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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

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

28

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

30

31 INTRODUCTION

32

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

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

52 enriched with cruciferous vegetables.^{1-3, 18,19} Cruciferous vegetables could protect
53 colonocytes against oxidative stress. Rychlik et al.²⁰ reported that undigested extracts of raw
54 broccoli sprouts and extracts subjected to an *in vitro* method of gastrointestinal digestion had
55 a similar ability to protect NCM460 colon cells against H₂O₂ induced oxidative DNA
56 damage. Cells were incubated with the extracts for only 1 hour before exposure to H₂O₂, so
57 protection was tentatively attributed to direct antioxidant effects of phenolic compounds such
58 as sinapic acid, which were resistant to gastrointestinal digestion. Bonnesen et al.¹⁵ reported
59 that the GLS breakdown products, sulforaphane and indolo[3,2-b]carbazole protected LS-174
60 colon cancer cells against H₂O₂ induced DNA damage, an effect that may have been
61 mediated by the induction of cytoprotective enzymes.^{15,21}

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

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

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

90

91

92 **MATERIALS AND METHODS**

93 *Chemicals*

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

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

104

105 *Broccoli*

106 Broccoli (*Brassica oleracea* L. var. *italica*, cv. Marathon) fed during the trial was supplied by
107 Sainsbury's Garthdee, Aberdeen. It was grown in Spain and new batches were delivered by
108 Kettle Produce, Cupar, Fife to Sainsbury's on Monday, Wednesday and Friday morning each
109 week. The supplier reported that the typical time lapse between harvesting in Spain and
110 delivery to Sainsbury's was 4 to 6 d. To conform to the regulatory procedures of the minimal
111 disease pig unit of the Rowett Institute of Nutrition and Health, broccoli was washed with a
112 1% Virkon solution and rinsed with cold water prior to preparation. Washed heads of
113 broccoli were trimmed to approximately 300 g and either stored at 4°C to be fed raw or
114 blanched and frozen before feeding. The blanching and freezing process was designed to
115 closely replicate the commercial preparation of frozen broccoli. Heads of broccoli were steam
116 blanched at 95°C for 4 min in a Convostar, Convotherm steamer (Eglfing, Germany) and then
117 rapidly immersed in ice water to halt the blanching process. After 5 min, the broccoli was
118 removed from the ice water, gently shaken to remove excess water, frozen for 50 min in a
119 blast freezer and then stored at -20°C. Each batch of raw and blanched-frozen broccoli was
120 stored for a maximum of 4 d. The adequacy of the blanching process was tested using a 60
121 sec peroxidase test (protocol supplied by J.Dempsey of Esk Frozen Foods, Montrose,
122 personal communication). Samples of blanched broccoli were removed from the ice water
123 and cut in half through the length of the stem and florets. Each half was covered evenly with
124 enzyme reagent (equal volumes of 0.5% w/v guaiacol solution and 1.5% hydrogen peroxide
125 (H_2O_2) solution). The blanching process was accepted as adequate if no brown colouration of
126 the enzyme reagent occurred in the broccoli florets within 60 sec.

127

128 *Preparation of broccoli for the determination of myrosinase activity*

129 Three raw broccoli heads (approx 300 g) were stored at 4°C and three were blanched (as
130 described earlier, except that a Hobart steamer (model CSD1012E, Ohio, USA) was used),
131 blast frozen and stored at -20°C. In both cases, the broccoli was stored for 4 d prior to
132 preparing extracts for measuring myrosinase activity. Samples of approximately 2 g in weight
133 were cut from the stalk and the outer and inner florets of 3 raw and 3 blanched-frozen
134 broccoli heads. Each sample was transferred to ice-cold 33 mM potassium phosphate buffer
135 (5 ml/g plant tissue) and then homogenised with 2 x 5 sec bursts of a rotor stator homogeniser
136 (Ystral D-79282, Ballrechten-Dottingen, Germany). Homogenates were centrifuged at 3000 g
137 x 10 min (Hermle Labortechnik Z36HK, Germany). Supernatants were carefully removed
138 and centrifuged again at 3000 g for a further 10 min. Myrosinase activity was determined
139 immediately in the resulting supernatants.

140

141 *Determination of myrosinase activity*

142 Myrosinase activity was determined by the spectrophotometric method of Shapiro *et al.*³⁶ as
143 modified by Rungapamestry *et al.*³⁷ The reaction was initiated by adding 100 µl of broccoli
144 extract to 1 ml of reaction mixture (150 µM sinigrin, 500 µM ascorbic acid, 1mM EDTA in a
145 33 mM phosphate buffer, pH 7.0) in a quartz cuvette (Hellma 114-QG). After a 1 min lag
146 time, the rate of reaction was measured as a linear decrease in absorbance at 227 nm for 3
147 min (Cecil CE9500 Super Aquarius, Cambridge, England). A standard curve was constructed
148 using isolated myrosinase (0.012 to 0.3 units, $R^2=0.998-0.999$) and the myrosinase activity in
149 each vegetable extract was calculated by linear regression and expressed as units/g of plant
150 tissue. Myrosinase standards and samples were measured in duplicate.

151

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°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.H₂O),
174 anaesthetised (with an intravenous injection of Somulose®; 1 ml per 10 kg BW), and then

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

177

178 *Collection and preparation of blood and tissue samples*

179 Immediately after slaughter, venous blood was collected into 10 ml lithium-heparin coated
180 vacutainers (SIS, Nottingham, UK) and plasma was isolated by centrifugation (2400 g x 15
181 min at 4°C). For the analysis of ascorbic acid, an aliquot of plasma was diluted 50:50 v/v with
182 10% metaphosphoric acid, snap frozen in liquid N₂ and stored at -80°C. The remaining
183 plasma was divided into aliquots, snap frozen in liquid N₂ and stored at -80°C for the analysis
184 of retinol, carotenoids and tocopherols.

185 The entire colon from the ileocaecal junction to the rectum was excised. A section of
186 the mid colon, approximately 200 mm in length was carefully flushed with modified
187 Ca²⁺/Mg²⁺-free Hanks Balanced Salt Solution (HBSS, 37°C) and then transferred to fresh
188 HBSS solution (37°C) for transportation from the post-mortem room to the laboratory for
189 isolation of colonocytes (described below). Adjoining sections of colon were rinsed with
190 H₂O, snap frozen in liquid N₂ and stored at -80°C for later preparation of cytosolic fractions.
191 Livers were excised and excess connective tissue was removed. Each liver was chopped into
192 smaller pieces, rapidly frozen in liquid N₂ and stored at -80°C for later preparation of
193 microsomes and cytosolic fractions.

194

195 *Isolation of colonocytes*

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

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

212

213 *Preparation of colonic cytosolic fractions*

214 Colonic cytosolic fractions for determining total glutathione S-transferase (GST) activity
215 were prepared as described by Nijhoff & Peters³⁹, with minor modifications. For each pig, a
216 section of tissue (sealed in a plastic bag) from the same location in the mid colon (directly
217 distal to the section used for the isolation of colonocytes) was defrosted under cold running
218 water. All subsequent steps were conducted at 4°C or on ice. Colons were cut longitudinally
219 and their lumens rinsed with homogenising buffer (0.25 M sucrose, 20 mM Tris, 1 mM DTT,
220 adjusted to pH 7.4 with 12 M HCl). Mucosae were removed by gentle scraping of the
221 exposed luminal wall with a glass microscope slide. Scrapings of mucosal cells were
222 homogenised in ice-cold homogenising buffer (4 ml/g of tissue) by 10-12 strokes of a motor
223 driven Potter-Elvehjem Teflon/glass homogeniser (Status R100C, CAT, Germany) (1000
224 rpm). Homogenates were centrifuged at $10,000 \text{ g} \times 20 \text{ min}$. The resulting supernatants were

225 centrifuged at 100,000 g x 60 min, decanted, rapidly snap frozen in liquid N₂ and stored at -
226 80°C for later analysis of GST activity.

227 Cytosolic fractions for QR determination were prepared as described for analysis of
228 total GST analysis except for the following modifications. Sections of mid colon (directly
229 distal to those used for the determination of GST) were allowed to thaw in KCl-potassium
230 phosphate buffer (0.15 M KCl, 0.25 M KH₂PO₄/K₂HPO₄, pH 7.25) at 4°C, lumens were
231 rinsed with 1.15% KCl and scrapings of mucosal cells were homogenised in KCl-potassium
232 phosphate buffer (4 ml/g of tissue).

233

234 *Preparation of hepatic cytosolic fractions and microsomes*

235 All steps were conducted on ice or at 0-4°C. Tissue from the left lobe of the liver of each pig
236 was used to prepare cytosolic fractions and microsomes. To minimise the degradation of
237 cytochrome P450 (CYP450), tissue was allowed to defrost slowly in ice-cold homogenising
238 buffer (0.25 M sucrose, 20 mM Tris, 1 mM dithiothreitol (DTT), pH 7.4).⁴⁰ Defrosted liver
239 segments were rinsed with ice cold buffer to remove blood, blotted dry, minced with scissors
240 and then homogenised in homogenising buffer (4ml/g tissue) with one 45-60 sec burst of a
241 rotor stator homogeniser (8000 rpm; Janke & Kunkel, Ultra-turrax T25, Germany).

242 Homogenates were centrifuged at 10,000 g x 20 min. Supernatants were decanted and
243 centrifuged at 100,000 g x 60 min. The fat layer was aspirated and resulting supernatants
244 were decanted, snap frozen in liquid N₂ and stored at -80°C for later analysis of phase 2
245 enzyme activity. A quantity of homogenising buffer equal to the amount of supernatant
246 decanted was added to the remaining pellet. The pellet was then re-suspended in the buffer
247 with 6-8 strokes of a motor driven Potter-Elvehjem Teflon/glass homogeniser (600 rpm). The
248 resulting homogenate was centrifuged for a further 60 min at 100,000 g. Supernatants were
249 discarded and the remaining pellet was re-suspended in freezing buffer (10 mM Tris, 20%

250 w/v glycerol, 1 mM EDTA, adjusted to pH 7.4 with acetic acid) with 6-8 strokes of the
251 Potter-Elvehjem Teflon/glass homogeniser (600 rpm). The resulting homogenate was snap
252 frozen in liquid N₂ and stored at -80°C for later analysis of total CYP450 and CYP1A1/2
253 activities.

254

255 *Analyses*

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

260

261 *Comet assay.* The colonocytes were thawed, suspended in 85 μ l of 1% LMP agarose and then
262 pipetted onto a frosted glass microscope slide pre-coated with 1% HMP agarose (3 slides per
263 animal, 2 gels per slide). The agarose was allowed to set by incubating at 4°C for 5 min.
264 Slides were then incubated in lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM Na₂EDTA
265 and 1% (v/v) Triton X-100, adjusted to pH 10 with 10 M NaOH) for at least 1 hour at 4°C to
266 remove membranes and soluble cellular constituents, including histones. Slides were then
267 placed in an electrophoresis tank (containing 0.3 M NaOH & 1 mM EDTA, approximately
268 pH 13) in continuous rows and incubated for 40 min to allow time for DNA unwinding.
269 Subsequently, slides were subjected to electrophoresis in the same solution at a fixed voltage
270 of 25 V and 300 mA for 30 min. The slides were then washed 3 times for 5 min with
271 neutralising buffer (0.4 M Tris, adjusted to pH 7.5 with HCl, 4°C), before staining with 20 μ l
272 of DAPI (1 μ g/ml).

273 DAPI stained nucleoids ('comets') were examined with a fluorescence microscope.
274 One hundred nucleoids on each slide were examined visually and scored according to tail
275 density and length using an integer scale between 0 (no damage) and 4 (maximal damage).
276 Therefore the total score for 100 comets could range from 0-400. The researcher scoring the
277 slides was blinded to the treatment allocation during the scoring process. Results from the
278 visual scoring method closely match those from computer image analysis.⁴³ In an attempt to
279 account for damage that occurred to colonocytes during isolation, a novel adjustment was
280 made to the total comet score. It was assumed that the number of class 4 comets in the
281 control group reflected the level of damage that occurred to colonocytes in all treatment
282 groups during the handling process. This number (which was 29) was subtracted from the
283 number of class 4 comets in all 3 groups and all remaining comet classes were divided by
284 0.71 to adjust for the removed comets and normalise the data to give a total score out of 400
285 (i.e. as if 100 comets were present).

286

287 *Total CYP, ethoxyresorufin O-dealkylation (EROD), methoxyresorufin O-dealkylation*
288 *(MROD), GST and QR.* Microsomal and cytosolic protein was measured by the method of
289 Lowry et al.⁴⁴ as modified by Ohnishi & Barr.⁴⁵ Total CYP content was determined as the
290 reduced-carbon monoxide (CO) versus oxidised-CO difference spectra⁴⁶ on a Kontron,
291 Uvikon 860 spectrophotometer (Kontron Instruments, Watford, UK). Each microsomal
292 sample (0.2 ml) was diluted with 0.1 M potassium phosphate buffer (pH 7.4) to a final
293 volume of 2 ml and divided into two quartz cuvettes. A baseline was recorded between 400
294 and 500 nm and then the reference cuvette was bubbled with CO for 30-40 sec and 5 µl of
295 NADH (20mM, prepared in potassium phosphate buffer) was added (to reduce cytochrome
296 b₅). The tip of a small spatula was used to add a few grains of sodium dithionite
297 (approximately 1 mg; to reduce CYP450 and cytochrome b₅) to the sample cuvette, which

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

302 EROD and MROD activities were determined by the method of Burke *et al.*⁴⁷ For
303 both assays, 0.2 mg of microsomal protein and 0.25 ml of an NADPH generating system (0.8
304 mg NADP, 1.2 mg isocitric acid, 50 µl 0.1M MgSO₄, 1 unit isocitrate dehydrogenase and 0.1
305 M Tris-HCl, pH 7.8) were added to 0.1 M Tris-HCl buffer (pH 7.8), total volume 1.99 ml.
306 Samples were incubated for 2 min at 37°C and the reaction was initiated by adding 10 µl of
307 ethoxyresorufin or methoxyresorufin (cuvette concentration 5 µM). The linear increase in
308 fluorescence was recorded for 10 min at an excitation wavelength 530 nm and an emission
309 wavelength 585 nm (Perkin-Elmer LS55, Massachusetts, USA) and calibrated by the addition
310 of a resorufin standard (10 µM in DMSO).

311 GST activity was determined spectrophotometrically at 25°C with 1-chloro-2, 4-
312 dinitrobenzene (CDNB) as substrate according to the method of Habig *et al.*⁴⁸ with minor
313 modifications. The reaction was initiated by the addition of 0.1 ml of cytosol to a cuvette
314 containing 0.1 ml glutathione (final concentration 5 mM) and 0.8 ml reaction mixture (final
315 concentration 0.1 M KH₂PO₄/K₂HPO₄ pH 6.5, and 1 mM CDNB, 2% ethanol). Cytosolic
316 fractions were diluted in PBS to ensure assay linearity (a 100 x dilution for liver and 5 x
317 dilution for colon). The linear increase in absorbance at 340 nm was recorded for 3 min
318 (Unicam 8700 series UV/VIS spectrophotometer, Cambridge, UK) and GST activity was
319 calculated using the molar extinction coefficient of 9.6 mM/cm and expressed as nmol of
320 product formed/min/mg cytosolic protein.

321 QR activity was determined by the spectrophotometric method of Ernster⁴⁹, as
322 modified by Benson *et al.*⁵⁰, using 2, 6-dichlorophenolindophenol (DPIP) as the electron

323 acceptor and NADPH as the electron donor. The reaction was initiated by the addition of 10
324 μl of 12 mM DPIP to 0.3 ml of cytosol (100 x dilution for liver, 20 x dilution for colon) and
325 2.65 ml of the reaction mixture (final concentrations: 25 mM Tris, 0.7 mg BSA, 0.01%
326 Tween 20, 5 μM FAD, 0.2 mM NADPH). The rate of reaction was measured as a linear
327 decrease in absorbance at 600 nm over 90 sec (Unicam 8700 series UV/VIS
328 spectrophotometer, Cambridge, UK). Activity was measured in triplicate in the presence and
329 absence of dicumarol (10 μM). Quinone reductase activity was calculated from the dicumarol
330 sensitive portion of the assay, using a molar extinction coefficient of 21 mM/cm and
331 expressed as nmol of product formed/min/mg cytosolic protein.

332

333 *Statistical analysis*

334 The myrosinase data was \log_{10} transformed after Levene's test indicated the data had unequal
335 variance. A two-way ANOVA using the log-transformed data was used to investigate the
336 effect of blanch-freezing on myrosinase activity, and whether myrosinase activity varied in
337 different sections of broccoli. Standardised residuals of the \log_{10} transformed data were
338 normally distributed (Shapiro-Wilk W test). Results are expressed as the geometric mean and
339 95% CIs of the antilog transformed data.

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

345 All statistical analyses were conducted on SPSS Release 19.0 (2011) and a P value of
346 <0.05 was used as the critical level of significance.

347

348 **RESULTS**

349 *Effect of blanch-freezing on myrosinase activity in broccoli*

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

354 *Animal Experiment*

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

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

363 The consumption of raw broccoli caused a significant 27% increase in DNA strand
364 breakage in comparison to the control group (Fig. 2; $P=0.025$). The frequency of DNA strand
365 breaks in the group that consumed blanched-frozen broccoli was not significantly different
366 from the control group (Fig 2 $P=0.243$). Analysis of individual comet classes revealed a
367 significantly greater percentage of maximally damaged class 4 comets (42 v 29%, $P=0.03$)
368 and a significantly lower percentage of colonocytes exhibiting no damage (class 0; 15 v 26%,
369 $P=0.019$) in pigs fed raw broccoli than in pigs fed the control diet. There were no other
370 significant differences in comet classes (Table 4). The number of maximally damaged class 4
371 comets in the control group was high (29%). In the absence of exposure to a genotoxin, cells

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

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

384

385 **DISCUSSION**

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

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

404 Although the current study seems to be the first to report an increase in DNA damage
405 in colonocytes after the consumption of raw broccoli, there are other reports of cruciferous
406 vegetables damaging DNA *in vivo*. In a rodent study, Sorensen et al.⁵⁵ reported that a cooked
407 Brussels sprouts extract increased 8-oxo-dG levels in the liver. Scaled for body weight, the
408 daily intake of Brussels sprouts was similar to the intake of broccoli in the present study (25.6
409 v 17.3 g/kg/body weight). In a recent human study, Charron et al.⁵⁶ reported a transient
410 increase in DNA damage in peripheral blood mononuclear cells isolated from individuals 3
411 hours after they had consumed either isolated allyl isothiocyanate (AITC) (114.7 μ mol)
412 incorporated into mayonnaise or a mustard/cabbage treatment (150 g of raw homogenised
413 cabbage plus 30 g of Grey Poupon Country Dijon Mustard). In contrast, the majority of
414 studies in humans have reported that modest intakes of raw and cooked cruciferous
415 vegetables (85-300 g/d) protect lymphocytes from DNA damage or reduce the urinary
416 excretion of 8-oxo-dG⁵⁷⁻⁶² (a possible marker of whole body oxidative stress).⁶³ Also, in a rat
417 study Kassie et al.⁶⁴ reported that juice prepared from raw garden cress reduced background
418 levels of DNA damage in colonocytes. Thus, it seems that the effects of cruciferous
419 vegetables on DNA damage may depend on the amount and type of cruciferous vegetables
420 fed, the timing of measurement, the tissue studied, as well as the method of preparation.

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

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

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

453 The induction of phase 2 detoxification enzymes is thought to be an important
454 mechanism through which broccoli consumption protects against chemically induced
455 carcinogenesis.⁷⁷ In the current study, neither, raw nor blanched-frozen broccoli altered the
456 activity of GST and QR in the liver and colon. In contrast, rodent studies have reported
457 significant inductions of hepatic and colonic QR^{78,79} or hepatic GST in response to diets
458 containing freeze-dried broccoli.^{80,81} This disparity probably reflects the quantity of broccoli
459 fed. Accounting for the water content of fresh broccoli, pigs in the current study consumed a
460 diet equivalent to a 5% w/w freeze-dried broccoli diet. Aspry & Bjeldanes⁸⁰ reported a
461 significant induction of hepatic GST in rodents fed a 25% w/w broccoli diet, but no
462 significant effect of a 10% w/w broccoli diet, whereas other studies reporting induction of
463 GST or QR fed 20-25% w/w broccoli diets.^{78,79,81}

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

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

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

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

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

505

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512

513 *Conflicts of interest*

514 The authors report no conflicts of interest.

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765 **Table 1** Composition of Rowett Standard Grower Feed

766	Component	kg/1000 kg
767	Barley	228
768	Wheat	430
769	Hipro Soya	225
770	Super Soya	50
771	Soya Oil	10
772	Salt	5
773	Grower vitamin and mineral mix*	30
774	Molasses	20
775	De-oderase®	2

776

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

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783 **Table 2** Mean values (SD) of weight gain and daily intake of basal diet and broccoli over the
 784 12 d test period.

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

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

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806 **Table 3** Mean plasma concentration of vitamins and carotenoids in groups of pigs fed cereal
 807 diets unsupplemented (control) or supplemented with 600 g of raw or blanched-frozen
 808 broccoli (cv. Marathon) for 12 d.
 809

810 Treatment	811 Retinol 812 µg/ml	813 Zeaxanthin/lutein 814 µg/ml	815 Ascorbic acid 816 µM	817 α-tocopherol 818 µg/ml	819 γ-tocopherol 820 µg/ml
821 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)
822 Blanched- 823 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)
824 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)

820 Non-transformed data are expressed as mean (SD), data that were log₁₀ transformed before analysis are
 821 expressed as geometric mean (95 % CI). Two way ANOVA was used to assess statistical differences, *n*=5 in
 822 each diet group for all variables, except zeaxanthin/lutein, where *n*=4.
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827 **Table 4** Comet classes in colonocytes isolated from pigs fed one of the following diets:
 828 cereal diet unsupplemented (control), or cereal diet supplemented with 600 g/d of raw and
 829 blanched-frozen broccoli for 12 d.

830 Treatment	831 Comet classes				
	832 0	833 1	834 2	835 3	836 4
837 Raw broccoli	15.0* (12.3)	11.5 (2.0)	9.3 (2.1)	22.1 (6.1)	42.2* (9.3)
838 Blanched-frozen 839 broccoli	17.7 (5.3)	15.2 (2.8)	12.4 (1.3)	21.6 (3.5)	33.2 (6.5)
840 Control	26.0 (6.8)	15.4 (4.6)	11.4 (3.3)	18.4 (3.2)	28.9 (8.5)

841

842 Results are expressed as the number of colonocytes in each class of damage and are the mean (SD) of 3 slides
 843 (each containing two gels) for each animal and 5 animals in each treatment group. Two way ANOVA and
 844 Dunnett's test were used to assess significance of differences. Within each column an asterisk indicates that the
 845 treatment group differs significantly from the control group (*P*<0.05).
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853 **Table 5** The effect of raw and blanched-frozen broccoli on total CYP450 content and specific
 854 activities of EROD and MROD in hepatic microsomes.

855		Total CYP450	EROD	MROD
856		nmol/mg	pmol/min/mg	pmol/min/mg
857		of protein	of protein	of protein
858	Raw broccoli	0.61 (0.08)	106.1 (31.9)	38.3 (9.7)
859				
860	Blanched-frozen broccoli	0.60 (0.08)	111.6 (33.8)	39.1 (9.2)
861				
862	Control	0.61 (0.14)	103.2 (20.8)	37.2 (5.0)
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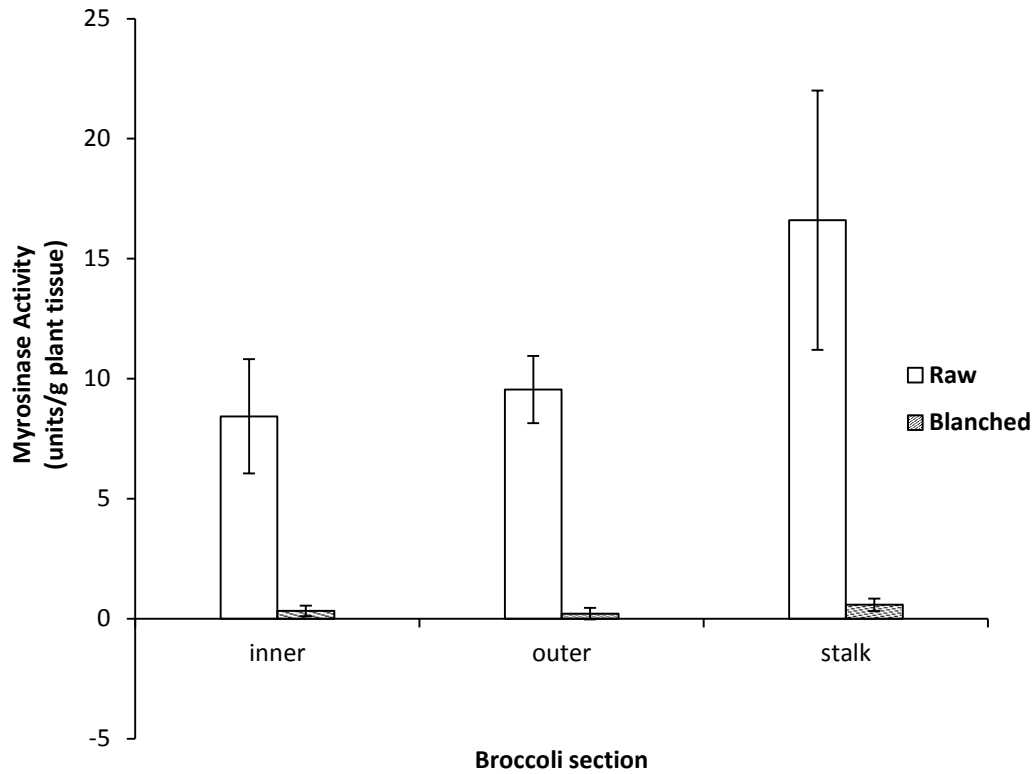
864 Values are means (SD), in all cases $n=5$ in each diet group. All analyses were conducted in triplicate. Two way
 865 ANOVA was used to assess significance of differences.
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 879 **Table 6** The effect of raw and blanched-frozen broccoli on the specific activities of GST and
 880 QR in hepatic and colonic cytosols.

881		Hepatic	Colonic	Hepatic	Colonic
882		GST	GST	QR	QR
883		nmol/mg	nmol/mg	nmol/mg	nmol/mg
884		of protein	of protein	of protein	of protein
885	Raw broccoli	2109 (549)	160.1 (89.2)	120.3 (15.7)	133.8 (25.9)
886					
887	Blanched-frozen broccoli	2178 (547)	171.3 (56.5)	137.6 (23.2)	130.8 (27.1)
888					
889	Control	2213 (255)	149.1 (94.3)	134.9 (24.5)	106.7 (30.8)
890					

891 Values are means (SD), in all cases $n=5$ in each diet group. All analyses were conducted in triplicate. QR
 892 activity was determined in the presence and absence of dicumarol. Two way ANOVA was used to assess
 893 significance of differences.
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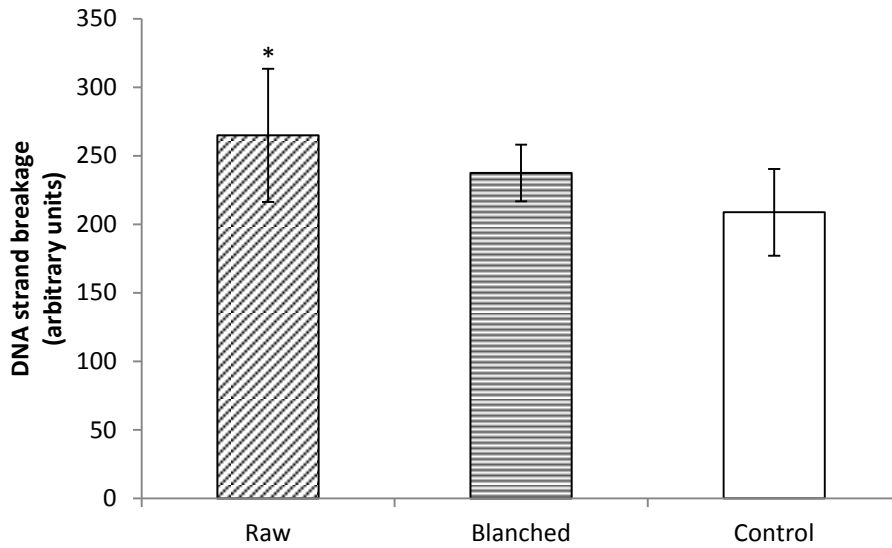
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908 **Fig 1** Effect of blanch-freezing on myrosinase activity in broccoli. Myrosinase activity is expressed as units/g of
 909 plant tissue in sections sampled from the stalk and the inner and outer regions of heads of whole raw and
 910 blanched-frozen 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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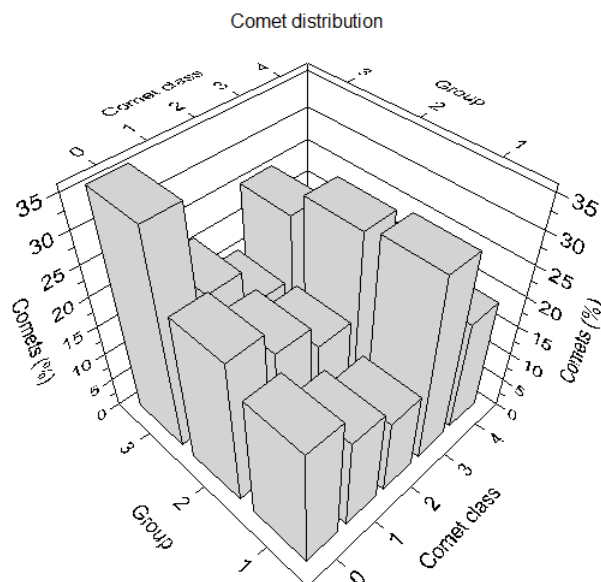


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920 **Fig 2** The level of DNA damage in colonocytes isolated from pigs fed cereal diets unsupplemented (control) or
 921 supplemented with raw or blanched-frozen broccoli (cv. Marathon) for 12 d. Results are expressed as mean
 922 values (in arbitrary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

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

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