydrolysis products by selective breeding of low-GSL cultivars. Canola (*Brassica napus L.*) for example, is just one species that has intentionally had the GSL content reduced through selective breeding in order to increase palatability as well as decrease toxicity [26].

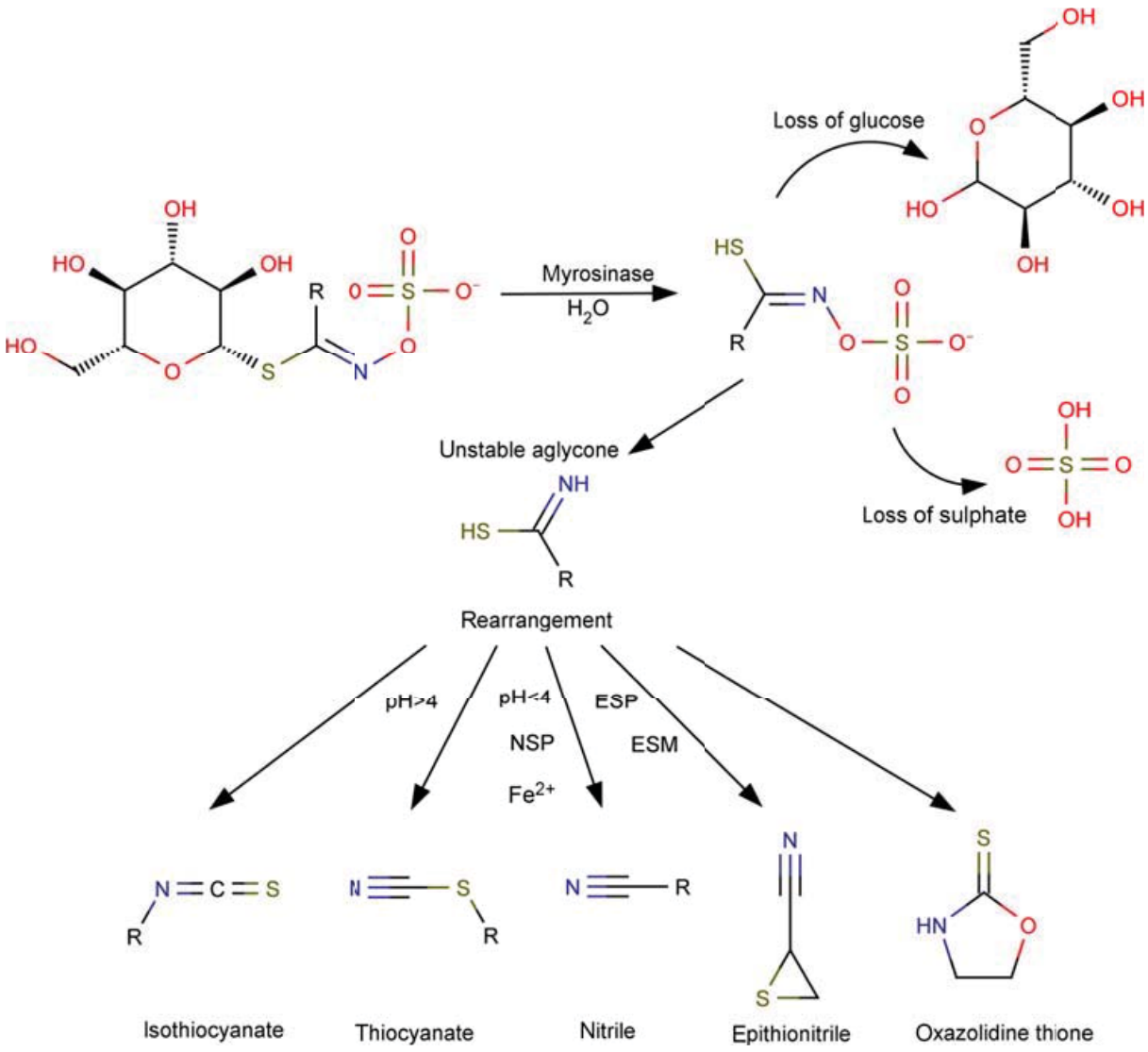

Figure 2 Hydrolysis of a GSL liberates a glucose molecule and an unstable aglycone intermediate shown in the centre. Rearrangement then releases a sulphate group and generates different products depending on the R-group characteristics, pH, ferrous ions (Fe^{2+}), and the presence of epithiospecifier (ESP), epithiomodifier (ESM) and nitrile specifier proteins (NSP).

The Host Response to ITCs

Phase II inducing, apoptosis inducing and anti-proliferative compounds

The body's detoxification system is responsible for the inactivation and elimination of toxins and xenobiotics and is a two-step process, usually referred to as the phase-I and phase-II system. Phase I and II enzymes, which catalyse the elimination of drugs and xenobiotics, are grouped according to their mode of action. Oxidative (phase I) enzymes include the dehydrogenases, oxidases and oxidoreductases, while the conjugative (phase II) enzymes include the reductases and transferases. A review of the molecular mechanisms of phase I and II enzymes was published in 2007 [37]. The detoxification system is dynamic, surveying everything that passes through the body and responding when necessary by producing enzymes to target and detoxify any molecules requiring elimination.

This has relevance to ITCs, which have been investigated extensively for their role in cancer chemoprevention [38–46]. ITCs from Brassicaceae exert either a chemopreventative (delaying or reversing damage) or a therapeutic effect by promoting cancer cell death (apoptosis) or both. They do this by modulation of metabolic pathways involved in the elimination of foreign compounds from the host, namely histone deacetylation systems, apoptotic pathways, antioxidant response pathways and the phase I and phase II enzymes.

Phase II enzymes may be induced, for example, one consequence of this induction by ITCs is that phase II

enzymes are able to directly and independently activate signalling pathways, which may have been switched off by developing cancer cells. One of these pathways is to apoptosis (programmed cell death) and once induced, cancer cells are destroyed [6, 7, 47–53]. Another consequence of phase II induction is that it is known to stimulate the antioxidant response pathways as well which are also protective [54].

Sulphoraphane and the phase I and phase II inducing potential of ITCs

While repeated intake of broccoli does not appear to be toxic [55] some have shown a mutagenic effect for sulphoraphane in cell culture, but for reasons that may be more the result of glutathione (GSH) depletion than cytotoxicity [48, 56]. Others show, however, that sulphoraphane is protective not mutagenic, elevating the concentration of nuclear response factor (Nrf2) dependent enzymes and GSH [57]. In another cell culture study, Nrf2 and GSH levels remained elevated in astrocytes for more than 20 h after stimulation [58]. Owing to their electrophilic properties, ITCs can damage DNA but the threshold for a damaging response depends on the reactivity of the species of ITC and the dosage. For example, when administered at between 10 and 20 μM, sulphoraphane can elicit intracellular reactive oxygen species (ROS) formation inducing single-stranded DNA breakage, leading to apoptosis [59] whereas single-stranded DNA breakage, generated by allyl isothiocyanate (AITC), has been found to be only temporary and is quickly resolved by the DNA excision repair systems [60]. The phase II induction and subsequent antioxidant capacity have also been assessed recently with sulphoraphane trialled as a protective agent against UV-light-induced skin cancer, with some success [61–63].

Although considered inactive, GSLs may be able to generate cellular responses just as ITCs do [64–66]. For example, it has been shown that GSLs can be absorbed intact [67] and *ex vivo*, glucoraphanin and glucoerucin have been found to elevate *O*-dealkylations of methoxy- and ethoxyresorufin cytochrome, which are markers for cytochrome P450 (Cyp450) proteins CYP1A1, CYP1A2 and CYP1B1. Both glucoraphanin and glucoerucin were also shown to elevate, if somewhat 'modestly', phase II enzymes, quinone reductase and GSTs [64].

Gene expression, regulation and damage

The silencing or un-silencing of genes can occur via changes in the DNA methylation state as well as through epigenetic modifications at the level of the histones, the proteins responsible for the packaging and order of DNA into nucleosomes. Although the mechanisms are not well understood, recent studies have implicated several candidates including ITCs that can cause accumulation of ROS and also induction of apoptosis proteins TRAIL, DR4 and DR5 [43, 50, 51, 68, 69].

Antioxidant response element, Nrf2 and Keap1

The antioxidant response element (ARE) mediates the transcriptional induction of a number of genes that make up the chemoprotective response system [70]. The role of Keap1 and how its oxidation status affects the stability and subsequent induction of Nrf2 has been elucidated [71–82]. A review of the mechanisms by which GSL breakdown products are thought to inhibit carcinogenesis including the ARE elements has been published recently [83].

Null genotype and GSH transferase

A person's genotype also determines the phase I and phase II response. A null genotype for GSH transferase genes *GSTM1* or *GSTT1* can affect the concentration of GSL metabolites in the blood and the rate of clearance from the body. In a 2005 study, it was found that while *GSTM1*-null genotypes did have significantly higher levels of sulphoraphane metabolites in plasma, they also had a greater rate of urinary excretion of sulphoraphane metabolites during the first 6 h after broccoli consumption, and also had a higher percentage of sulphoraphane excretion 24 h after ingestion [84]. In another study, it was shown that there was a positive association between ITC levels and lung cancer for *GSTM1*-null genotypes among women who had never smoked [85]. While the causes of this are as yet unknown, the authors suggested that this could be related to increased exposure to indoles leading to an unintended increased activation of phase I enzymes and ROS.

The biotransformation of GSLs

Apart from providing essential nutrients and fibre, cruciferous vegetables such as broccoli contain glucoraphanin as their major GSL, and this can be transformed by enzymatic hydrolysis into bioactive sulphoraphane. As discussed, sulphoraphane is a potent inducer of phase II enzymes in the host.

When broccoli is chewed or the tissue damaged, the broccoli myrosinase enzyme is released from within the cell compartment and is available to hydrolyse any GSLs it finds. However, myrosinase may be inactive as a result of storage or cooking [26, 86–90], so for GSLs to be transformed into ITCs requires other means of hydrolysis.

That other means could be by the bacteria that inhabit the gut.

The role of bacteria in the bioconversion of GSLs: a timeline from the past to the present

In 1949, the anti-thyroid GSL progoitrin was identified and isolated [35] and in 1959 Greer and co-workers were surprised to find that even after inactivating myrosinase, progoitrin was still being transformed to goitrin when eaten. They conceded that the current view (crucifers would be made safe by cooking) would 'need some revision' but did not speculate or comment why this hydrolysis might still be occurring [91]. In 1965, Oginsky *et al.* linked bacterial activity to the conversion of progoitrin to goitrin, introducing the bacterial myrosinase concept [33]. *Aspergillus niger* was added to the list of myrosinase producing organisms in 1969 [92]. Then, in 1974, bacteria found growing in a sinigrin solution were isolated, further characterized and taxonomically identified as *Enterobacter cloacae*. Following this, a bacterial myrosinase enzyme was purified and its myrosinase activity compared with the plant [93, 94]. The following year myrosinase activity was discovered in the yeast *Geotrichum candidum* [95] and in soil dweller *Bacillus cereus* in 1983 [96]. Between 1988 and 1993, germ-free or gnotobiotic rats were used by researchers to demonstrate that intestinal bacteria possessed myrosinase activity. Whether colonized with whole human microbiota or mono-associated, myrosinase activity could be observed [97–101].

In 1995 lactic acid bacteria were screened for sinigrin degrading potential: one strain, later identified as *Lactobacillus agilis* R16, demonstrated 'considerable' sinigrin degrading activity producing AITC as one of the end products [102].

By 1998, the Nugon–Baudon group, had followed their gnotobiotic rat studies with a study of the changes in Cyp450 enzymes resulting from intestinal microbiota in the presence of GSLs from myrosinase-free rapeseed [98]. They found that some phase I Cyp450 enzymes were modulated by GSLs and that microbiota were a prerequisite for a decrease in total Cyp450 to be observed. However, they identified that other unknown factors were involved because there were isoforms of Cyp450s that were elevated or depressed only in germ-free rats. Their results highlighted the complex interactions occurring within the host.

Also in 1998, Shapiro *et al.* showed that when the bowel microbiota was reduced by mechanical cleansing and antibiotics, the conversion of GSLs became negligible [3], confirming the important role of bacteria in the biotransformation of GSLs. In 1999, GSLs were shown to be converted to ITCs even when plant myrosinase was completely inactivated. Getahun *et al.* incubated cooked watercress juice with fresh human faeces under anaerobic conditions and found 18% of GSLs were hydrolysed to ITCs within 2 h: they attributed the hydrolysis to the microbiota (enteric bacteria) present [103].

In 2001, the types of GSLs and bacteria being investigated were extended. Gnotobiotic rats were colonized with a human digestive strain of *Bacteroides thetaiotaomicron* and dosed with pure sinigrin (allyl GSL) which yielded AITC or allyl cyanide (ACN) after hydrolysis [104]. In this case, the appearance of AITC or ACN could only be the result of bacterial degradation and these were found only in the gastrointestinal tracts of the rats' monoassociated with *B. thetaiotaomicron*. This year also, nuclear magnetic resonance spectroscopy (NMR) was employed to enable more sensitive analysis and identification of the degradation products that sinigrin and glucotropaeolin were converted to by human microbiota [105].

In 2002, an *in vitro* large intestine model was used in combination with pooled human colonic microbiota containing species of *Enterococcus, Enterobacter*, *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Clostridium* to demonstrate the conversion of sinigrin to AITC although not all of the ITC was accounted for [106]. Gnotobiotic rats were used again in 2003 to determine the influence of plant and bacterial myrosinase activity: the fate of sinigrin and benzyl GSL as they passed through the gastrointestinal tract was compared, with or without microbiota [107]. They concluded that the bacterial contribution was minimal, so the bacterial status of the host was irrelevant and plant myrosinase was the key to the generation of ITCs. In rats colonized with whole human microbiota, the recovery of ITCs in urine was less than the germ-free animals. It was theorized that not all ITCs were able to be accounted for because in the colonized hosts the bacteria were able to transform ITCs into other products that were not ITCs. Supporting this hypothesis was the observation that benzyl isothiocyanate (BITC) that was added to the GSLs and fed as a biomarker or internal standard to enable quantitative analysis of the recovery of ITCs had 'disappeared' during the process. The authors commented that it was hard to quantitatively detect anything because of background excretion products. As *E. cloacae*, a commensal gut bacteria found in humans is known to degrade BITC into benzyl amine and hydrogen sulphide and was almost certainly present in the +flora group, this may explain the disappearance of BITC [108]. In the bacterial transformation of GSLs, not all of the products are accounted for, and those that are detected may not be the expected products. In a study of intestinal bacteria using human strains of *Bifidobacterium* (*B. pseudocatenulatum* JCM 7040, *B. adolescentis* JCM 7045 and *B. longum* JCM 7050), sinigrin and benzyl GSL were added and resulted in the production of their corresponding nitriles 3-butenenitrile and 2-phenylacetonitrile, rather than the expected ITCs AITC and BITC that were barely detectable in the broth [109].

Is bacterial bioconversion significant?

So is the role of the bacterial bioconversion of GSLs a significant one? Vermeulen *et al.* downplayed the bacterial contribution [110] because they found from their human

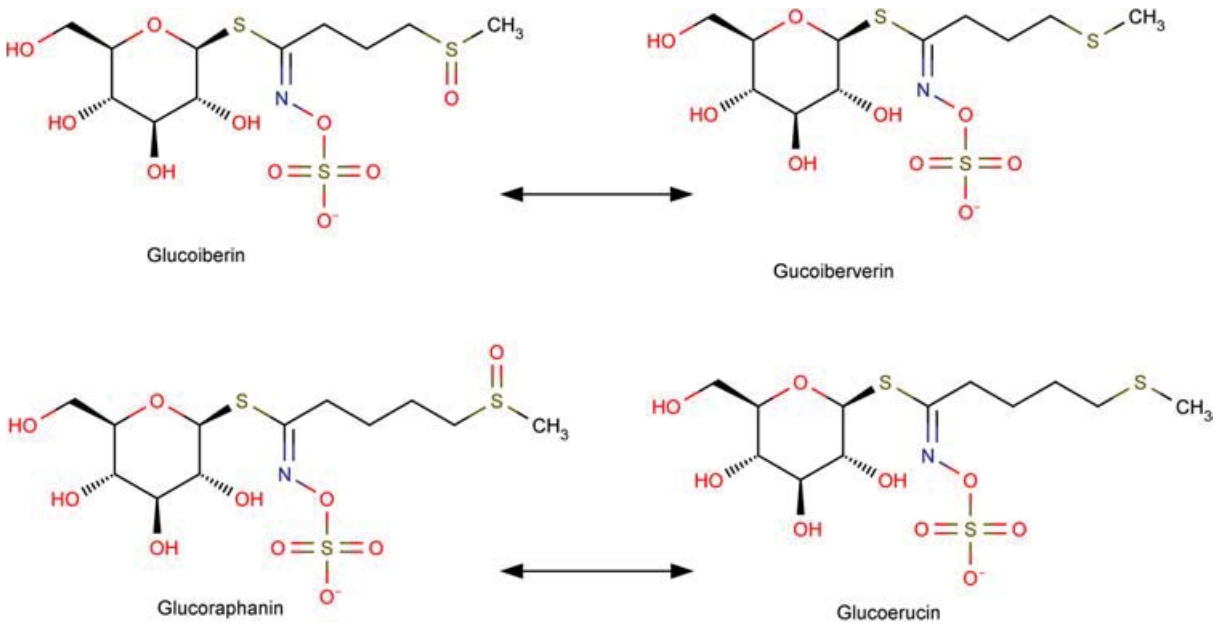

Figure 3 Top from left to right: Glucoiberin and glucoiberverin are the same molecules in different states of redox. Below from left to right: Similarly, but with one extra carbon in their alkane chain glucoraphanin and glucoerucin are a redox pair. Arrows indicate that molecules can shift between redox states.

trials that sulphoraphane 'bioavailability' from raw broccoli was 37% compared with cooked broccoli at 3.4%. The cooked broccoli presumably had no active myrosinase and was reliant on bacterial hydrolysis. Although they determined the GSL concentration of the broccoli and verified that no glucoraphanin remained after 'crushing and incubation', they also acknowledged that not all the glucoraphanin had converted to sulphoraphane. An unexpected finding was that the raw broccoli hydrolysed by myrosinase, had less sulphoraphane generated than expected when compared with the glucoraphanin content of the same amount of cooked broccoli. This, they conceded, could be the result of production of other metabolites such as sulphoraphane nitrile but did not consider this product for analysis presumably because it is not known to induce phase II enzymes. So they only looked for sulphoraphane and sulphoraphane conjugates and found that there was a lower concentration of sulphoraphane mercapturic acid conjugates present than expected. Another epidemiological study in 2008 examined chemoprevention by consumption of crucifers using data from a hospital-based case-controlled environment. They found that chemoprevention was associated with the consumption of raw rather than cooked crucifers [111] lending support to the earlier study of Vermeulen *et al.*

Bacterial bioconversion produces erucin nitrile as the major product

Lactobacillus species (*L. gasseri*, *L. acidophilus*, *L. casei* and two *L. plantarum*) also contribute to GSL metabolism in rats [112]. A degradation capability of up to 49% after 24 h incubation was seen, with the major hydrolytic product a nitrile, although one group colonized with caecal microbiota generated some sulphoraphane as well as the nitriles. When glucoraphanin was introduced directly into the caecum of live rats, sulphoraphane could be detected in the portal blood stream within 2 h. However, while conversion in the caecum by caecal microbiota led to absorption, at the same time the conversion yielded erucin nitrile rather than sulphoraphane or sulphoraphane nitrile [113]. Glucoraphanin was becoming glucoerucin, which was being transformed into erucin nitrile and Lai *et al.* suggested that the reduction of GSLs and their nitriles could explain this. Recently however, it was shown that glucoraphanin becomes glucoerucin and is metabolized to erucin nitrile by Enterobacteriaceae and this was because Enterobacteriaceae converted GSLs to nitriles differently to lactic acid bacteria, reducing alkylsulphinyl GSLs and their nitriles to alkylthiol GSLs and nitriles [114]. A schematic of the possible reactions is given in Figures 3 and 4.

Bacterial conversion of GSLs into good, bad or indifferent compounds?

The bioactivity of the hydrolysis products of GSLs is affected not only by how much bioactive compound is available for absorption but also its degree of bioactivity. While nitriles are thought to be less bioactive than ITCs, each one has some level of bioactivity as determined by its ability to induce a phase II host response. Substitution of an atom within a reactive group may impact on bioactivity; for example, when selenium becomes substituted

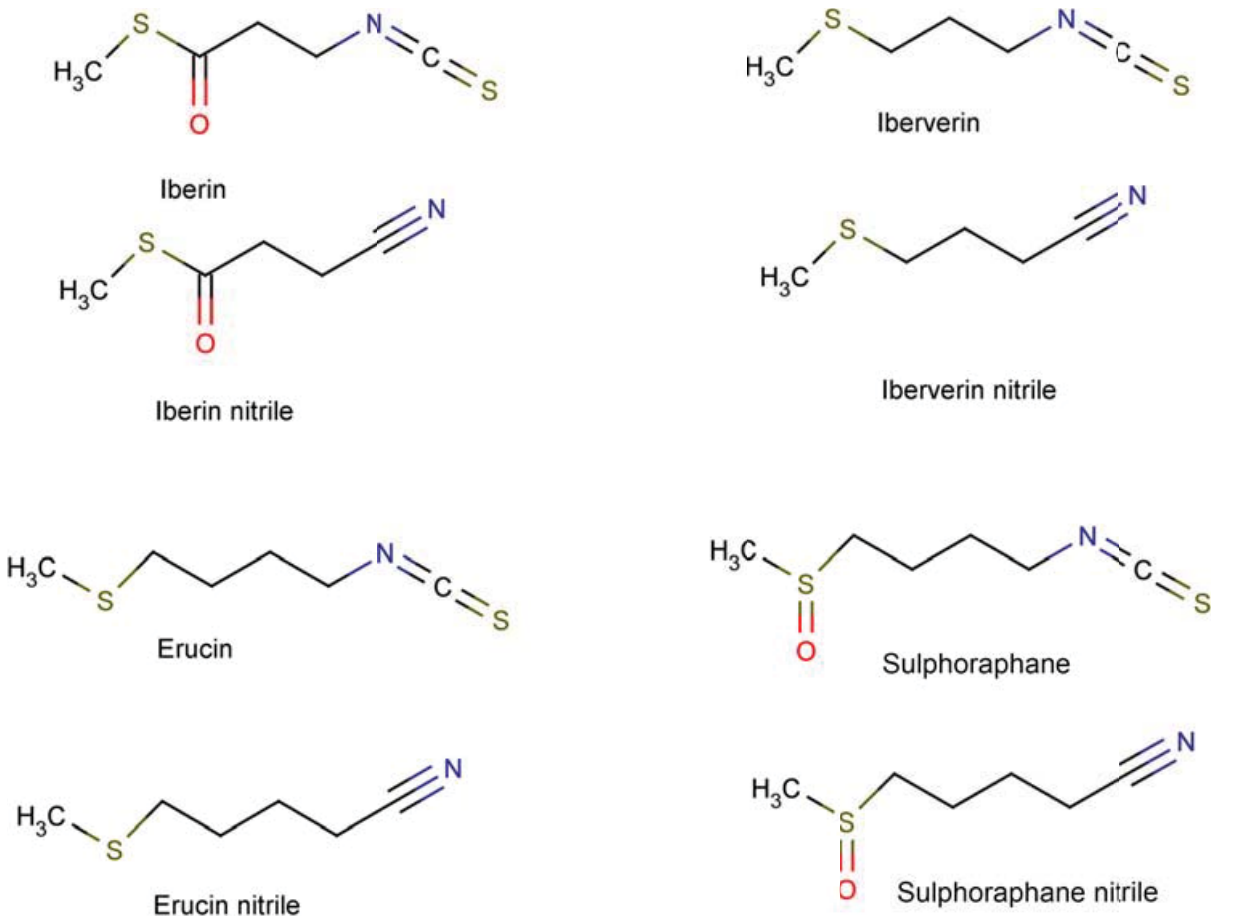

Figure 4 The GSL hydrolysis products of glucoraphanin, glucoerucin, glucoiberin and glucoiberverin are also redox pairs.

for sulphur. In one study, when high-selenium broccoli was given to rats, it reduced the number of preneoplastic lesions (cancers) significantly more than selenium or broccoli alone and the authors' suggested that the selenium in the broccoli was the cause but did not speculate further [115]. In another study it was observed that fertilising broccoli with selenium had an impact on phase II induction which was thought to be due to selenium becoming incorporated into GSLs [116]. In yet another study it was found that seleno-GSLs could produce isoselenocyanates that were more bioactive than ITCs [117]. However, producing seleno-GSLs reduced the concentration of all of the GSLs in a dose-dependent manner; further, modification to the GSLs seemed to occur at the expense of GSLs suggesting that the quality of the product may have been at the expense of quantity [117]. The structure is given in Figure 5(C).

For selenium analogues, the position the selenium occupies matters. Recently, chemically synthesized isoselenocyanates were found to have more potency as inducers of phase II enzymes than sulphoraphane [75, 118]. However, the synthesized isoselenocyanates were synthesized from 1-amino-4-(methylsulphinyl)butane and the selenium added to the isothiocyanate group as shown in Figure 5 and it has been since shown that this is not the way selenium is replaced for sulphur in the plant GSL biosynthesis [119]. Matich *et al.* have recently analysed selenium fertilized broccoli using NMR to confirm the position selenium occupies in seleno-GSLs and their hydrolysed products either nitriles or ITCs [119]. As Figure 5 shows, there are two main differences between the chemically synthesized sulphoraphane analogue and the plant biosynthesis product. First, as (D) shows, the chemically synthesized species is similar to sulphoraphane (B) in that it has a sulphinyl group, but differs in that selenium is incorporated into an isoselenocyanate group. Secondly, the plant-derived seleno-isothiocyanate (C) has selenium replacing sulphur at the sulphinyl group, and the selenium is not oxidized, making it more like a thiol group similar to erucin (A). Recently comparison of phenylalkyl isoselenocyanates with phenylalkyl ITCs suggests that the thiol groups may be contributing to the reactivity of ITCs [116]. Further studies are required to determine how bioactive or phase II inducing plant synthesized seleno-GSLs and their hydrolysis products are. As selenium is taken up by plants and incorporated into methionine (selenomethionine), cysteine (selenocysteine) and other organic molecules, these may also be exerting an effect independent of any seleno-GSLs and their derivatives [116–118].

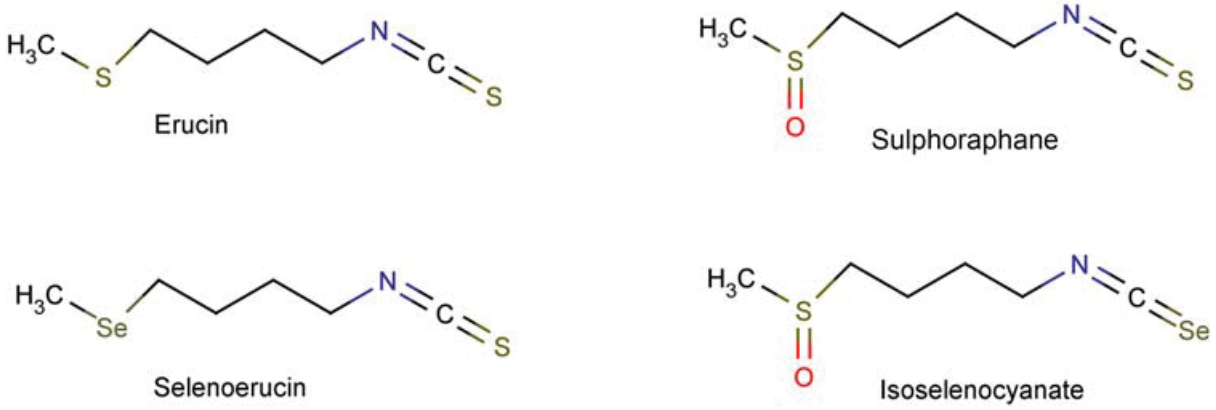

Figure 5 A: Erucin, the ITC derived from glucoerucin; B: Sulphoraphane the ITC of glucoraphanin. C: the selenoisothiocyanate produced by plants shows that the sulphur–selenium substitution is at the methyl end of the molecule. There is no oxidation of selenium making it more like erucin (A) than sulphoraphane (B). D: the chemically synthesized isoselenocyanate places selenium at the cyanate end and has the sulphinyl group similar to sulphoraphane

Do nitriles possess any of the bioactive properties of ITCs?

Of the ITCs from GSLs that have been tested, all have shown potent phase II inducing capability and many have shown promise as therapeutic treatments against cancers but ITCs are not the only transformation product resulting from hydrolysis of GSLs, nor are they the predominant species [25, 27, 29, 113, 120–122]. It is mostly nitriles that are produced when bacteria are incubated with GSLs. Nitriles are considered poor inducers of phase II enzymes [1, 32, 123, 124] so how does the consumer benefit from consumption of dietary GSLs if nitriles are the main product from GSLs; and are cell culture and *in vitro* systems valid when considering GSL to nitrile metabolism *in vivo*? Keck *et al.* found that their results may have cast doubt of the validity of *in vitro* systems. They noted that in cell culture, the doses of crambene needed for induction of quinone reductase were ~100-fold greater than effective doses of sulphoraphane and yet when administered to Fischer 344 rats, crambene performed nearly as well as sulphoraphane (1.5- and 1.7-fold induction, respectively) [125].

Crambene (1-cyano-2-hydroxy-3-butene), formed from the cruciferous GSL progoitrin, has apoptotic properties similar to sulphoraphane [126]. Crambene's major metabolite is the *N*-acetyl-cysteine conjugate derived from GSH with two other minor metabolites identified but as yet unassigned, which may also be bioactive [126, 127] (Figure 6). Crambene elevates GSH and GSTs, which serves to demonstrate that for crambene, the detoxification pathway to excretion is via the phase II pathway and GSTs. Other nitriles such as 4-hydroxybenzyl cyanide and 1-isothiocyanato-3-methylsulphonylpropane (cheirolin nitrile) have demonstrated antiproliferative properties in an *in vitro* cell culture model with an inhibitory concentration value (IC_{50}) of 104 and 43 μM, respectively [125, 128].

Bacteria may be the key to biotransformation of GSLs in the intestine

Previous studies have shown that the enzyme myrosinase not only loses its activity through cooking, but its activity also declines over time with the rate of loss dependent on temperature and the ambient storage conditions of the vegetables [86, 87, 90, 129–131]. In the absence of active myrosinase, our gut microbiota is evidently able to carry out this process, and bacteria possess genes encoding glycoside hydrolases, which may have specificity to GSLs. There is some evidence that the myrosinase enzyme is likely to be cell-associated as previous experiments using *Lactobacillus* bacterial cells found no GSL transformation capability unless the cells were intact [102] but as yet, a GSL uptake mechanism has not been identified. It is not known whether the bacteria are actually using the GSLs as a source of glucose in the gut environment (taking them in through an unknown transporter system) or whether breakdown of GSLs with the liberation of glucose and sulphate is simply a consequence (by-product) of metabolic activity by bacteria. The order in which bacteria remove key components of the GSLs might provide insight as to whether it is the glucose that is targeted and also might explain why nitriles are always the preferred products over ITCs. As ITCs exhibit antimicrobial properties [5, 114,132–137], bacteria may recognize ITCs as toxic and be directing the transformation in favour of nitriles instead.

The bacterial metabolism of GSLs may be beneficial to our health

Dietary GSLs in combination with intestinal bacteria may offer health benefits through the bacterial metabolism of these GSLs into bioactives. The degree of bioactivity will vary depending on several factors: the population

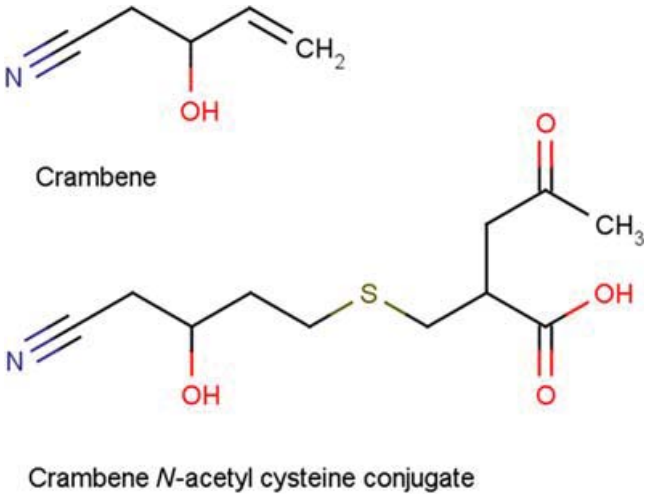

Figure 6 Chemical structure of (a) 1-cyano-2-hydroxy-3-butene (crambene) and the *N*-acetyl-cysteine conjugate of crambene (2-(acetylamino)-3-(4-cyano-3-hydroxybuty(sulphinyl)propionate.

dynamics and metabolic activity of the gut microbes, the GSLs that are in the food, the hydrolysis products from their transformation and the presence or absence of plant-based myrosinases. Something we can be confident about is that we need our cruciferous vegetables to contain plenty of GSLs so choosing cultivars high in this compound is desirable as long as it does not lead to a lack of other nutrients in the process (sometimes over production of one nutrient in a cultivar leads to underproduction of others, e.g., selenium fertilized broccoli has already been shown to have a reduced GSL profile). Another approach is to increase the ITC component of our food by combining GSL supplements with fresh vegetables containing active myrosinase or mixing GSLs together with ITCs [138–140]. However the location of hydrolysis may be critical for effect as the bacterial hydrolysis is most likely to occur in the lower gut while myrosinase-assisted hydrolysis is most likely to take place in the upper digestive tract such as the oesophagus, stomach and duodenum. From here a different path to elimination may follow as already shown by others [141].

Since ITCs are so much more potent as phase II inducers, it may be preferable that GSLs are converted into ITCs rather than nitriles. However, indications are that some of the most potent phase II inducing ITCs are not actually produced *in vivo* from the corresponding GSL [67, 113, 120]. Nitriles derived from GSLs are still biologically relevant because, due to their increased stability over ITCs, the effects observed could be due to accumulation which is associated with potency.

There are several paths GSLs can take via intermediaries to nitriles. Thus, there may be varying degrees of bioactivity, related to redox capacity, and there may also be a relationship between inducer potency and the length of time the weak inducing product is in contact with the host tissue [66, 128, 142–144]. The nitrile end is a cyanide group and the best characterized cyanide is hydrogen cyanide, known which is known to be metabolized to thiocyanate by the addition of a sulphur donor [145]. If this is occurring, and GSH conjugates are not possible, perhaps nitriles are able to be converted to thiocyanates that are able to be conjugated with GSH and induce phase II enzymes just as ITCs do.

There is also evidence for microbial thiocyanate degradation which, if occurring, would bypass this route entirely [146, 147]. We have been unable to find any published work that describes and characterizes host metabolic degradation of GSL nitriles to date. Aliphatic nitriles, on the other hand, have been studied for toxicity; this is related to their conversion to cyanide and causing GSH depletion [148].

Understanding how bacteria metabolize GSLs is important because in the absence of myrosinase, GSLs pass through our body intact [113]. However, if our microbiota possesses myrosinase activity, GSLs can still be transformed into GSL hydrolysis products which are bioactive. Also, the products of bacterial GSL metabolism differ from the products of plant myrosinase-mediated hydrolysis [149], and the effects of these bacterial products have not yet been studied.

The by-products of bacterial metabolism, the short-chain fatty acids (SCFAs) can have significant effects on our health [150–155]. Some metabolic diseases have their own 'signature' of microbiota population dynamics and the ratios of the top three, acetate, butyrate and propionate correlate with the individual's health status [156, 157]. Dietary beneficial bacteria are associated also with an increase of propionate and butyrate while acetate levels are reduced [158]. Butyrate from bacterial SCFAs has also been associated with phase II induction capacity [153].

Even though nitriles do not have the phase II quinone reductase or GST-inducing power of the ITCs in cell culture, this does not mean they do not make a difference,

especially considering that nitriles are the main product from hydrolysis of GSLs in the gut.

Even knowing which GSLs are eaten does not necessarily allow us to be certain which products will be produced from the process of GSL biotransformation when microbiota are involved. Nor does it allow us to predict how bioactive they will be because the interactions that occur between host and microbiota are so complex.

One should never underestimate the contribution bacteria can make in the biotransformation of GSLs. Cruciferous vegetables deliver dietary GSLs safely to our body and if they are subsequently transformed into bioactives by our own microbiota, they have the potential to protect us from cancers formed in and near our gut. Eating these vegetables combined with an appropriate host response to these compounds, well-functioning gut microbiota and supportive gene expression may be the best ongoing protection we can rely on.

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