JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Lactic Acid Bacteria Convert Glucosinolates to Nitriles Efficiently Yet Differently from Enterobacteriaceae

Jane A. Mullaney,[†,⊥] William J. Kelly,[‡] Tony K. McGhie,[§] Juliet Ansell,[†,⊥] and Julian A. Heyes*[,∥]

[†]Riddet Institute, Massey University, Private Bag 11222, Palmerston North, New Zealand

[‡]AgResearch, Animal Nutrition & Health, Grasslands Research Centre, Palmerston North, New Zealand

[§]Food Innovation—Biological Chemistry & Bioactives, The New Zealand Institute for Plant & Food Research Limited, Palmerston North, New Zealand

[⊥]Food Innovation—Food and Nutrition Group, The New Zealand Institute for Plant & Food Research Limited, Palmerston North, New Zealand

[∥]Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11222, Palmerston North, New Zealand

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.,

Received: December 19, 2012
Revised: March 5, 2013
Accepted: March 6, 2013
Published: March 6, 2013

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

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).

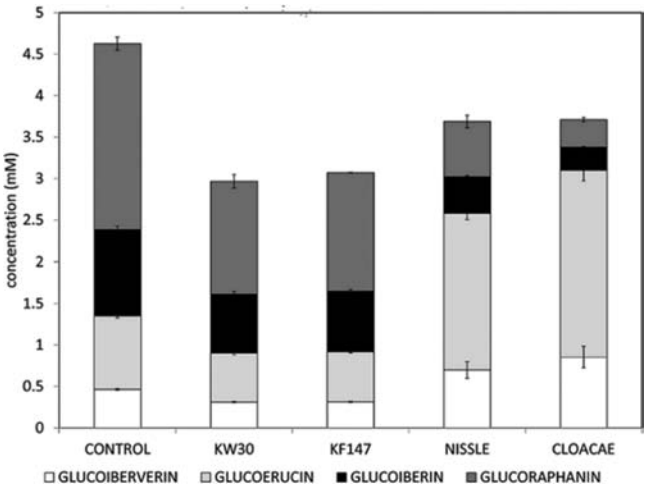

Figure 1. Glucosinolate profile of culture medium as determined by LC-QTOF-HRMS after 24 h anaerobic incubation of named bacteria with broccoli seed powder extract (BSP) at 37 °C. Control samples had broccoli seed extract added to uninoculated culture medium. The Enterobacteriaceae interconversion of 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

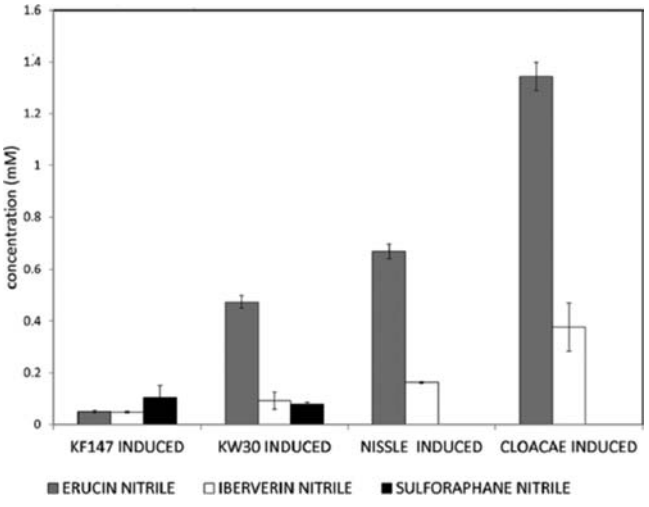

Figure 2. Concentration (mM) of individual nitriles in culture medium after 24 h incubation with named bacteria that had been induced by prior anaerobic culture with broccoli seed extract for 24 h. Erucin nitrile was the predominant product followed by iberverin nitrile, with *E. cloacae* producing the 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.

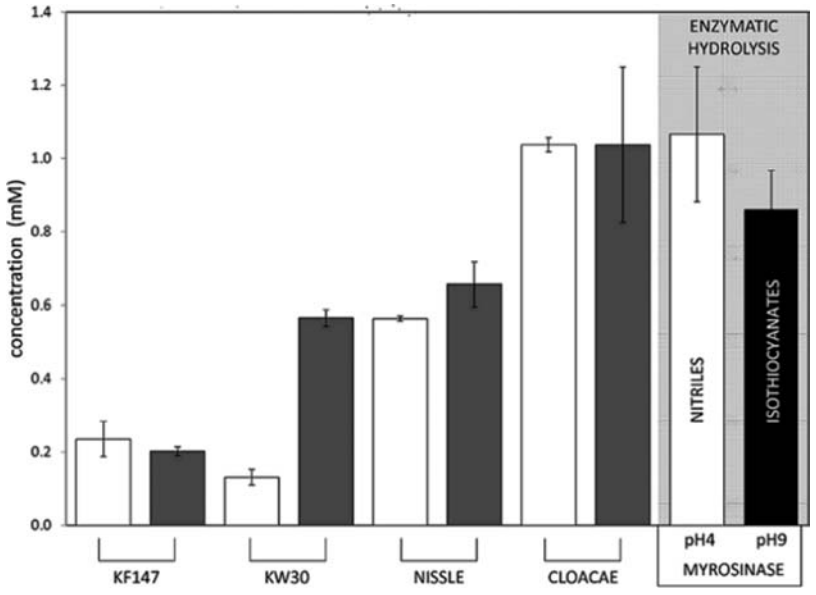

Figure 3. Total nitrile concentration in culture medium after anaerobic incubation of BSP with named bacteria after 24 h prior to incubation with or without BSP as assessed by GC-MS. An adaptive response was found only for KW30. Dark gray bars represent those precultured with GSLs (adapted); white bars 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).

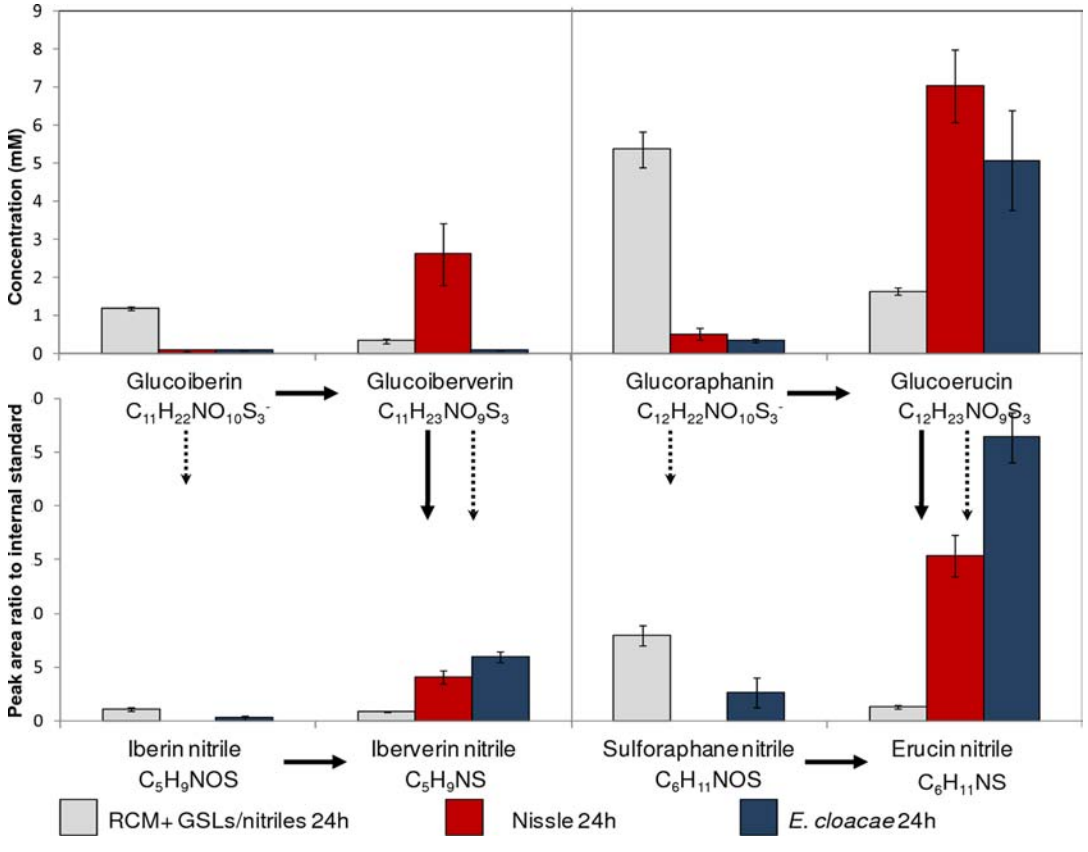

Figure 4. Comparison of the GSLs and nitriles shows that interconversion of GSLs occurred only for Nissle and *E. cloacae*. There were more of the reduced species iberverin nitrile and erucin nitrile than could be accounted for by hydrolysis of glucoerucin and glucoiberverin. This is because sulforaphane 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).

dx.doi.org/10.1021/jf305442j | J. Agric. Food Chem. 2013, 61, 3039−3046

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.

AUTHOR INFORMATION

Corresponding Author

*Phone: +64 6 350 5963. E-mail: J.A.Heyes@massey.ac.nz.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors wish to acknowledge Comvita NZ Ltd, Paengaroa, New Zealand, for providing us with the ground freeze-dried broccoli seed, and Martin B. Hunt, Plant & Food Research, Palmerston North, New Zealand, for his invaluable skill and expertise with GC-MS. Funding for this research was provided by the Food Centre of Research Excellence Platform 2.

■ 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

■ REFERENCES

(1) Cohen, J. H.; Kristal, A. R.; Stanford, J. L. Fruit and vegetable intakes and prostate cancer risk. *J. Natl. Cancer Inst. (Bethesda)* **2000**, *92*, 61−68.

(2) Das, S.; Tyagi, A. K.; Kaur, H. Cancer modulation by glucosinolates: a review. *Curr. Sci. (Bangalore)* **2000**, *79*.

(3) Jones, R. B.; Faragher, J. D.; Winkler, S. A review of the influence of postharvest treatments on quality and glucosinolate content in broccoli *Brassica oleracea* heads. *Postharvest Biol. Technol.* **2006**, *41*, 1−8.

(4) Hanschen, F. S.; Platz, S.; Mewis, I.; Schreiner, M.; Rohn, S.; Kroh, L. W. Thermally-induced degradation of sulfur-containing aliphatic glucosinolates in broccoli sprouts (*Brassica oleracea* var *italica*) and model systems. *J. Agric. Food Chem.* **2012**, *60*, 2231−2241.

(5) Hanschen, F. S.; Bauer, A.; Mewis, I.; Keil, C.; Schreiner, M.; Rohn, S.; Kroh, L. W. Thermally-induced degradation of aliphatic glucosinolates: identification of intermediary breakdown products and proposed degradation pathways. *J. Agric. Food Chem.* **2012**, *60*, 9890−9899.

(6) Rampal, G.; Khanna, N.; Thind, T. S.; Arora, S.; Vig, A. P. Role of isothiocyanates as anticancer agents and their contributing molecular and cellular mechanisms. *Med. Chem. Drug Discovery* **2012**, *3*, 79−93.

(7) Fahey, J. W.; Zhang, Y.; Talalay, P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10367−10372.

(8) Oginsky, E. L.; Stein, A. E.; Greer, M. A. Myrosinase activity in bacteria as demonstrated by the conversion of progoitrin to goitrin. *Proc. Soc. Exp. Biol. Med.* **1965**, *119*, 360−364.

(9) Tani, N.; Ohtsuru, M.; Hata, T. Purification and general characteristics of bacterial myrosinase produced by *Enterobacter cloacae*. *Agric. Biol. Chem.* **1974**, *38*, 1623−1630.

(10) Huber, J.; Kranz, G.; Kreibich, G.; Beining, K.; Kruger, M.; Weissbach, F. Mikrobieller abbau von glucosinolaten im rapsextraktionsschrot. *Die Nahrung* **1983**, *27*, 257−263.

(11) Palop, M. L.; Smiths, J. P.; ten Brink, B. Degradation of sinigrin by *Lactobacillus agilis* strain R16. *Int. J. Food Microbiol.* **1995**, *26*, 219−229.

(12) Lai, R.-H.; Miller, M. J.; Jeffery, E. H. Glucoraphanin is hydrolyzed by lactobacilli in vitro and rat cecal microbiota in vitro and in situ. *FASEB J.* **2009**, *23*, (Meeting Abstract Supplement 561.564).

(13) Cheng, D. L.; Hashimoto, K.; Uda, Y. In vitro digestion of sinigrin and glucotropaeolin by single strains of *Bifidobacterium* and identification of the digestive products. *Food Chem. Toxicol.* **2004**, *42*, 351−357.

(14) Elfoul, L.; Rabot, S.; Khelifa, N.; Quinsac, A.; Duguay, A.; Rimbault, A. Formation of allyl isothiocyanate from sinigrin in the digestive tract of rats monoassociated with a human colonic strain of *Bacteroides thetaiotaomicron*. *FEMS Microbiol. Lett.* **2001**, *197*, 99−103.

(15) Kelly, W. J.; Asmundson, R. V.; Huang, C. M. Isolation and characterization of bacteriocin-producing lactic acid bacteria from ready-to-eat food products. *Int. J. Food Microbiol.* **1996**, *33*, 209−218.

(16) Aukrust, T.; Blom, H. Transformation of *Lactobacillus* strains used in meat and vegetable fermentations. *Food Res. Int.* **1992**, *25*, 253−261.

(17) Schillinger, U.; Lücke, F. K. Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl. Environ. Microbiol.* **1989**, *55*, 1901−1906.

(18) Kelly, W. J.; Davey, G. P.; Ward, L. J. H. Characterization of lactococci isolated from minimally processed fresh fruit and vegetables. *Int. J. Food Microbiol.* **1998**, *45*, 85−92.

(19) Song, L.; Iori, R.; Thornalley, P. J. Purification of major glucosinolates from *Brassicaceae* seeds and preparation of isothiocyanate and amine metabolites. *J. Sci. Food Agric.* **2006**, *86*, 1271−1280.

(20) Rochfort, S.; Caridi, D.; Stinton, M.; Trenerry, V. C.; Jones, R. The isolation and purification of glucoraphanin from broccoli seeds by solid phase extraction and preparative high performance liquid chromatography. *J Chromatogr. A* **2006**, *1120*, 205−210.

(21) Wegkamp, A.; Teusink, B.; De Vos, W. M.; Smid, E. J. Development of a minimal growth medium for *Lactobacillus plantarum*. *Lett. Appl. Microbiol.* **2010**, *50*, 57−64.

(22) Stepper, J.; Shastri, S.; Loo, T. S.; Preston, J. C.; Novak, P.; Man, P.; Moore, C. H.; Havlícek, V.; Patchett, M. L.; Norris, G. E. Cysteine S-glycosylation, a new post-translational modification found in glycopeptide bacteriocins. *FEBS Lett.* **2011**, *585*, 645−650.

(23) Tani, N.; Ohtsuru, M.; Hata, T. Isolation of myrosinase producing microorganism. *Agric. Biol. Chem.* **1974**, *38*, 1617−1622.

(24) Ren, Y.; Ren, Y.; Zhou, Z.; Guo, X.; Li, Y.; Feng, L.; Wang, L. Complete genome sequence of *Enterobacter cloacae* subsp. *cloacae* type strain ATCC 13047. *J. Bacteriol.* **2010**, *192*, 2463−2464.

(25) Hancock, V.; Dahl, M.; Klemm, P. Probiotic *Escherichia coli* strain Nissle 1917 outcompetes intestinal pathogens during biofilm formation. *J. Med. Microbiol.* **2010**, *59*, 392−399.

(26) Blodgett, R. Most probable number from serial dilutions. In *Bacteriological Analytical Manual*; F.U.S.F.A.D. Administration, 2006; pp 1−15.

(27) McLafferty, F. W. Mass spectrometric analysis. aliphatic nitriles. *Anal. Chem.* **1962**, *34*, 26−30.

(28) Spencer, G. F.; Daxenbichler, M. E. Gas chromatography-mass spectrometry of nitriles, isothiocyanates and oxazolidinethiones derived from cruciferous glucosinolate. *J. Sci. Food Agric.* **1980**, *31*, 359−367.

(29) Vaughn, S. F.; Berhow, M. A. Glucosinolate hydrolysis products from various plant sources: pH effects, isolation, and purification. *Ind. Crops Prod.* **2005**, 193−202.

(30) Janowitz, T.; Trompetter, I.; Piotrowski, M. Evolution of nitrilases in glucosinolate-containing plants. *Phytochemistry* **2009**, *70*, 1680−1686.

(31) Barillari, J.; Iori, R.; Broccoli, M.; Pozzetti, L.; Canistro, D.; Sapone, A.; Bonamassa, B.; Biagi, G. L.; Paolini, M. Glucoraphasatin and glucoraphenin, a redox pair of glucosinolates of *Brassicaceae*, differently affect metabolizing enzymes in rats. *J. Agric. Food Chem.* **2007**, *55*, 5505−5511.

(32) Clarke, J. D.; Hsu, A.; Riedl, K.; Bella, D.; Schwartz, S. J.; Stevens, J. F.; Ho, E. Bioavailability and inter-conversion of sulforaphane and erucin in human subjects consuming broccoli sprouts or broccoli supplement in a cross-over study design. *Pharmacol. Res.* **2011**, *64*, 456−463.

(33) Iori, R.; Bernardi, R.; Gueyrard, D.; Rollin, P.; Palmieri, S. Formation of glucoraphanin by chemoselective oxidation of natural glucoerucin: a chemoenzymatic route to sulforaphane. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1047−1048.

(34) Dawes, E. A. Production of indole by *Escherichia coli*. *Nat.* **1948**, *162*, 229−231.

(35) Lai, R. H.; Miller, M. J.; Jeffery, E. Glucoraphanin hydrolysis by microbiota in the rat cecum results in sulforaphane absorption. *Food Funct.* **2010**, *1*, 161−166.

(36) Boyd, D. R.; Sharma, N. D.; King, A. W. T.; Shepherd, S. D.; Allen, C. C. R.; Holt, R. A.; Luckarift, H. R.; Dalton, H. Stereoselective reductase-catalysed deoxygenation of sulfoxides in aerobic and anaerobic bacteria. *Org. Biomol. Chem.* **2004**, *2*, 554−561.
(37) Zhao, H.; Kim, G.; Levine, R. L. Methionine sulfoxide reductase contributes to meeting dietary methionine requirements. *Arch. Biochem. Biophys.* **2012**, *522*, 37−43.
(38) Tête-Favier, F.; Cobessi, D.; Boschi-Muller, S.; Azza, S. d.; Branlant, G.; Aubry, A. Crystal structure of the *Escherichia coli* peptide methionine sulphoxide reductase at 1.9 Å resolution. *Structure* **2000**, *8*, 1167−1178.
(39) Hayes, J.; Kelleher, M.; Eggleston, I. The cancer chemopreventive actions of phytochemicals derived from glucosinolates. *Eur. J. Nutr.* **2008**, *47*, 73−88.
(40) Takaya, K.; Suzuki, T.; Motohashi, H.; Onodera, K.; Satomi, S.; Kensler, T. W.; Yamamoto, M. Validation of the multiple sensor mechanism of the Keap1-Nrf2 system. *Free Radical Biol. Med.* **2012**, *53*, 817−827.
(41) Matusheski, N. V.; Jeffery, E. H. Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile. *J. Agric. Food Chem.* **2001**, *49*, 5743−5749.
(42) Basten, G. P.; Bao, Y.; Williamson, G. Sulforaphane and its glutathione conjugate but not sulforaphane nitrile induce UDP-glucuronosyl transferase (UGT1A1) and glutathione transferase (GSTA1) in cultured cells. *Carcinogenesis* **2002**, *23*, 1399−1404.
(43) Keck, A.-S.; Staack, R.; Jeffery, E. H. The cruciferous nitrile crambene has bioactivity similar to sulforaphane when administered to Fischer 344 rats but is far less potent in cell culture. *Nutr. Cancer* **2002**, *42*, 233−240.
(44) Kensler, T.; Egner, P.; Agyeman, A.; Visvanathan, K.; Groopman, J.; Chen, J.-G.; Chen, T.-Y.; Fahey, J.; Talalay, P. Keap1−Nrf2 signaling: a target for cancer prevention by sulforaphane. In *Topics in Current Chemistry*; Springer: Berlin, 2012; pp 1−15.
(45) Ahn, Y.-H.; Hwang, Y.; Liu, H.; Wang, X. J.; Zhang, Y.; Stephenson, K. K.; Boronina, T. N.; Cole, R. N.; Dinkova-Kostova, A. T.; Talalay, P.; Cole, P. A. Electrophilic tuning of the chemoprotective natural product sulforaphane. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 9590−9595.
(46) Talalay, P. A fascination with enzymes: the journey not the arrival matters. *J. Biol. Chem.* **2005**, *280*, 28829−28847.
(47) Barillari, J.; Canistro, D.; Paolini, M.; Ferroni, F.; Pedulli, G. F.; Iori, R.; Valgimigli, L. Direct antioxidant activity of purified glucoerucin, the dietary secondary metabolite contained in rocket (*Eruca sativa* Mill.) seeds and sprouts. *J. Agric. Food Chem.* **2005**, *53*, 2475−2482.
(48) Bheemreddy, R. M.; Jeffery, E. H. The metabolic fate of purified glucoraphanin in F344 rats. *J. Agric. Food Chem.* **2007**, *55*, 2861−2866.
(49) Melchini, A.; Costa, C.; Traka, M.; Miceli, N.; Mithen, R.; De Pasquale, R.; Trovato, A. Erucin, a new promising cancer chemopreventive agent from rocket salads, shows anti-proliferative activity on human lung carcinoma A549 cells. *Food Chem. Toxicol.* **2009**, *47*, 1430−1436.