Influence of cabbage processing methods and prebiotic manipulation of colonic microflora on glucosinolate breakdown in man

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Glucosinolate consumption from brassica vegetables has been implicated in reduction of cancer risk. The isothiocyanate breakdown products of glucosinolates appear to be particularly important as chemoprotective agents. Before consumption, brassica vegetables are generally cooked, causing the plant enzyme, myrosinase, to be denatured, influencing the profile of glucosinolate breakdown products produced. Some human intestinal microflora species show myrosinase-like activity (e.g. bifidobacteria). We aimed to increase bifidobacteria by offering a prebiotic (inulin) in a randomised crossover study. Six volunteers consumed inulin (10 g/d) for 21 d followed by a 21 d control period (no inulin). Treatment periods were reversed for the remaining six volunteers. During the last 5 d of each period two cabbage-containing meals were consumed. Total urine output was collected for 24 h following each meal. Cabbage was microwaved for 2 min (lightly cooked) or 5.5 min (fully cooked). Faecal samples were collected at the start and after the inulin and control treatments. Bifidobacteria were enumerated by real-time PCR. Allyl isothiocyanate production was quantified by measuring urinary excretion of allyl mercapturic acid (AMA). Bifidobacteria increased following prebiotic supplementation (P<0.001) but there was no impact of this increase on AMA excretion. AMA excretion was greater following consumption of lightly cooked cabbage irrespective of prebiotic treatment (P<0.001). In conclusion, the most effective way to increase isothiocyanate production may be to limit the length of time that brassica vegetables are cooked prior to consumption.

Isothiocyanate: Prebiotic: Bifidobacteria: Cooking

Increased intake of brassica vegetables and brassica-containing foods (e.g. mustard, horse radish) may be of benefit for cancer prevention1,2. This is thought to be partly due to the presence of glucosinolates (thioglucoside phytochemicals) in brassica vegetables. They are accompanied in the plant by myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1). Following tissue disruption (during cutting or chewing), glucosinolates come in contact with myrosinase and the resulting hydrolysis yields a range of active aglycone products including isothiocyanates, nitriles and cyano-epithioalkanes3. Isothiocyanates influence xenobiotic metabolising enzyme expression and apoptosis4–6 and may explain the cancer-protective effect of brassica vegetable consumption1. However, not all epidemiological studies demonstrate a protective role for brassica vegetables8,9 and this may be due to differences in release of breakdown products in different situations.

In general, man consumes brassica vegetables following processing. Myrosinase is denatured by the application of heat during cooking10 and this has implications for the amount and composition of breakdown products available to man. Consumption of raw vegetables containing plant-derived myrosinase activity produces rapid hydrolysis of glucosinolates and the release of isothiocyanates may primarily occur in the upper digestive tract11. The isothiocyanates are absorbed and rapidly excreted as conjugates in the urine. Consumption of cooked brassica with denatured myrosinase permits intact glucosinolates to pass through the upper digestive tract and enter the colon12,13 where they may be hydrolysed by the gut microbiota. The different sites of release and absorption of hydrolysis products of glucosinolates may have implications for the cancer-protective properties of brassica vegetables.

Apoptosis was increased in the colon crypts of dimethylhydrazine-treated rats following absorption of allyl isothiocyanate from fresh Brussels sprout juice or raw sprouts in the upper intestine. Therefore a systemic delivery of glucosinolate metabolites to the colonic epithelium may be effective for chemoprotection6. However, the release of allyl isothiocyanate from purified sinigrin (with no plant myrosinase) into the colon has also been shown to enhance apoptosis and reduce the formation of aberrant crypt foci in dimethylhydrazine-treated rats, indicating that a localised production of allyl isothiocyanate may also be of benefit14. The most effective route

Abbreviations: AMA, allyl mercapturic acid.

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of delivery of glucosinolate metabolites is not yet clear. Indeed it is probable that both systematic and luminal delivery are important for maximising the chemoprotective effect of brassica vegetables.

Some genera of the human intestinal microflora (e.g. *Bifidobacterium*, *Lactobacillus* and *Bacteroides*) possess myrosinase-like activity. Three species of bifidobacteria of human origin have been shown to be capable of degrading the glucosinolates sinigrin and glucotropaeolin *in vitro*. *Lactobacillus agilis* and *Bacteroides thetaiotaomicron*, isolated from human faeces, have also been reported to convert sinigrin to allyl isothiocyanate *in vitro*. Incubation of human faeces with cooked watercress (in which plant myrosinase had been denatured) *in vitro* also yielded isothiocyanates. Bacteria may hydrolyse glucosinolates consumed in a cooked vegetable meal and release hydrolysis products directly into the lumen of the colon. Enhancement of selected microbial populations in the colon of human faecal flora-associated rats alters the biological effects of glucosinolates consumed in rapeseed meal and may also alter vegetable glucosinolate effects. Bifidobacteria residing in the colon may be enhanced following the consumption of prebiotics. Prebiotics are fermentable, indigestible food ingredients that selectively stimulate growth and/or activity of a limited number of bacterial species resident in the colon. Given the availability of pre- and probiotic supplements and the potential for myrosinase-like activity in lactobacilli and bifidobacteria, an investigation into the effect of enhanced microflora populations on glucosinolate metabolism in man is needed.

In the current study we sought to enhance colonic bifidobacterial populations in human subjects by the use of a commercially available prebiotic to investigate the influence of changes to the colonic bacterial population on the hydrolysis of glucosinolates. We hypothesised that any changes to glucosinolate hydrolysis brought about through prebiotic supplementation would be manifest following consumption of cooked cabbage but not after consumption of cabbage retaining myrosinase activity.

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**Experimental methods**

**Subjects**

Twelve healthy, Caucasian, non-smoking adult volunteers (three male, nine female; mean age 38·1 (SEM 2·43), range 25–51 years; mean BMI 25·0 (SEM 1·09), range 19·8–31·5 kg/m²) were recruited from academic institutions in Aberdeen. After discussion with the study investigator the volunteers provided a record of informed consent. The volunteers did not consume medical drugs or supplements during the study period. The protocol and all procedures were approved by the NHS Trust Grampian Research Ethics Committee.

**Study design**

The subjects were randomly split into two groups prior to participating in a crossover study design. Group 1 consumed the recommended amount of a commercially available prebiotic formulation (92 % inulin–oligofructose +8 % glucose–fructose–sucrose; Beneo®, DKSH/Orafti Great Britain Ltd, Beckenham, Kent, UK) for a 21 d treatment period which included a test meal period during the last 5 d. A 21 d control period, during which no supplement was consumed, followed immediately and this control period also concluded with a 5 d test meal period. The second group completed the control and treatment periods in reverse order (Fig. 1). During the prebiotic treatment period, volunteers were provided with sachets of pre-weighed quantities (5 g) of the prebiotic supplement. The 10 g daily prebiotic dose contained approximately 0·04 MJ (10·7 kcal) which could lead to an increase in energy intake of 0·5 % assuming a normal energy intake of 8·3 MJ/d (2000 kcal/d). This increase in energy intake was much lower than the natural day-to-day variation in energy intake of 20–30 % and was not considered sufficient to challenge...
the interpretation of the results. In general, compliance to the study protocol was good. Out of the forty-two inulin doses one volunteer missed four and two volunteers each missed two doses.

**Vegetable treatments**

Within each 5 d test meal period each volunteer was offered a test meal on day 1 and again on day 4 at 12.30 hours. The second test meal was consumed 72 h after the first to permit clearance of urinary metabolites between the cabbage meals. Clearance has been shown to occur within 24 h in previous work. For 2 d prior to consumption of the first test meal and until the end of each test meal period, subjects were asked to avoid consumption of potential sources of glucosinolates. A list of glucosinolate-containing vegetables and foodstuffs was provided at the outset of the study and verbal reminders were given at each test meal. To check volunteer compliance, a simple food diary was completed for 2 d prior to, and during, each test meal period.

Each test meal consisted of a 150 g portion of white cabbage with a standard meal of chicken and rice (containing no glucosinolates), followed by a glucosinolate-free dessert course (summer fruit and ice cream). Alongside each cabbage meal each volunteer was provided with a 70 ml drink consisting of an extract made from 50 ml water and 9 g ground broccoli seeds (GEO organic broccoli seeds; UK Juicers Ltd, York, UK), in which myrosinase had been denatured by heating (80°C for 10 min). Solid matter was removed by sieving the seed and water mixture before 20 ml fresh orange juice and 10 g Canderel (aspartame) artificial sweetener were added to improve palatability. The drink contained high concentrations of the nitrile hydrolysis product of glucoraphanin, the metabolism of which is being investigated in a further study.

A homogeneous source of large white cabbage (var. Colmar; Kettle Produce, Cupar, UK) was used. Each cabbage was divided into a series of nine longitudinal wedges. Each wedge weighed 150 g and contained consistent proportions of inner and outer leaves. Residual cabbage was discarded. Each cabbage wedge was roughly chopped. Four portions were microwave cooked for 2 min (750 W; Whirlpool UK Ltd, Croydon, Surrey, UK) in a dish containing 16 ml water and covered with pierced PVC cooking film while a further four were microwave cooked for 5.5 min under the same conditions. Cooking times were chosen to provide meals with similar sinigrin content but different myrosinase activities (2 min – lightly cooked, retaining active, naturally available plant myrosinase; 5.5 min – fully cooked, containing denatured natural myrosinase) and were chosen based on the results of a previous study. Cabbage treatments were offered in a randomised order to each individual. One portion from each cooking treatment per cabbage along with the remaining raw portion was reserved for subsequent analysis. These portions were placed on a plate and left at room temperature for the duration of the volunteers’ meal. At the end of each meal, these cabbage portions were snap-frozen in liquid nitrogen and maintained at −20°C for analysis of glucosinolate concentrations and myrosinase activity. As the type and quantity of active ingredients may vary between individual cabbage heads, the identity of the cabbage head consumed by each volunteer was recorded to permit accurate determination of the intake of glucosinolates and myrosinase by each volunteer.

Cabbage samples were freeze-dried, ground and maintained at −20°C prior to glucosinolate and myrosinase analyses. The glucosinolate content of the cabbage samples was determined using HPLC by the methods described in Rungapamestry et al. The myrosinase activity of the cooked cabbage was determined using a UV visible spectrophotometer (Cary 50; Varian Ltd, Yarnton, Oxford, UK) to measure the rate of disappearance of sinigrin from a test mixture. Standard curves were run each day using commercially prepared myrosinase isolated from Sinapis alba (white mustard seed; Sigma Aldrich, Poole, UK). One unit of myrosinase activity is defined as that which will produce 1.0 µmol glucose/min from sinigrin at pH 6.0 and 25°C.

**Urine collection and analysis**

Prior to consumption of the test meal each volunteer provided a spot sample of urine to enable the determination of background concentrations of glucosinolate metabolites. Total urine collections were obtained for 24 h after each cabbage test meal. The 24 h collection was separated into four time periods (0–4, 5–9, 10–19 and 20–24 h). Since volunteers were free-living, urine samples were kept in insulated bags containing cool blocks to reduce possible degradation of the compounds of interest during the collection period. The pH of the urine was not modified. For each collection period, urine volume was measured by a study investigator and recorded. Four 20 ml aliquots from each collection receptacle were placed in sealed tubes (Sterilin, Stone, Staffordshire, UK) and maintained at −20°C until analysis.

Urinary metabolites of isothiocyanates derived from glucosinolates contained in the cabbage meals were quantified in the urine samples. The major aliphatic glucosinolate in cabbage is sinigrin which, on hydrolysis by myrosinase, forms allyl isothiocyanate. Allyl isothiocyanate is metabolised by the mercapturic acid pathway prior to urinary excretion as N-acetyl (allylthiocarbamoyl)-l-cysteine (hereafter referred to as allyl mercapturic acid; AMA). AMA excreted in the urine was measured using the method as adapted from Mennicke et al. AMS and N-acetyl (phenylthiocarbamoyl)-l-cysteine (phenyl mercapturic acid) were synthesised according to Mennicke et al. for use as standards in the analysis. Phenyl mercapturic acid (dissolved in deionised water–ethanol, 50:50, v/v) was used as an internal standard and calibration curves for AMA (R^2 = 0.9993 (SEM 0.0002)) were analysed within each batch of samples for quantification of the amount of AMA excreted. Each sample was analysed in duplicate. Discrepancy between duplicate samples was always less than 5%.

**Faecal collection and analysis**

At three points during the study volunteers provided a faecal sample to enable changes in bifidobacterial numbers relative to the total bacteria population to be monitored. Faecal samples were obtained at the outset of the study for determination of baseline bifidobacterial population ratios and also at day 16 of both the prebiotic treatment and control periods (Fig. 1). At each time-point, the subjects placed a 10 g
sub-sample of faeces into a collection tube. Samples were further aliquoted before being maintained at −20°C for later analysis by real-time PCR.

**Quantitative real-time PCR**

Standard template DNA was prepared from the 16S rRNA gene of *Bifidobacterium pseudocatenulatum* DSM 20 438 by amplification with primers 27F and RP2 and purification as described previously30. Standard curves were prepared as described previously31 with either universal primers UniF (GTGSGTGCAAYGGYGTCTGCA32, modified) and UniR (ACGTTCGTCMCNCCCTTCCTC32, modified), or *Bifidobacterium*-specific primers BifF (TCGGTCYGTGTTAAGAG33) and g-Bifid-R (GGTGTCTTCCCCGATATCACA34). Both primer sets resulted in very similar standard curves (PCR efficiency 95-9 % for universal primers and 96-7 % (SEM 1-34) % for *Bifidobacterium*-specific primers).

DNA from faecal samples was extracted using the DNA Spin Kit for Soil (MP Biomedicals Europe, Illkirch, France). DNA concentrations were determined with a NanoDrop ND 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was diluted to 0·5 ng/µl in 5 µg/ml herring sperm DNA for amplification with universal primers UniF and UniR and 5 ng/µl in 5 µg/ml herring sperm DNA for amplification with *Bifidobacterium*-specific primers BifF and g-Bifid-R. Herring sperm DNA (5 µg/ml) was used for all DNA dilutions as carrier DNA, as it significantly increased the accuracy of the data, especially at low DNA concentrations. The absence of cross-reactivity with either primer pair was confirmed by real-time PCR under the same conditions as described later.

PCR reactions were performed as described previously31. Starting quantities of all bacterial and bifidobacterial 16S rRNA genes were determined using the iCycler IQ software version 3.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA) to calculate the percentage of bifidobacterial genes. The detection limit was 0-01 % of total bacterial 16S rRNA genes.

**Results**

**Glucosinolate concentrations in cabbage**

Raw cabbage contained the following glucosinolates (mean µmol/g DM): sinigrin, 9.54 (SD 4.01); glucobrassicin, 2.56 (SD 0.91); progoitrin, 1.39 (SD 0.47); glucoraphanin, 0.02 (SD 0.02). Glucosinolate concentrations in cooked cabbage did not differ from raw values (Fig. 2). The proportion of sinigrin to total glucosinolates did not differ between the 2 and 5·5 min cooking treatments (2 min = 43·7 %; 5·5 min = 43·1 %).

![Fig. 2. Total glucosinolate (■) and sinigrin (▲) concentrations in raw (0 min), lightly cooked (2 min) and fully cooked (5·5 min) cabbage. Values are means with their standard errors depicted by vertical bars (n 9 for lightly cooked and fully cooked cabbage; n 5 for raw cabbage).](https://doi.org/10.1017/S0007114507709091)
**Myrosinase activity in cabbage treatments**

Myrosinase activity was reduced from 21·5 (SEM 13·29) units/g DM (291·8 units/150 g fresh weight portion consumed) in raw cabbage to 2·44 (SEM 0·25) (35·5 units/150 g fresh weight portion consumed) and 0·87 (SEM 0·05) units/g DM (17·67 units/150 g fresh weight portion consumed) in cabbage cooked for 2 and 5·5 min, respectively. Myrosinase activity was higher in cabbage following a 2 min period of microwave cooking than following the 5·5 min cooking period (\(P=0·043\)).

**Faecal bifidobacterial population**

Inulin supplementation increased the relative proportion of bifidobacteria in faecal samples by approximately two-fold (\(P<0·001\), Fig. 3). In two volunteers the percentage of bifidobacterial 16S rRNA genes was below the detection limit in baseline samples. The presence of a small population of bifidobacteria was detected in one volunteer after inulin supplementation but no change was noted for the other. Although retained in the analysis these results were not sufficient to eliminate the increase shown overall. Baseline bifidobacterial populations varied markedly between individuals as indicated by a large range in values (the minimum population was below detection and maximum was 4·6 % of the total bacterial genes) and standard error (mean 1·80 (SEM 0·45) bifidobacterial genes as a percentage of total genes) but the stimulatory effect of inulin supplementation was not dependent on the baseline population (\(P=0·236\)) or the order in which the prebiotic and control periods were experienced (\(P=0·162\)).

**Urinary excretion of glucosinolate metabolites**

All mercapturic acid excretion data were corrected for individual glucosinolate intakes. Following the 48 h period of abstinence from brassica vegetables and glucosinolate-containing foods immediately prior to each cabbage meal, mercapturic acid concentrations in baseline urine samples were essentially zero (Fig. 4). AMA excretion in the 24 h following the meal was greater following consumption of lightly cooked cabbage (mean recovery 23·3 (SEM 1·9) %) than fully cooked cabbage (mean recovery 7·7 (SEM 0·76) %, \(P<0·001\)). Prebiotic supplementation did not significantly influence AMA excretion after either cabbage cooking treatment (prebiotic mean recovery 14·6 (SEM 2·01) %, control mean recovery 15·8 (SEM 2·40) %, \(P=0·880\)). The pattern of AMA excretion over time was different between the cooking treatments (\(P<0·001\)). Consumption of lightly cooked cabbage resulted in a peak of mercapturic acid excretion between 4 and 9 h following the cabbage meal in contrast to the excretion after consumption of cooked cabbage which peaked between 9 and 19 h (Fig. 4). Excretion of AMA in the urine was negligible by the end of the 24 h period irrespective of prebiotic treatment or cabbage cooking time.

Despite the significant increase in the proportion of faecal bifidobacteria during the prebiotic treatment period there was no association between the change in bifidobacteria and the change in AMA excretion after consumption of the fully cooked cabbage meal (\(R = 0·416; P=0·179\)). Although ten of the eleven volunteers that completed both the prebiotic and control periods demonstrated an increase in faecal bifidobacteria in response to inulin supplementation, the extent of change had no relation to either the extent or even direction of change of AMA excretion between periods (Fig. 5).

**Discussion**

Hydrolysis of glucosinolates in the colon or caecum has been known for some time\(^{12}\). The gastrointestinal microflora of rats and poultry have the ability to hydrolyse glucosinolates.\(^{15,35,36}\)
as demonstrated by the development of negative side-effects of Isothiocyanate exposure such as goitre following excessive progoitrin consumption even in the absence of dietary myrosinase. The myrosinase activity of the intestinal microflora is physiologically relevant as the biological effects of consumption of cruciferous vegetables do not occur when germ-free animals are provided with a glucosinolate-rich but myrosinase-free diet. This activity is not confined to laboratory animals; human microflora also show myrosinase-like activity as demonstrated when mechanical and antibiotic bowel cleansing in man decreased the isothiocyanate excreted following consumption of a cooked brassica homogenate from 11 to 1% of the dose. As the intake of cooked brassica (i.e. glucosinolate consumption) leads to a low delivery of isothiocyanates to the gastrointestinal tract relative to consumption of the raw vegetable (i.e. isothiocyanate consumption) but is a late consumption) leads to a low delivery of isothiocyanates to absorption. It is possible that rather than forming isothiocyanate or nitrile breakdown products, glucosinolates could be hydrolysed to amines, or that isothiocyanates formed in the colon could be degraded to amines prior to absorption.

Despite the strong, positive influence of inulin consumption on faecal bifidobacteria there was no effect of this increase on urinary output of mercapturic acid following consumption of the fully cooked cabbage meal. This may have been because the additional fermentable carbohydrate (inulin) could have led to a decrease in colonic pH due to the increased production of fermentation acids by the gut microbiota. A reduction in pH has been demonstrated when human flora-associated rats were fed a diet containing 10% inulin in one study, although not shown in a second study by the same group. A decrease in the pH of the hydrolysation environment may be of importance since nitrile rather than isothiocyanate formation is favoured in an acidic environment. It is possible that rather than forming isothiocyanate or nitrile breakdown products, glucosinolates could be hydrolysed to amines, or that isothiocyanates formed in the colon could be degraded to amines prior to absorption.

There is some evidence that inulin supplementation can influence liver detoxification enzyme activity. This appears to be associated with changes to the SCFA profile of the colon. Our use of urinary mercapturic acids as markers of isothiocyanate production in the digestive tract assumed consistent post-absorptive metabolism of isothiocyanates via the mercapturate pathway across treatments; treatment-related alterations in isothiocyanate metabolism could have undermined this assumption. Since we found no evidence that inulin altered urinary concentrations of mercapturic acids, this was not a major concern for this experiment but in further work this possible interference could be excluded by simultaneously monitoring post-absorptive metabolism of homologous isothiocyanates as has been done previously.

Isothiocyanates are highly reactive compounds and could react rapidly with compounds present in the colon to form other derivatives or could have been metabolised to different final products by the microflora prior to absorption by the host. It is also likely that inulin has positive effects on other, perhaps currently unidentified, bacterial species within the complex colonic environment. However, as inulin supplementation did not lead to a statistically significant effect on AMA excretion, microbial changes in the microflora were not investigated further. Differences in glucosinolate-mediated toxic effects observed in different animal species
most effective way of increasing isothiocyanate production is to limit the length of time of cooking for brassica vegetables prior to consumption.

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