Comparison of the effect of raw and blanched-frozen broccoli on DNA damage in colonocytes

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Abstract

Consumption of cruciferous vegetables may protect against colorectal cancer. Cruciferous vegetables are rich in a number of bioactive constituents including polyphenols, vitamins and glucosinolates. Before consumption, cruciferous vegetables often undergo some form of processing that reduces their content of bioactive constituents and may determine whether they exert protective effects. The aim of this study was to compare the ability of raw and blanched-frozen broccoli to protect colonocytes against DNA damage, improve antioxidant status and induce xenobiotic metabolising enzymes (XME). Fifteen Landrace x Large White male pigs were divided into five age and weight matched sets (79 days, SD 3, and 34.7 kg, SD 3.9 respectively). Each set consisted of siblings to minimise genetic variation. Within each set, pigs received a cereal-based diet, unsupplemented (control) or supplemented with 600 g/d of raw or blanched-frozen broccoli for 12 d. The consumption of raw broccoli caused a significant 27% increase in DNA damage in colonocytes (P=0.03) relative to the control diet, whereas blanched-frozen broccoli had no significant effect. Both broccoli diets had no

significant effect on plasma antioxidant status or hepatic and colonic XME. This study is the first to report that the consumption of raw broccoli can damage DNA in porcine colonocytes.

KEY WORDS: broccoli; glucosinolates; comet assay; DNA damage

INTRODUCTION

Experimental evidence indicates that cruciferous vegetables could protect against colorectal cancer.[1-3] Cruciferous vegetables contain a number of potentially protective constituents such as folate, vitamin C, tocopherols, carotenoids, and polyphenols.[4-6] The anticarcinogenic effects of cruciferous vegetables are however commonly attributed to their content of a group of sulphur containing secondary plant metabolites called glucosinolates (GLS).[7] Intact GLS are relatively biologically inert, but can be hydrolysed to products with greater bioactivity such as isothiocyanates (ITC), indoles, epithionitriles and nitriles.[8] The hydrolysis of GLS is largely dependent on the activity of a plant based β-D-thioglucosidase enzyme called myrosinase.[9] GLS and myrosinase are located in vacuoles in different cell types within the plant tissue. On disruption of the plant tissue, GLS and myrosinase come into contact with each other and GLS are hydrolysed.[8] If myrosinase is denatured by thermal processing, then GLS are thought to be hydrolysed by colonic bacteria with β-thioglucosidase activity, but the yield of ITC is much lower.[10-12] The identity of the other compounds formed *in vivo* from the bacterial degradation of GLS has yet to be fully clarified, but they seem to include nitriles and simple amines.[13-14]

In cultured colorectal cancer cells, various ITC and indoles have been shown to modulate xenobiotic metabolising enzymes (XME), arrest the cell cycle and induce apoptosis.[15-17] They have also been shown to induce XME in the colonic tissue of rodents and typically inhibit the development of chemically induced aberrant crypt foci, as have diets

enriched with cruciferous vegetables. [1-3, 18,19] Cruciferous vegetables could protect colonocytes against oxidative stress. Rychlik et al. [20] reported that undigested extracts of raw broccoli sprouts and extracts subjected to an *in vitro* method of gastrointestinal digestion had a similar ability to protect NCM460 colon cells against H_2O_2 induced oxidative DNA damage. Cells were incubated with the extracts for only 1 hour before exposure to H_2O_2, so protection was tentatively attributed to direct antioxidant effects of phenolic compounds such as sinapic acid, which were resistant to gastrointestinal digestion. Bonnesen et al. [15] reported that the GLS breakdown products, sulforaphane and indolo[3,2-b]carbazole protected LS-174 colon cancer cells against H_2O_2 induced DNA damage, an effect that may have been mediated by the induction of cytoprotective enzymes.[15,21]

Before consumption, cruciferous vegetables are often subjected to a range of treatments such as cutting, blanching, freezing and cooking that may alter their biological effects. Most forms of heat treatment result in the loss of bioactive constituents, with high temperatures, and prolonged exposure to water causing the greatest losses.[22-25] Processing may also alter the bioavailability of bioactive constituents. [11, 26, 27] Cruciferous vegetables are often over-cooked before consumption.[28] The bioavailability of ITC from over-cooked cruciferous vegetables is substantially lower than from raw and lightly cooked cruciferous vegetables. [11, 27] Therefore, it is possible that the chemoprotective effects of cruciferous vegetable may depend on how they are processed. We previously reported that whole raw broccoli consumption protected colonocytes in pigs against DNA damage, whereas broccoli that had been homogenised or cooked in a microwave did not.[29,30] Smith et al. [2] found that juice prepared from raw Brussels sprouts conferred protection against 1,2 dimethylhydrazine induced aberrant crypt foci in rats whereas juice prepared from blanched tissue had no effect. Zhu et al.[31] reported that heating significantly reduced the ability of raw broccoli to induce quinone reductase (QR) (a phase II detoxification enzyme) in the liver of rats, but did not

significantly reduce its ability to induce QR in the colonic mucosa. Collectively these studies indicate that it may be more beneficial to consume raw cruciferous vegetables than cooked cruciferous vegetables.

Broccoli is the most commonly consumed cruciferous vegetable in the UK.[32] A substantial proportion of broccoli is purchased frozen, but it is uncertain whether frozen broccoli retains the bioactivity of raw broccoli.[33] The commercial preparation of frozen broccoli involves a steam or water blanching step that causes the loss of bioactive constituents and inactivates plant myrosinase.[22,34,35] So it follows that the consumption of frozen broccoli may result in a lower exposure to GLS breakdown products and other bioactive constituents than raw broccoli. In the present study we aimed to compare the ability of raw and blanched-frozen broccoli to protect colonocytes against DNA damage, improve plasma antioxidant status, and induce hepatic and colonic xenobiotic metabolising enzymes (XME) using young male pigs as a model system.

MATERIALS AND METHODS

Chemicals

Somulose was from Arnolds, London, UK; zoletil was from Virbac, Glasgow, UK; collagenase/dispase were from Roche Diagnostics (Mannheim, Germany); low melting point (LMP) and high melting point (HMP) agarose from Gibco Life Technologies (Paisley, UK); NaCl and NaOH from Fisher Scientific (Loughborough, UK); modified RPMI 1640 from ICN Flow (Irvine, UK); DAPI (4'6-diamidine-2-phenylindole dihydrochloride) from Boehringer Mannheim (Lewes, UK); acetic acid, acetonitrile, ascorbic acid, benzene, EDTA, ethanol, metaphosphoric acid, n-hexane from Merck Chemicals (Nottingham, UK); European and US vitamin standards from Promochem Ltd (Welwyn Garden city, Herts, UK); KCl,

K_2HPO_4 and K_2HPO_4 from BDH Laboratory Supplies (Poole, UK). All other chemicals were purchased from Sigma-Aldrich (Poole, UK).

Broccoli

Broccoli (*Brassica oleracea* L. var. *italica*, cv. Marathon) fed during the trial was supplied by Sainsbury's Garthdee, Aberdeen. It was grown in Spain and new batches were delivered by Kettle Produce, Cupar, Fife to Sainsbury's on Monday, Wednesday and Friday morning each week. The supplier reported that the typical time lapse between harvesting in Spain and delivery to Sainsbury's was 4 to 6 d. To conform to the regulatory procedures of the minimal disease pig unit of the Rowett Institute of Nutrition and Health, broccoli was washed with a 1% Virkon solution and rinsed with cold water prior to preparation. Washed heads of broccoli were trimmed to approximately 300 g and either stored at 4^{o}C to be fed raw or blanched and frozen before feeding. The blanching and freezing process was designed to closely replicate the commercial preparation of frozen broccoli. Heads of broccoli were steam blanched at 95^{o}C for 4 min in a Convostar, Convotherm steamer (Eglfing, Germany) and then rapidly immersed in ice water to halt the blanching process. After 5 min, the broccoli was removed from the ice water, gently shaken to remove excess water, frozen for 50 min in a blast freezer and then stored at -20^{o}C. Each batch of raw and blanched-frozen broccoli was stored for a maximum of 4 d. The adequacy of the blanching process was tested using a 60 sec peroxidase test (protocol supplied by J.Dempsey of Esk Frozen Foods, Montrose, personal communication). Samples of blanched broccoli were removed from the ice water and cut in half through the length of the stem and florets. Each half was covered evenly with enzyme reagent (equal volumes of 0.5% w/v guaiacol solution and 1.5% hydrogen peroxide (H_2O_2) solution). The blanching process was accepted as adequate if no brown colouration of the enzyme reagent occurred in the broccoli florets within 60 sec.

Preparation of broccoli for the determination of myrosinase activity

Three raw broccoli heads (approx 300 g) were stored at 4^{o}C and three were blanched (as described earlier, except that a Hobart steamer (model CSD1012E, Ohio, USA) was used), blast frozen and stored at -20^{o}C. In both cases, the broccoli was stored for 4 d prior to preparing extracts for measuring myrosinase activity. Samples of approximately 2 g in weight were cut from the stalk and the outer and inner florets of 3 raw and 3 blanched-frozen broccoli heads. Each sample was transferred to ice-cold 33 mM potassium phosphate buffer (5 ml/g plant tissue) and then homogenised with 2 x 5 sec bursts of a rotor stator homogeniser (Ystral D-79282, Ballrechten-Dottingen, Germany). Homogenates were centrifuged at 3000 **g** x 10 min (Hermle Labortechnik Z36HK, Germany). Supernatants were carefully removed and centrifuged again at 3000 **g** for a further 10 min. Myrosinase activity was determined immediately in the resulting supernatants.

Determination of myrosinase activity

Myrosinase activity was determined by the spectrophotometric method of Shapiro *et al.*[36] as modified by Rungapamestry *et al.* [37] The reaction was initiated by adding 100 µl of broccoli extract to 1 ml of reaction mixture (150 µM sinigrin, 500 µM ascorbic acid, 1mM EDTA in a 33 mM phosphate buffer, pH 7.0) in a quartz cuvette (Hellma 114-QG). After a 1 min lag time, the rate of reaction was measured as a linear decrease in absorbance at 227 nm for 3 min (Cecil CE9500 Super Aquarius, Cambridge, England). A standard curve was constructed using isolated myrosinase (0.012 to 0.3 units, R^2=0.998-0.999) and the myrosinase activity in each vegetable extract was calculated by linear regression and expressed as units/g of plant tissue. Myrosinase standards and samples were measured in duplicate.

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152 *Animals and Experimental Design*

153 A total of fifteen male Landrace X Large White pigs (Rowett Institute of Nutrition and
154 Health, Aberdeen, Scotland) consisting of 5 sets of age (79 (SD 3) d) and weight (34.7 (SD
155 3.9) kg) matched siblings were used. Siblings were used to minimise the effect of genetic
156 variation on subsequent treatment outcome. Within each set, siblings were randomly assigned
157 to one of three diet groups: (1) control diet (Rowett Grower feed, see Table 1); (2) the control
158 diet plus 600 g/d of whole raw broccoli; (3) the control diet plus 600 g/d of blanched-frozen
159 broccoli. Before feeding, the blanched-frozen broccoli was allowed to defrost at room
160 temperature and any water released was added to the feed.

161 Each pig was individually housed in pens in a temperature-controlled room (20^{o}C)
162 with a 12 h light-dark cycle with the light phase beginning at 07.00 h. At the start of the
163 experiment, the pigs had a 3 day adaptation period, during which their intake of vegetables
164 was gradually increased to 600 g/d. All pigs were then maintained on their respective
165 experimental diets for a further 12 d. The control diet and broccoli were provided in two daily
166 feeds of equal size at 08.00 h and 16.00 h, with each pig receiving the control diet at a level
167 of 5% of their bodyweight. Within each cohort, feed intake was monitored and corrected
168 where necessary to ensure a similar intake between animals. No correction was made to the
169 control diet to account for the macronutrient content of the supplemental vegetables.
170 Throughout the trial, pigs were allowed access to water *ad libitum*. The starting date of each
171 cohort was staggered to allow time at the end of the experimental period to remove and
172 process samples. At the end of the experimental period, the pigs were weighed, sedated
173 (intramuscular injection of Zoletil 100®; 1 vial reconstituted in 5 ml of di.H_2O),
174 anaesthetised (with an intravenous injection of Somulose®; 1 ml per 10 kg BW), and then

killed by exsanguination. Within each cohort, pigs were killed in a randomised sequence, at hourly intervals, commencing approximately 1 h after the morning feed.

Collection and preparation of blood and tissue samples

Immediately after slaughter, venous blood was collected into 10 ml lithium-heparin coated vacutainers (SIS, Nottingham, UK) and plasma was isolated by centrifugation (2400 g x 15 min at 4^{o}C). For the analysis of ascorbic acid, an aliquot of plasma was diluted 50:50 v/v with 10% metaphosphoric acid, snap frozen in liquid N_2 and stored at -80^{o}C. The remaining plasma was divided into aliquots, snap frozen in liquid N_2 and stored at -80^{o}C for the analysis of retinol, carotenoids and tocopherols.

The entire colon from the ileocaecal junction to the rectum was excised. A section of the mid colon, approximately 200 mm in length was carefully flushed with modified Ca^{2+}/Mg^{2+}-free Hanks Balanced Salt Solution (HBSS, 37^{o}C) and then transferred to fresh HBSS solution (37^{o}C) for transportation from the post-mortem room to the laboratory for isolation of colonocytes (described below). Adjoining sections of colon were rinsed with H_2O, snap frozen in liquid N_2 and stored at -80^{o}C for later preparation of cytosolic fractions. Livers were excised and excess connective tissue was removed. Each liver was chopped into smaller pieces, rapidly frozen in liquid N_2 and stored at -80^{o}C for later preparation of microsomes and cytosolic fractions.

Isolation of colonocytes

Colonocytes were removed from the section of mid colon by a modification of the method of Brendler-Schwaab *et al.*[38] One end of the colon was clamped shut and filled with approximately 50 ml of freshly prepared ice-cold HBSS solution containing 30 mM dithiothreitol (DTT). The open end was tied shut and excess connective tissue was removed.

The colon was then opened at both ends, flushed with ice cold HBSS, re-tied at the base, filled with 40 ml of digestion buffer (HBSS containing 1 mg/ml collagenase/dipase, pH 7.3), re-tied at the top, and suspended in a beaker containing HBSS. The beaker was placed in a shaking water-bath (37^{o}C, 60 rpm x 30 min) to gently agitate the colon. The colon was cut longitudinally and the colonocyte cell suspension from inside was centrifuged at 200 **g** for 6 min at 20^{o}C. The supernatant was decanted and the pellet was resuspended in RPMI 1640-glutamine (1% w/v). Cell membrane integrity (a rough indicator of viability) was determined at the time of cell counting, by trypan blue exclusion (≥80% trypan blue negative). The cell suspension was centrifuged at 200 **g** for 3 min at room temperature, the supernatant was decanted and cells were resuspended in freezer mix (90% FCS, 5% DMSO, 5% RPMI) at a cell concentration of 3 x 10^6 per ml. Cells were frozen slowly to –80^{o}C and then stored in liquid N_2 until analysis.

Preparation of colonic cytosolic fractions

Colonic cytosolic fractions for determining total glutathione S-transferase (GST) activity were prepared as described by Nijhoff & Peters[39], with minor modifications. For each pig, a section of tissue (sealed in a plastic bag) from the same location in the mid colon (directly distal to the section used for the isolation of colonocytes) was defrosted under cold running water. All subsequent steps were conducted at 4^{o}C or on ice. Colons were cut longitudinally and their lumens rinsed with homogenising buffer (0.25 M sucrose, 20 mM Tris, 1 mM DTT, adjusted to pH 7.4 with 12 M HCl). Mucosae were removed by gentle scraping of the exposed luminal wall with a glass microscope slide. Scrapings of mucosal cells were homogenised in ice-cold homogenising buffer (4 ml/g of tissue) by 10-12 strokes of a motor driven Potter-Elvehjem Teflon/glass homogeniser (Status R100C, CAT, Germany) (1000 rpm). Homogenates were centrifuged at 10, 000 **g** x 20 min. The resulting supernatants were

centrifuged at 100, 000 **g** x 60 min, decanted, rapidly snap frozen in liquid N_2 and stored at -80^{o}C for later analysis of GST activity.

Cytosolic fractions for QR determination were prepared as described for analysis of total GST analysis except for the following modifications. Sections of mid colon (directly distal to those used for the determination of GST) were allowed to thaw in KCl-potassium phosphate buffer (0.15 M KCl, 0.25 M KH_2PO_4/K_2HPO_4, pH 7.25) at 4^{o}C, lumens were rinsed with 1.15% KCl and scrapings of mucosal cells were homogenised in KCl-potassium phosphate buffer (4 ml/g of tissue).

Preparation of hepatic cytosolic fractions and microsomes

All steps were conducted on ice or at 0-4^{o}C. Tissue from the left lobe of the liver of each pig was used to prepare cytosolic fractions and microsomes. To minimise the degradation of cytochrome P450 (CYP450), tissue was allowed to defrost slowly in ice-cold homogenising buffer (0.25 M sucrose, 20 mM Tris, 1 mM dithiothreitol (DTT), pH 7.4).[40] Defrosted liver segments were rinsed with ice cold buffer to remove blood, blotted dry, minced with scissors and then homogenised in homogenising buffer (4ml/g tissue) with one 45-60 sec burst of a rotor stator homogeniser (8000 rpm; Janke & Kunkel, Ultra-turrax T25, Germany). Homogenates were centrifuged at 10,000 **g** x 20 min. Supernatants were decanted and centrifuged at 100,000 **g** x 60 min. The fat layer was aspirated and resulting supernatants were decanted, snap frozen in liquid N_2 and stored at -80^{o}C for later analysis of phase 2 enzyme activity. A quantity of homogenising buffer equal to the amount of supernatant decanted was added to the remaining pellet. The pellet was then re-suspended in the buffer with 6-8 strokes of a motor driven Potter-Elvehjem Teflon/glass homogeniser (600 rpm). The resulting homogenate was centrifuged for a further 60 min at 100,000 **g**. Supernatants were discarded and the remaining pellet was re-suspended in freezing buffer (10 mM Tris, 20%

w/v glycerol, 1 mM EDTA, adjusted to pH 7.4 with acetic acid) with 6-8 strokes of the Potter-Elvehjem Teflon/glass homogeniser (600 rpm). The resulting homogenate was snap frozen in liquid N_2 and stored at -80^{o}C for later analysis of total CYP450 and CYP1A1/2 activities.

Analyses

Plasma antioxidant levels. Plasma vitamin C levels were determined by reverse phase HPLC using an ion-pairing reagent with UV detection.[41] Plasma levels of retinol, α-carotene, β-carotene zeaxanthin/lutein, α-tocopherol and γ-tocopherol were measured simultaneously using reverse phase HPLC with visual and fluorescence detection .[42]

Comet assay. The colonocytes were thawed, suspended in 85 μl of 1% LMP agarose and then pipetted onto a frosted glass microscope slide pre-coated with 1% HMP agarose (3 slides per animal, 2 gels per slide). The agarose was allowed to set by incubating at 4^{o}C for 5 min. Slides were then incubated in lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA and 1% (v/v) Triton X-100, adjusted to pH 10 with 10 M NaOH) for at least 1 hour at 4^{o}C to remove membranes and soluble cellular constituents, including histones. Slides were then placed in an electrophoresis tank (containing 0.3 M NaOH & 1 mM EDTA, approximately pH 13) in continuous rows and incubated for 40 min to allow time for DNA unwinding. Subsequently, slides were subjected to electrophoresis in the same solution at a fixed voltage of 25 V and 300 mA for 30 min. The slides were then washed 3 times for 5 min with neutralising buffer (0.4 M Tris, adjusted to pH 7.5 with HCl, 4^{o}C), before staining with 20 μl of DAPI (1 μg/ml).

DAPI stained nucleoids ('comets') were examined with a fluorescence microscope. One hundred nucleoids on each slide were examined visually and scored according to tail density and length using an integer scale between 0 (no damage) and 4 (maximal damage). Therefore the total score for 100 comets could range from 0-400. The researcher scoring the slides was blinded to the treatment allocation during the scoring process. Results from the visual scoring method closely match those from computer image analysis .[43] In an attempt to account for damage that occurred to colonocytes during isolation, a novel adjustment was made to the total comet score. It was assumed that the number of class 4 comets in the control group reflected the level of damage that occurred to colonocytes in all treatment groups during the handling process. This number (which was 29) was subtracted from the number of class 4 comets in all 3 groups and all remaining comet classes where divided by 0.71 to adjust for the removed comets and normalise the data to give a total score out of 400 (i.e. as if 100 comets were present).

Total CYP, ethoxyresorufin O-dealkylation (EROD), methoxyresorufin O-dealkylation (MROD), GST and QR. Microsomal and cytosolic protein was measured by the method of Lowry et al.[44] as modified by Ohnishi & Barr. [45] Total CYP content was determined as the reduced-carbon monoxide (CO) versus oxidised-CO difference spectra[46] on a Kontron, Uvikon 860 spectrophotometer (Kontron Instruments, Watford, UK). Each microsomal sample (0.2 ml) was diluted with 0.1 M potassium phosphate buffer (pH 7.4) to a final volume of 2 ml and divided into two quartz cuvettes. A baseline was recorded between 400 and 500 nm and then the reference cuvette was bubbled with CO for 30-40 sec and 5 µl of NADH (20mM, prepared in potassium phosphate buffer) was added (to reduce cytochrome b_5). The tip of a small spatula was used to add a few grains of sodium dithionite (approximately 1 mg; to reduce CYP450 and cytochrome b_5) to the sample cuvette, which

was then bubbled with CO for 30-40 sec. Spectra were repeatedly recorded until the maximum peak at 450 nm was reached. Total CYP450 was calculated as the change in absorbance at 450 nm relative to the absorbance at 490 nm converted to a concentration using the extinction coefficient of 106 mM/cm.

EROD and MROD activities were determined by the method of Burke *et al*.[47] For both assays, 0.2 mg of microsomal protein and 0.25 ml of an NADPH generating system (0.8 mg NADP, 1.2 mg isocitric acid, 50 µl 0.1M $MgSO_4$, 1 unit isocitrate dehydrogenase and 0.1 M Tris-HCl, pH 7.8) were added to 0.1 M Tris-HCl buffer (pH 7.8), total volume 1.99 ml. Samples were incubated for 2 min at 37^{o}C and the reaction was initiated by adding 10 µl of ethoxyresorufin or methoxyresorufin (cuvette concentration 5 µM). The linear increase in fluorescence was recorded for 10 min at an excitation wavelength 530 nm and an emission wavelength 585 nm (Perkin-Elmer LS55, Massachusetts, USA) and calibrated by the addition of a resorufin standard (10 µM in DMSO).

GST activity was determined spectrophotometrically at 25^{o}C with 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate according to the method of Habig *et al.*[48] with minor modifications. The reaction was initiated by the addition of 0.1 ml of cytosol to a cuvette containing 0.1 ml glutathione (final concentration 5 mM) and 0.8 ml reaction mixture (final concentration 0.1 M KH_2PO_4/K_2HPO_4 pH 6.5, and 1 mM CDNB, 2% ethanol). Cytosolic fractions were diluted in PBS to ensure assay linearity (a 100 x dilution for liver and 5 x dilution for colon). The linear increase in absorbance at 340 nm was recorded for 3 min (Unicam 8700 series UV/VIS spectrophotometer, Cambridge, UK) and GST activity was calculated using the molar extinction coefficient of 9.6 mM/cm and expressed as nmol of product formed/min/mg cytosolic protein.

QR activity was determined by the spectrophotometric method of Ernster[49], as modified by Benson *et al.*[50], using 2, 6-dichlorophenolindophenol (DPIP) as the electron

acceptor and NADPH as the electron donor. The reaction was initiated by the addition of 10 μl of 12 mM DPIP to 0.3 ml of cytosol (100 x dilution for liver, 20 x dilution for colon) and 2.65 ml of the reaction mixture (final concentrations: 25 mM Tris, 0.7 mg BSA, 0.01% Tween 20, 5 μM FAD, 0.2 mM NADPH). The rate of reaction was measured as a linear decrease in absorbance at 600 nm over 90 sec (Unicam 8700 series UV/VIS spectrophotometer, Cambridge, UK). Activity was measured in triplicate in the presence and absence of dicumarol (10 μM). Quinone reductase activity was calculated from the dicumarol sensitive portion of the assay, using a molar extinction coefficient of 21 mM/cm and expressed as nmol of product formed/min/mg cytosolic protein.

Statistical analysis

The myrosinase data was $\log_{10}$ transformed after Levene's test indicated the data had unequal variance. A two-way ANOVA using the log-transformed data was used to investigate the effect of blanch-freezing on myrosinase activity, and whether myrosinase activity varied in different sections of broccoli. Standardised residuals of the $\log_{10}$ transformed data were normally distributed (Shapiro-Wilk *W* test). Results are expressed as the geometric mean and 95% CIs of the antilog transformed data.

The significance of differences between treatment means was tested by two way ANOVA (diet as the fixed factor and cohort as the random factor); when a significant effect was found this was followed by post hoc Dunnett's test. Standardised residuals for each outcome variable were assessed for normality (Shapiro Wilk *W* test); when they deviated from normality, ANOVA was conducted on $\log_{10}$ transformed data.

All statistical analyses were conducted on SPSS Release 19.0 (2011) and a *P* value of <0.05 was used as the critical level of significance.

RESULTS

Effect of blanch-freezing on myrosinase activity in broccoli

The blanch-freezing protocol caused a significant decrease in myrosinase activity ($P<0.001$). Myrosinase activity did not differ significantly between inner florets, outer florets and stalk ($P=0.147$) and there was no significant interaction between the effects of blanch-freezing and the part of the plant that each section was sampled from ($P=0.765$) (Fig 1.).

Animal Experiment

The mean intake of basal diet (1273 (SD 167.1) g/d) did not differ by dietary group and the raw broccoli and blanched-frozen broccoli groups consumed similar amounts of broccoli. All groups of pigs gained weight, but the raw broccoli ($P=0.028$) and blanched-frozen broccoli ($P=0.043$) groups gained significantly more weight (approximately 1 kg) than the control group (Table 2).

Broccoli supplementation failed to have any significant effect on plasma concentrations of retinol, lutein/zeaxanthin, ascorbic acid, α-tocopherol or γ-tocopherol (Table 3).

The consumption of raw broccoli caused a significant 27% increase in DNA strand breakage in comparison to the control group (Fig. 2; $P=0.025$). The frequency of DNA strand breaks in the group that consumed blanched-frozen broccoli was not significantly different from the control group (Fig 2 $P=0.243$). Analysis of individual comet classes revealed a significantly greater percentage of maximally damaged class 4 comets (42 v 29%, $P=0.03$) and a significantly lower percentage of colonocytes exhibiting no damage (class 0; 15 v 26%, $P=0.019$) in pigs fed raw broccoli than in pigs fed the control diet. There were no other significant differences in comet classes (Table 4). The number of maximally damaged class 4 comets in the control group was high (29%). In the absence of exposure to a genotoxin, cells

should contain few if any class 4 comets, so it seems that a substantial amount of DNA damage had occurred during isolation of the colonocytes. In an attempt to gain a clearer picture of the influence of the broccoli diets on DNA damage we adjusted the comet class data assuming that the level of class 4 comets in the control group reflected the level of damage that occurred to colonocytes in all treatment groups during the handling process (see methods for explanation). The adjusted data shown in Fig 3 illustrate the clearly different populations of individual comets in the raw broccoli (group 1) and control (group 3) groups and also provide some evidence that the distribution of comets in the blanched-frozen broccoli group is closer to the raw broccoli group than to the control group.

Neither broccoli supplemented diet increased the total content of CYP450 in the liver or altered the activity of hepatic, EROD and MROD (Table 5). Moreover hepatic and colonic GST and QR activities were also unaffected (Table 6).

DISCUSSION

The present study found that pigs fed a diet supplemented with raw broccoli had a greater amount of DNA damage in their colonocytes than pigs fed a control diet. This contrasts with our earlier work where we reported that raw broccoli protected colonocytes from DNA damage.[29, 30] This disagreement is difficult to explain because the present work used the same experimental design as our earlier studies, but one possibility is that the composition of the broccoli differed. It is uncertain which constituents of the raw broccoli were responsible for damaging DNA, but indolyl GLS are potential candidates. Baasanjev et al. [51] reported that the indolyl GLS, neoglucobrassicin, gluconeobrassicin and 4-methoxyglucobrassicin (all typically present in broccoli) exhibited mutagenicity in *S.typhimurium* TA100 and TA104 and formed adducts with herring sperm DNA, whereas glucoraphanin (the major aliphatic GLS in broccoli) was not mutagenic and produced few DNA adducts. The ratio of indolyl

GLS to aliphatic GLS in broccoli varies several-fold between cultivars and even within the same cultivar grown under different conditions.[52-54] It is possible that such differences explain the contrast between our present work and earlier studies.[29,30] The GLS in the broccoli fed in the current study degraded before a successful analysis could be completed; however, published data indicate that the Marathon cultivar typically has a high ratio of indolyl GLS to aliphatic GLS. [24,54] Differences in the content of other bioactive constituents in the broccolis may also have contributed to the contrasting results of our experiments.

Although the current study seems to be the first to report an increase in DNA damage in colonocytes after the consumption of raw broccoli, there are other reports of cruciferous vegetables damaging DNA *in vivo*. In a rodent study, Sorensen et al.[55] reported that a cooked Brussels sprouts extract increased 8-oxo-dG levels in the liver. Scaled for body weight, the daily intake of Brussels sprouts was similar to the intake of broccoli in the present study (25.6 v 17.3 g/kg/body weight). In a recent human study, Charron et al.[56] reported a transient increase in DNA damage in peripheral blood mononuclear cells isolated from individuals 3 hours after they had consumed either isolated allyl isothiocyanate (AITC) (114.7 μmol) incorporated into mayonnaise or a mustard/cabbage treatment (150 g of raw homogenised cabbage plus 30 g of Grey Poupon Country Dijon Mustard). In contrast, the majority of studies in humans have reported that modest intakes of raw and cooked cruciferous vegetables (85-300 g/d) protect lymphocytes from DNA damage or reduce the urinary excretion of 8-oxo-dG[57-62] (a possible marker of whole body oxidative stress).[63] Also, in a rat study Kassie et al.[64] reported that juice prepared from raw garden cress reduced background levels of DNA damage in colonocytes. Thus, it seems that the effects of cruciferous vegetables on DNA damage may depend on the amount and type of cruciferous vegetables fed, the timing of measurement, the tissue studied, as well as the method of preparation.

In contrast to the effect of raw broccoli, blanched-frozen broccoli did not cause a statistically significant increase in colonocyte DNA damage. Similar blanching protocols to ours have been shown to cause a substantial loss of GLS and other water soluble compounds from broccoli.[22,34] So, the failure of the blanched-frozen broccoli to significantly increase DNA damage could reflect a lower exposure to the genotoxic component(s). Latte et al.[65] recently speculated that the genotoxicity of broccoli required active plant myrosinase and epithiospecifier protein (a cofactor that promotes the formation of nitriles over ITC during GLS hydrolysis). Our blanching protocol caused an almost complete loss of myrosinase activity and would have also inactivated the more heat sensitive epithiospecifier protein (not measured).[26] When cruciferous vegetables with inactive myrosinase are consumed the colonic microflora can hydrolyse GLS; however, the yield of ITCs is approximately 10 fold lower.[66] It is therefore likely that the colonocytes of pigs fed the blanched-frozen broccoli were exposed to lower levels of ITC and possibly indole derivatives (although we are unaware of data on the yield of indoles from bacterial catalysed degradation of indolyl GLS) than the colonocytes of pigs fed raw broccoli. It is also probable that they were exposed to different GLS metabolites produced by the colonic bacteria.[13-14]

We determined the plasma concentrations of ascorbic acid, α-tocopherol, γ-tocopherol, lutein/zeaxanthin and retinol as markers of a change in antioxidant status in pigs fed the broccoli diets. Published data indicate that 600 g of broccoli (cv. Marathon) would be expected to contain an average of 8.2 mg of total carotenoids (5.5 mg β-carotene, 2.6 mg lutein, 0.1 mg other carotenoids), 8.7 mg α-tocopherol, 5.4 mg γ-tocopherol, and 730 mg of ascorbic acid.[67,68] Despite this, neither broccoli-supplemented diet increased the concentration of any of the putative plasma markers of antioxidant status. The lack of effect on retinol and α-tocopherol may be because the basal diet was enriched with substantial amounts of retinol (average intake 4.3 mg/d) and α-tocopherol (143 mg/d). Pro-vitamin A

carotenoids are relatively ineffective at increasing plasma retinol when vitamin A status is adequate.[69] Studies investigating the effect of supplemental ascorbic acid in pigs are inconsistent with some reporting an elevation and others no effect.[70-73] The reason for this is unclear, but there is evidence that the feeding of high intakes of ascorbate to pigs can inhibit the ascorbic acid synthesising enzyme, l-gulono-gamma-lactone oxidase.[74] The inertia in plasma lutein contrasts with human studies that have reported that 200-300 g/d of broccoli significantly increased plasma lutein.[75-76]

The induction of phase 2 detoxification enzymes is thought to be an important mechanism through which broccoli consumption protects against chemically induced carcinogenesis.[77] In the current study, neither, raw nor blanched-frozen broccoli altered the activity of GST and QR in the liver and colon. In contrast, rodent studies have reported significant inductions of hepatic and colonic QR[78,79] or hepatic GST in response to diets containing freeze-dried broccoli.[80,81] This disparity probably reflects the quantity of broccoli fed. Accounting for the water content of fresh broccoli, pigs in the current study consumed a diet equivalent to a 5% w/w freeze-dried broccoli diet. Aspry & Bjeldanes[80] reported a significant induction of hepatic GST in rodents fed a 25% w/w broccoli diet, but no significant effect of a 10% w/w broccoli diet, whereas other studies reporting induction of GST or QR fed 20-25% w/w broccoli diets.[78,79,81]

The EROD and MROD assays are thought to be fairly specific probes of hepatic CYP1A1 and CYP1A2 activities respectively.[82,83] Diets containing 10 to 25% w/w broccoli have been shown to induce hepatic EROD and MROD in rodents, whereas in the current study we failed to find an effect of either broccoli supplemented diet.[31,53,78] We are unaware of any studies that have reported on EROD or MROD activity in animals fed similar amounts of broccoli to our study; however, in a human study, the consumption of a proportionally lower intake of broccoli (500 g/d; cv. Marathon) for 12 d caused a modest induction of

CYP1A2 as determined by a significant 19% increase in the metabolism of a defined dose of caffeine.[84] The disagreement with the current study may reflect differences in response between humans and pigs, variations in the GLS content/profile of the fed broccoli or differences in the sensitivity of measuring CYP1A2 induction with the MROD assay in liver microsomes versus the *in vivo* metabolism of caffeine.

This study has several limitations. First, the high level of DNA damage observed in colonocytes isolated from the control pigs indicates that a substantial amount of damage occurred during the isolation process. It is possible that this high level of damage may have masked a greater genotoxic effect of raw broccoli or a modest genotoxic effect of the blanched-frozen broccoli. In an attempt to determine the impact of this damage we adjusted the comet class data by subtracting the percentage of class 4 comets in the control group from all groups and normalised the data (see results). This arguably provided more compelling evidence of the genotoxic effect of the raw broccoli, but also illustrated that the comet distribution of the blanched-frozen broccoli seemed closer to that of the raw broccoli than the control group. Second, we did not adjust the diet of the control pigs to account for the additional energy intake the treatment pigs received from the broccoli (approx. 11.3 MJ over the complete 15 d trial period). This resulted in a slightly greater weight gain in the pigs fed the broccoli supplemented diets. It is uncertain whether weight gain influenced the effect of the broccoli diets. Third, to be consistent with our earlier studies, the pigs were sedated with Zoletil and anaesthetised with Somulose before being killed. It is possible that these drugs may have masked a small induction of phase 1 and phase 2 enzyme activities by the broccoli diets, but the short time lapse between administration of the drugs and tissue removal (approx. 30 min) probably precluded a substantial effect. Furthermore, others have reported significant inductions of colonic and hepatic QR in rats fed broccoli and anaesthetised with ketamine/xylazine prior to killing.[31,79] Fourth, we were unable to characterise the GLS profile

of the broccoli, so we can only speculate on its probable composition of indolyl and aliphatic GLS.

In summary, the present study demonstrated that raw broccoli consumption can increase DNA damage in the colonocytes of pigs whereas broccoli that has been blanched and frozen prior to consumption does not. The contrast with our earlier work that found raw broccoli protected colonocytes against DNA damage is difficult to explain but raises the possibility that different cultivars of broccoli exert opposite effects. Further studies are needed to clarify whether the genotoxicity of broccoli varies by cultivar and/or GLS composition.

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Conflicts of interest

The authors report no conflicts of interest.

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Table 1 Composition of Rowett Standard Grower Feed

Component	kg/1000 kg
Barley	228
Wheat	430
Hipro Soya	225
Super Soya	50
Soya Oil	10
Salt	5
Grower vitamin and mineral mix*	30
Molasses	20
De-oderase®	2

* The vitamin and mineral mix contained (per kg) calcium 250 g, copper 8.925 g, phosphorus 7 g, sodium 5 g, selenium 13 mg, retinol 113, 636 μg, α-tocopherol acetate 3750 mg, vitamin D, 1875 μg. De-oderase® is a preparation that contains glycocomponents derived from the *Yucca shidigera* plant. It is thought to reduce odour and ammonia emissions from livestock (Amon *et al.* 1995).[85]

Table 2 Mean values (SD) of weight gain and daily intake of basal diet and broccoli over the 12 d test period.

Treatment	Basal diet g/d	Broccoli g/d	Initial body weight kg	Final body weight kg	Body weight gain kg
Raw broccoli	1273 (167)	577 (40)	33.9 (1.8)	41.6 (3.5)	7.7* (1.8)
Blanched-Frozen broccoli	1273 (167)	585 (29)	34.3 (6.3)	41.9 (7.0)	7.6* (1.2)
Control	1273 (167)	—	35.9 (2.9)	42.6 (3.9)	6.8 (2.0)

Basal diet, Rowett standard grower feed (see Table 1 for composition). Two way ANOVA and Dunnett's test were used to assess statistical differences in body weight gain. An asterisk within a column indicates a significant difference between a treatment group and the control group ($P<0.05$); $n=5$ in each diet group in all cases.

Table 3 Mean plasma concentration of vitamins and carotenoids in groups of pigs fed cereal diets unsupplemented (control) or supplemented with 600 g of raw or blanched-frozen broccoli (cv. Marathon) for 12 d.

Treatment	Retinol µg/ml	Zeaxanthin/lutein µg/ml	Ascorbic acid µM	α-tocopherol µg/ml	γ-tocopherol µg/ml
Raw broccoli	0.348 (0.211, 0.572)	0.0031 (0.0006)	28.6 (15.6, 52.8)	1.19 (0.21)	0.021 (0.005)
Blanched-frozen broccoli	0.280 (0.170, 0.461)	0.0029 (0.0006)	43.2 (23.4, 79.4)	1.52 (0.33)	0.024 (0.006)
Control	0.304 (0.185, 0.501)	0.0027 (0.0002)	29.4 (16.0, 54.2)	1.68 (0.64)	0.025 (0.012)

Non-transformed data are expressed as mean (SD), data that were $\log_{10}$ transformed before analysis are expressed as geometric mean (95 % CI). Two way ANOVA was used to assess statistical differences, *n*=5 in each diet group for all variables, except zeaxanthin/lutein, where *n*=4.

Table 4 Comet classes in colonocytes isolated from pigs fed one of the following diets: cereal diet unsupplemented (control), or cereal diet supplemented with 600 g/d of raw and blanched-frozen broccoli for 12 d.

Treatment	Comet classes				
	0	1	2	3	4
Raw broccoli	15.0*(12.3)	11.5 (2.0)	9.3 (2.1)	22.1 (6.1)	42.2*(9.3)
Blanched-frozen broccoli	17.7 (5.3)	15.2 (2.8)	12.4 (1.3)	21.6 (3.5)	33.2 (6.5)
Control	26.0 (6.8)	15.4 (4.6)	11.4 (3.3)	18.4 (3.2)	28.9 (8.5)

Results are expressed as the number of colonocytes in each class of damage and are the mean (SD) of 3 slides (each containing two gels) for each animal and 5 animals in each treatment group. Two way ANOVA and Dunnett's test were used to assess significance of differences. Within each column an asterisk indicates that the treatment group differs significantly from the control group ($P<0.05$).

Table 5 The effect of raw and blanched-frozen broccoli on total CYP450 content and specific activities of EROD and MROD in hepatic microsomes.

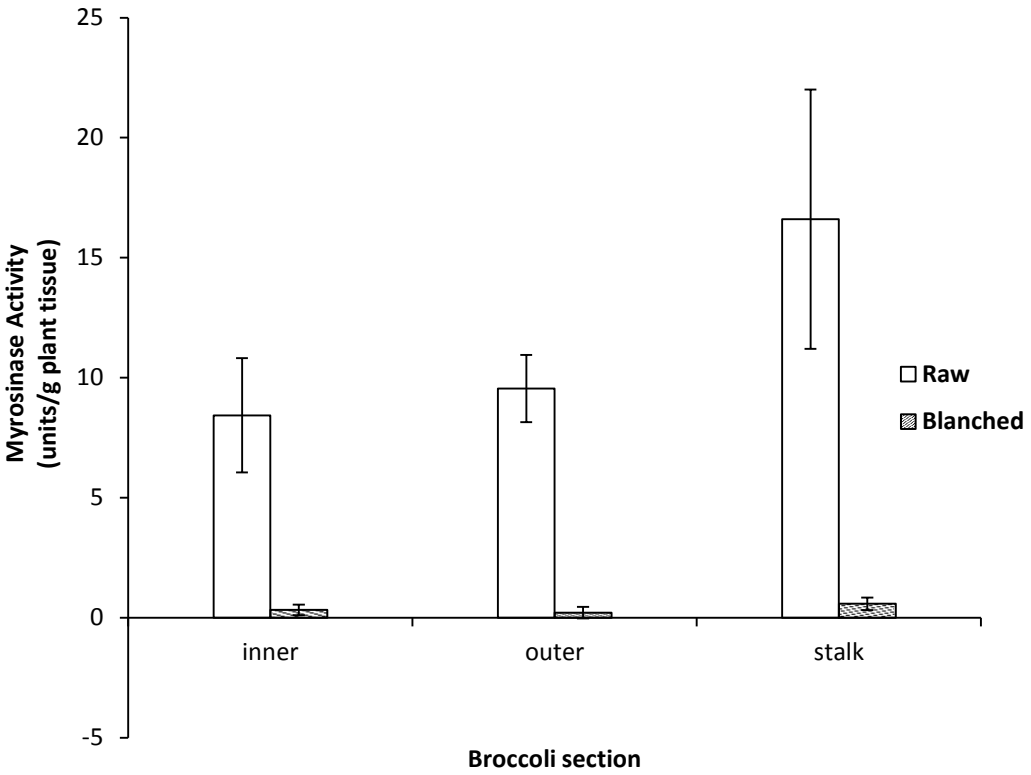
	Total CYP450 nmol/mg of protein	EROD pmol/min/mg of protein	MROD pmol/min/mg of protein
Raw broccoli	0.61 (0.08)	106.1 (31.9)	38.3 (9.7)
Blanched-frozen broccoli	0.60 (0.08)	111.6 (33.8)	39.1 (9.2)
Control	0.61 (0.14)	103.2 (20.8)	37.2 (5.0)

Values are means (SD), in all cases *n*=5 in each diet group. All analyses were conducted in triplicate. Two way ANOVA was used to assess significance of differences.

Table 6 The effect of raw and blanched-frozen broccoli on the specific activities of GST and QR in hepatic and colonic cytosols.

	Hepatic GST nmol/mg of protein	Colonic GST nmol/mg of protein	Hepatic QR nmol/mg of protein	Colonic QR nmol/mg of protein
Raw broccoli	2109 (549)	160.1 (89.2)	120.3 (15.7)	133.8 (25.9)
Blanched-frozen broccoli	2178 (547)	171.3 (56.5)	137.6 (23.2)	130.8 (27.1)
Control	2213 (255)	149.1 (94.3)	134.9 (24.5)	106.7 (30.8)

Values are means (SD), in all cases *n*=5 in each diet group. All analyses were conducted in triplicate. QR activity was determined in the presence and absence of dicumarol. Two way ANOVA was used to assess significance of differences.



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908 **Fig 1** Effect of blanch-freezing on myrosinase activity in broccoli. Myrosinase activity is expressed as units/g of
909 plant tissue in sections sampled from the stalk and the inner and outer regions of heads of whole raw and
910 blanched-frozen broccoli. Bars represent 95% CI of the geometric means of three replicates. Two way ANOVA
911 with interaction was used to assess the significance of differences. Blanched-frozen broccoli had significantly
912 less myrosinase activity than raw broccoli (P<0.001). Myrosinase activity did not differ significantly in samples
913 from different parts of the broccoli plant (P=0.147). There was no interaction between the effects of blanch-
914 freezing and part of the plant that the sample was taken from (P=0.765).
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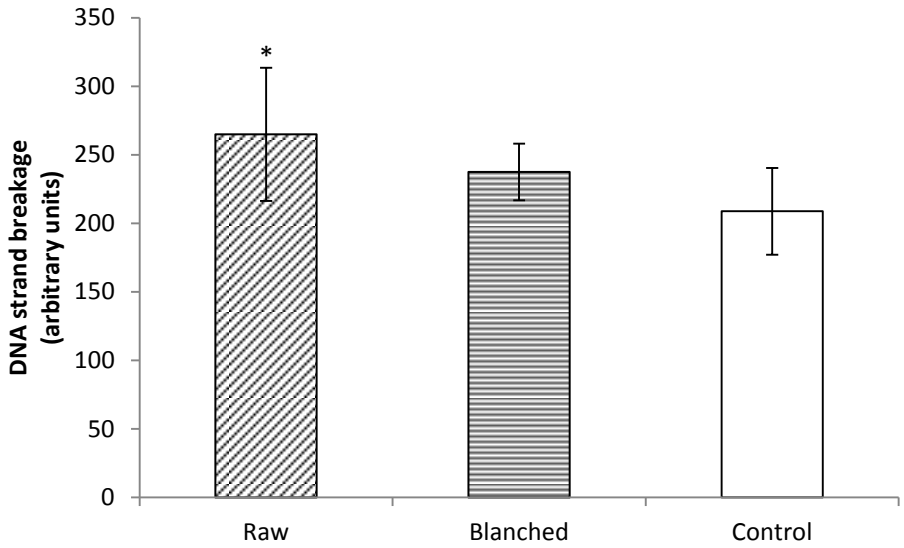

Fig 2 The level of DNA damage in colonocytes isolated from pigs fed cereal diets unsupplemented (control) or supplemented with raw or blanched-frozen broccoli (cv. Marathon) for 12 d. Results are expressed as mean values (in arbitrary units, scored 0-400) for each treatment group; vertical bars represent standard deviations. Two way ANOVA (for a randomised block design) and Dunnett's test were used to assess statistical difference. An asterisk indicates that the treatment group differs significantly from the control group (P<0.05).

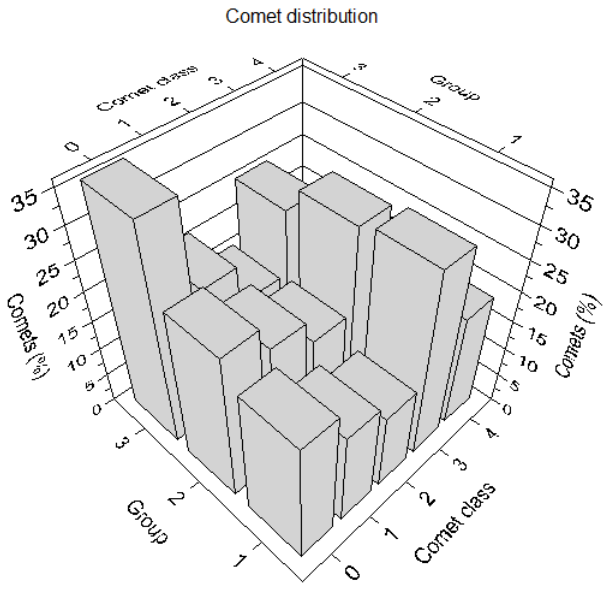

Fig 3 Distribution of comets in pigs fed cereal diets unsupplemented (control; group 3) or supplemented with raw broccoli (group 1) or blanched-frozen broccoli (group 2). Data have been adjusted for estimated damage that occurred during the isolation process (see text).